Dissertation zur Erlangung des Doktorgrades
der Fakultaet fuer Chemie und Pharmazie
der Ludwig-Maximillians-Universitaet Muenchen

Khd1p, a protein with multiple roles in mRNA localization and Telomeric Silencing

Gonçalo Nuno Nunes da Silva Rebelo de Andrade

aus

Lissabon

2008

Erklaerung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Herrn Professor Doktor Ralf Peter Jansen betreut.

Ehrenwoertliche Versicherung

Diese Dissertation wurde selsbtaendig, ohne unerlaubte Hilfe erarbeitet.

Muenchen, am 20. Juni 2008

_____

Dissertation eingereicht am 24. Juni 2008

1.Gutachter Herr Professor Doktor Ralf-Peter Jansen
2.Gutachter Herr Professor Doktor Klaus Foerstemann

Muendliche Pruefung am 28. Juli 2008

Table of Contents

Acknowledgements:

I would like to specially thank,

– **Ralf Peter Jansen, for being a considerate but yet, demanding boss, for reading this PhD thesis and making insightful comments and for putting up with me and my jokes and singing for the past 4,5 years.**
– **Heidi Feldmann, for constant support in the development of the telomeric story.**
– **my Jansen lab colleagues: Susanne, Maria, Heidrun, Hanna, Miguel, Valérie, Stephan, Birgit, Tung, Andreas, Steffanie and Anja for all the fun and hilarious moments we shared.**
– **the Straesser laboratory, Emanuel, Susanne, Lina, Anja, Petra, Britta, Patricia, Sittinan and Nina, for all the fun and also for the unforgettable radio choice.**
– **Andreas, Tung and Valérie, for the wonderful times in the Jansen "outstation".**
– **Susanne Lange for expanding my German language knowledge.**
– **both the Straesser and Jansen groups for the group seminars and group dynamics.**
– **Laurent Lariviere and Alessandro Vannini for making me understand better X-Ray christallography and for all the other offcampus events.**
– **my Genecenter colleagues, that make it a truly interesting place to work in.**
– **FCT, for the PhD scholarship.**
– **my girlfriend, Inês, for being there those days...**
– **my mother, for their never wavering support.**
– **my friends in Munich, specially to the Comité Lateral, for the offcampus anchor to reality and for the never ending stories together.**
– **the Portuguese National Football Team, for overperforming in the last 4 years.**
– **Carlota, the She2p-antibody producing hare.**
– **Leila, our most cooperative lab member.**

Part I

Khd1p involved in *ASH1* mRNA biology?

1. INTRODUCTION

A cell is the most basic unit of life. When a cell divides, it gives rise to two exactly like it, or so we were told during our basic biological studies. Later, we began to understand that this is not always the case, specially during development. A central issue in developmental biology is to explain the ability of a mother cell to divide into two daughter cells with different cell fates. These different cell fates are determined by different activated genetic programs. Differences in gene expression can be determined by the environment (external stimuli), or even by differential segregation of cell fate determinants. mRNA localization and asymmetric cell division are two mechanisms that to achieve this goal. Certain cell fate determinants are sorted onto different sister cells (reviewed Du *et al.*, 2007). One of the most important events in this process deals with the timing of expression. The onset of protein synthesis needs to occur at the right time and this will also mean at the right place. Proposedly, intracellular trafficking of a cell fate determinant is coupled to its translation repression and upon arrival at the destination, this repression is removed and the cell fate determinant expressed, thus creating a cell with a different cell fate (Farina and Singer, 2002; St. Johnston, 2005).

The process of mRNA localization plays an instrumental role in development of the fruit-fly, *Drosophila melanogaster*. For example the determination of the embryonic axes occurs by the cytoplasmic localization and local translation of specific maternally derived mRNAs within the oocyte. Segmentation is also a process that involves mRNA localization and spatially defined protein expression (reviewed in Johnstone and Lasko, 2001; Riechmann and Ephrussi, 2001; St Johnston, 2005). Although it is a conserved processed throughout evolution *per se*, the mechanisms and the implications of this localization and spatial organization can be quite different.

mRNA localization is also a very important mechanism in determining cell polarity and polarized growth (Farina and Singer, 2002; Farina *et al.*, 2003; Zarnack and Feldbruegge, 2007).

1.1 Mechanisms of mRNA localization

Intracellular mRNA localization is a widespread mechanism in the eukarya domain (St. Johnston, 2005). Although the list of known localized messages is ever increasing, the mechanisms are somewhat conserved. They range from directional mRNA export from the nucleus, general degradation and selective spatial and localized protection, diffusion coupled to specific retention and motor-protein driven directed cargo transport along the cytoskeleton. Examples of these mechanisms include: i, the localization of the *nanos* mRNA in *Drosophila melanogaster*, stabilized

by the localized binding of Smaug and destabilized and degraded everywhere else in the embryo (St Johnston *et al.*, 1992; Rongo *et al.*, 1995; Jeske *et al*., 2006) or the localization of *prospero* and *numb* to the basal pole of the neuroblasts by ubiquitylation and destruction of Miranda (Slack *et al.*, 2007), ii, targeting of *Vg1* mRNA to the vegetal pole of *Xenopus laevis* oocytes dependent on both actin and microtubules (Yisraeli *et al.*, 1990; Alarcon and Elinson, 2001), iii, microtubule dependent directed transport of *bicoid* mRNA (Pokrywka and Stephenson, 1991; Schnoerrer *et al.*, 2000; reviewed in Saxton, 2001). Recently, *bicoid* mRNA localization was linked to endosomal and vesicle trafficking (Irion and St. Johnston, 2007). The role of the cytoskeleton in mRNA localization process is still poorly characterized, but in recent years several well defined examples have been studied and it is not unlikely that the cytoskeleton is the driver of the mRNA localization process.

1.2 Conceptual Mechanism of cytoplasmic mRNA localization by active transport

During transcription the nascent transcript is processed to mRNA and packed to an export-competent ribonucleoprotein complex, an mRNP. This complex, once reaching the cytoplasm is recognized by the transport machinery that mediates the delivery to a defined destination. Having reached the final cellular address, the mRNA becomes effectively retained and concentrated at this location.

1.3 From the birth of a transcript to nuclear export of a mature mRNA

As the RNA polymerase II transcribes the genes into RNA, several factors, from splicing factors to export factors, are loaded onto the RNA making it export competent. For a localized mRNA, one supposes that a signal, supposedly a protein, is also loaded onto this mRNP so that it can be differentiated from the bulk mRNP particles that are not localized. This sorting is believed to take place early in the maturation process. This maturation process is characterized by the sequential deposition and removal of several heteregeneous nuclear ribo-nucleoproteins (hnRNP). Some hnRNPs are restricted to the nucleus, whereas others escort the RNA during nuclear export and remain associated until correctly localized in the cytoplasm (Hoek *et al.*, 1998; reviewed in Aguilera *et al.*, 2005; Hieronymus and Silver, 2004). One function for hnRNPs is to provide the nascent RNAs a signal for a specific location. It is conceivable that this latter hnRNP is responsible for the translation repression of the moving particle, mechanism that ensures the proper localization of the protein, only after the mRNA has been localized (Farina and Singer, 2002). For example,

hnRNP A2 binds to myelin basic protein (MBP) mRNA. This mRNA localizes to the distal ends of dendrites of oligodendrocytes (Hoek *et al.*, 1998). Another example is the Fragile X sindrome, caused by loss of function of FMR1, a protein with 2 KH-domains that binds to polyribosomes and to a few hundreds mRNAs, thus shaping synaptical activity by on-off translation regulation of synaptic localized mRNAs such as *MAP1*, *CaMKII* and others (Zalfa *et al.*, 2003; Brown *et al.*, 2001; Reviewed in Kaytor and Orr, 2001). In addition, dFMR, the Drosophila homolog of FMRP, may act as a translational repressor of *futsch*, the homolog of MAP1B (Zhang *et al.*, 2001).

Maturation of a nascent transcript into mRNA includes several co-transcriptional processes, such as 5'-capping, splicing and 3'end processing. The cap-binding complex binds to the 5' monomethylated cap structure, the exon junction complex marks the religated splice-sites and poly(A) binding protein binds the already processed 3' poly(A) tail. As any properly processed and matured mRNA will eventually lead to a protein and will then exert a function, this process of maturation has safeguards, to ensure that the mRNA produced is correctly capped, spliced and rejoined, and polyadenylated. Transcripts that lack this correct processing are retained at the nuclear periphery (Jensen *et al.*, 2003; Galy *et al.*, 2004) and are not exported, becoming then targets for the nuclear exosome (Saguez *et al.*, 2005).

The sequential maturation process indicates that the machinery involved is not only functionally but also physically linked. Increasing evidence point to the existence of mRNA factories (Iborra *et al.*, 1996; Szentirmay and Sawadogo, 2000) or large protein assemblies that are connected by a set of adaptor proteins such as Npl3p, Sub2p or Yra1p (Lei *et al.*, 2001; Erkmann and Kutay, 2004; Stutz and Izaurralde, 2003; Stewart, 2007) that ensure the swift maturation of mRNAs.

1.4 Reaching the cytoplasm

The mature mRNA upon reaching the cytoplasm changes its protein inventory, as several factors are released and re-enter the nucleus whereas others, cytoplasmic RNA-binding factors and the translation machinery, bind to the mature mRNA. This mRNA undergoes the first round of translation, circularizes and could then be sorted and loaded onto specialized ribosomes, as has been proposed for *ASH1* mRNA in budding yeast (Komili *et al.*, 2007). It can be translated immediately into protein, be targeted to the proper organelle (Corral-Debrinsky *et al.*, 2000; Marc *et al.*, 2002; Margeot *et al.*, 2003) or even be localized via the cytoskeleton to a specific intracellular destination and only then become translated, as has been shown for several mRNAs in different organisms, being *ASH1* mRNA in budding yeast (Paquin *et al.*, 2007; Deng *et al.*, 2008) and *nanos* mRNA in fruitfly (Gavis and Lehmann 1994) just two examples. Another example involves localized

destruction of a protein complex and its cargo. One such example is Miranda assymetrical basal localization in neuroblast. Miranda is ubiquitylated via its C-terminal domain; removal of this domain disrupts Miranda localisation and replacement of this domain with a ubiquitin moiety restores normal asymmetric Miranda localisation. These results demonstrate that APC/C activity and ubiquitylation of Miranda, in a proteasomal-independent process, are required for the asymmetric localisation of Miranda and its cargo proteins to the NB cortex (Slack *et al.*, 2007).

These movements in the cytoplasm can occur either just by diffusion or by active cytoskeletal-dependent transport. Whenever the cytoskeleton is involved a reorganization of the microtubules or actin cables has to occur and polarization must take place. The existence of mutants that fail to polarize or displace cargo and thus fail also to localize mRNAs to their destinations has proven the essential nature of the cytoskeleton on mRNA localization.

1.5 mRNPs travelling along the cytoskeleton

Directed mRNA transport within the cytoplasm is generally mediated through the interaction of trans-acting factors, the localization machinery, with some or several localization element(s), or "zipcodes" (Kislauskis and Singer, 1992), present within the mRNA sequence (Chartrand *et al.*, 2002; Jambhekar and DeRisi, 2007). In most cases the localization elements are located within the 3'-untranslated region, 3'UTR, of the mRNA (for review see Bashirullah *et al.*, 1998; reviewed in Jambhekar and DeRisi, 2007). Most zipcodes are characterized by extended secondary structure elements like stems, bulges, loops and hairpins (Jambhekar *et al.*, 2005; Olivier *et al.*, 2005). This suggests that it is the secondary structure rather than the primary sequence that confers the RNA-protein specificity. (reviewed in Jambhekar and DeRisi, 2007).

The minimal localization complex is thought to include the mRNA bound by its zipcode to a RNA binding protein, an adaptor and a motor protein.

In *Drosophila* it has been hinted that *bicoid* mRNA is linked to cytoplasmic dynein via the zipcode-binding protein Swallow, being the adaptor protein the dynein light chain (Schnoerrer *et al.*, 2000; Arn *et al.*, 2003). In addition, *bicoid* mRNA has also been shown to be bound by Staufen, which is necessary but not sufficient for proper targetting of *bicoid* mRNA to the anterior pole of the embryo (Ferrandon *et al.*, 1997; Snee *et al.*, 2005). Recently, *oskar* mRNA/Staufen complexes were shown to be linked to *bicoid* mRNA localization via Miranda protein (Irion *et al.*, 2006). Miranda has been shown to bind Staufen in neuroblasts and ganglion mother cells, ensuring assymetrical distribution of *numb* and *prospero* mRNAs in neuroblast-ganglion mother cells cellular division, thus assisting in proper brain development (Schuldt *et al.*, 1998). The observation that Miranda is ubiquitylated

(Slack *et al.*, 2007) indicates that coupled to the positive and directed transport a localized protection could also contribute to *oskar* and *bicoid* Staufen based mRNA localization.

In *Xenopus laevis*, a set of mRNAs are localized to the vegetal pole. These include the *Vg1*, *Xvelo* (Claussen and Pieler, 2004), *Xlsirt* (Allen *et al*., 2003), *XNIF* (Claussen *et al.*, 2004), *Xcat2* (Zhou and King, 1996) and *fatvg* (Chan *et al.*, 1999). Targeting of *Vg1* mRNA to the vegetal pole of *Xenopus laevis* oocytes is dependent on both actin and microtubules (Yisraeli *et al.*, 1990; Alarcon and Elinson, 2001).

1.5.1 Role of the cytoskeleton

Cytoplasmic mRNA transport has been described in a range of organisms, although the distance of transport can vary greatly, from extremely long in oocytes and neurons to relatively short in fibroblasts and yeast. Since cytoskeletal requirements can be determined with the help of drugs that either specifically depolymerize microfilaments or microtubules, long range transport has been associated to microtubules whereas short range transport to actin microfilaments (Nasmyth and Jansen, 1997). As an example, the localization of MBP mRNA in oligodendrocytes requires an intact microtubule cytoskeleton (Carson *et al.*, 1997), whereas targeting of β-actin mRNA to the leading edge in chicken fibroblasts requires microfilaments (Sundell and Singer, 1991).

Coupled to the selection of the type of cytoskeleton is the selection of the type of motor. In case of microtubule directed movement, two types of motors are associated, dynein, for minus-end directed movement as in *bicoid*, *gurken* and *wingless* mRNA localization (Januschke *et al.*, 2002b; MacDougall *et al.*, 2003; Delnaoue and Davis, 2005; Clark *et al.*, 2007) and kinesin, for plus-end directed movement as in *oskar* mRNA localization (Brendza *et al.*, 2000; Januschke *et al.*, 2002a; Arn *et al.*, 2003). In the case of actin filaments, myosin is the associated molecular motor (e.g. *ASH1* mRNA localization). So, coupled to the sort of cytoskeletal structure involved in mRNP transport, a specific type of motor can be found.

1.5.2 Role of the molecular motor binding adaptor proteins in determining cargo specificity

RNA is not the only cargo that is actively transported. It is generally assumed that different loads encompass different adaptor proteins, bound to the motor proteins (reviewed by Bretscher, 2003). In budding yeast, Myo2p has been implicated in numerous bud directed targetting processes, such as organelle inheritance, vesicle transport (Govindan *et al.*, 1995) or even mitotic spindle

orientation (Yin *et al.*, 2000). Cargo differentiation is a crucial event to the sorting of the localization of the load. The Myo2p motor, for example, has a wide variety of adaptor proteins, one for each cargo, which become tethered to separable cargo-binding regions within the Myo2p globular tail. Vacuole sorting involves adaptor proteins Vac17p/Vac8p (Catlett *et al.*, 2000; Tang *et al.*, 2003; Ishikawa *et al.*, 2003), transport of secretory vesicles involves Sec4p (Pruyne *et al.*, 1998; Schott *et al.*, 1999), and segregation of mitochondria requires Ypt11p (Itoh *et al.*, 2002). Sec4p and Ypt11p are organelle-binding specific Rab proteins with intrinsec GTPase activity that are located on the transported organelle membrane.

In yeast Sec4p has been shown to be targeted to the incipient bud site and this has been shown to be dependent on ER inheritance factors, such as Sec3p (Aronov *et al.*, 2007). Endoplasmic reticulum inheritance in yeast has been shown to require She3p and Myo4p (Estrada *et al.*, 2003). Both are required for cortical ER retention at the bud and contribute with Sec3 and other proteins to achieve proper ER inheritance (Aronov *et al.*, 2007). Interestingly, She3p and Myo4p have also been shown to involved in She2p-mRNP cargo transport (Boehl *et al.*, 2000; Schmid *et al.*, 2006), indicating that mRNA localization and ER inheritance are intimately linked. Evidence for this linkage is also the observation that *Xenopus laevis Vg1* mRNA is localized to the vegetal pole by VgRBP/VERA, an ER associated protein (Dresher *et al*., 1997).

1.6 Reaching the destination

In order to allow the concentration of a specific transcript against the diffusion gradient, it is generally assumed that an anchorage mechanism exists at the target site, thus preventing the mRNA to diffuse away from the delivery site. The mechanisms underlying this process are still poorly understood. One can imagine that the cargo, upon reaching the destination has to be handed over from the transport machinery to the molecular anchor and there be retained. After delivery, recycling back to the nuclear envelope of some components must occur whereas other components will remain associated to the mRNP.

At the anchoring site, perhaps with the help of RNA helicases, the mRNA is unraveled into a more accessible form that facilitates ribosome recruitment and the onset of protein synthesis. This event is normally described as local translation, coupling mRNA localization with protein synthesis. It is conceivable that the translation process itself can trigger the anchorage of a localized mRNA. In yeast, *ASH1* mRNA localization has been shown to require translation (Gonzalez *et al.*, 1999; Paquin *et al.*, 2007; Deng *et al*., 2008). Furthermore, recently it has been reported that the *ASH1* mRNA particle travels with Endoplasmic Reticulum (ER) tubules to the bud tip (Schmid *et al.*,

2006). The fact that the ribosomes are intrinsically a part of the localizing mRNP seems to strengthen the concept that it is translation itself that triggers the anchorage. The onset of *ASH1* mRNA translation has been proposed to occur upon removal of the translation repressor, Khd1p, due to phosphorylation by Yck1p (Paquin *et al.*, 2007). Similarly, another translation repressor, Puf6p has also been shown to act on *ASH1* mRNA localization and after phosphorylation by Yck2p, releasing the translation block (Deng *et al.*, 2008).

Similarly, in fruit flies *oskar* mRNA localization depends on local synthesis of Oskar protein (Rongo *et al,*, 1995).

In addition to polyribosome mediated retention, mRNAs can be anchored by other mRNAs, due to complementary base pairing or by other RNA-binding proteins. Such is the case for *Xlsirts* RNA, a short non protein coding RNA from *Xenopus laevis* oocytes that is required for *Vg1* mRNA anchorage at the vegetal pole (Kloc and Etkin, 1994). In *Drosophila* oocytes, the double stranded RNA binding protein Staufen anchors both *oskar* mRNA to the posterior pole (Ephrussi *et al.*, 1991; Rongo *et al.*, 1995) and *bicoid* mRNA to the anterior pole (St Johnston *et al.*, 1992; Ferrandon *et al.*, 1997; Snee *et al.*, 2005). Anterior Miranda localization requires microtubules, rather than actin, and depends on the function of Exuperantia and Swallow, indicating that Miranda links Staufen/*oskar* mRNA complexes to the *bicoid* mRNA localization pathway (Irion *et al.*, 2006).

For some transcripts the cytoskeleton has been proposed to play a role not only in their transport but also in their anchorage. The most clear example is the localization of *Vg1* mRNA. While the cytoplasmic transport process requires microtubules, the cortical anchorage seems to depend on a network of actin filaments and cytokeratin (Yisraeli *et al.*, 1990; Alarcon and Elinson, 2001). This mRNA is bound by VgRBP/VERA (Deshler *et al.*, 1997) and this protein has been shown to be bound to the ER, indicating that in this organism mRNA transport can be linked to organelle trafficking as has been proposed for *ASH1* mRNA in budding yeast (Schmid *et al.*, 2006).

1.7 mRNA Localization in S. cerevisiae

In budding yeast mRNA localization is an important regulatory process aswell. In this organism, a set of mRNAs is actively transported into the tip of growing buds, by a set of proteins, the SHE machinery (Jansen *et al.*, 1996; Boehl *et al.*, 2000; Long *et al.*, 2000). These SHE-proteins were identified in a screen designed to detect mutants that failed to generate asymmetric HO expression, an indicator of efficient *ASH1* mRNA localization. The set of mRNAs, that are actively transported to the bud-tip in a SHE-dependent manner, encode proteins that are restricted to daughter cells (e.g.

Ash1p), bud-enriched (e.g. Ist2p) or equally distributed (e.g. Tpo1p) (Shepard *et al.*, 2003). After bud localization and once translated, these proteins cannot freely diffuse back between mother and daughter cell, equilibrating the protein concentration between the two compartments, since this diffusion is prevented by the formation of the septin ring (Kozubowski *et al.*, 2005). The fact that only in the case of *ASH1* a clear correlation between RNA and protein localization occurs, shows that the underlying biological significance of this SHE-dependent localization process is still unclear. In addition, it has been suggested that SHE-dependent mRNA localization is not anaphase restricted (as in *ASH1*) and can take place independently of the cell cycle (Long *et al.*, 1997).

Recently, SHE dependent mRNA localization of polarized growth determinants was uncovered (Aronov *et al.*, 2007). *SEC4*, *CDC42* mRNAs that encode membrane associated cell polarization factors are localized to the incipient bud site prior to nuclear division in an mRNA transport independent manner but *SRO7* mRNA, encoding a non-anchored cell polarization factor is targeted in an mRNA transport dependent manner to the incipient bud site, prior to nuclear division, leading to local protein synthesis and enrichment. This work showed that this process, that relies on functional cortical ER inheritance, is Sec3p and She2p dependent, indicating that cortical ER inheritance and mRNA localization via She2p are both required for cell polarity establishment.

mRNA localization in yeast can also be SHE-independent, as observed for the targeting of mRNAs encoding a subset of mitochondrial proteins to polysomes located directly at the organelle surface (Corral-Debrinsky *et al.*, 2000; Marc *et al.*, 2002; Margeot *et al.*, 2003; Aronov *et al.*, 2007; Saint-Georges *et al.*, 2008). At this moment it is still unclear if a conserved mitochondria targeting sequence exists at the nucleotide level or if on the other hand, a mitochondria peptide signal really exists.

1.8 ASH1 mRNA localization

Budding yeast alternates between a diploid cell division cycle and, under conditions of nutrient deprivation, an haploid cell division cycle. The mating of two haploid cells with opposing mating type (a and alpha) leads to a diploid cell. Upon budding a mother cell and a daughter cell are generated. Mother cells are capable of switching their mating type, while daughter cells are not. Mating type switching is regulated by HO endonuclease (Nasmyth *et al.*, 1993), a protein that is expressed only in mother cells. HO initiates a genomic rearrangement of the MAT locus, converting it from a to alpha or vice versa. In daughter cells the expression of HO is repressed by Ash1p (asymmetric synthesis of HO), which is asymmetrically distributed to daughter cell nuclei (Bobola *et al.*, 1996; Sil and Herskowitz, 1996; Cosma *et al.*, 1999; Maxon and Herskowitz, 2001). This

asymmetric sorting of Ash1p to the daughter cell nuclei correlates with the localization of *ASH1* mRNA to the distal tip of daughter cells during the anaphase stage of cell cycle (Long *et al.*, 1997; Takizawa *et al.*, 1997). Ash1p serves a model for studying asymmetric segregation of cell fate determinants due to mRNA localization (reviewed in Darzacq *et al.*, 2003).

ASH1 is only transcribed in anaphase (Bobola *et al.*, 1996; Spellman *et al.*, 1998). Ash1p is not only recruited to the HO promoter but also interacts with *SGA1* and *PCL1* promoters, two loci implicated in sporulation and cell cycle control (Lee *et al.*, 2002). Pcl1p is one of the cyclins of the Pho85p kinase complex (McBride *et al.*, 2001). Deletion of *pho85* leads to a stabilization of Ash1p, so that its activity persists in the daughter cell during the cell cycle and thus represses mating type switching in the following cell division.

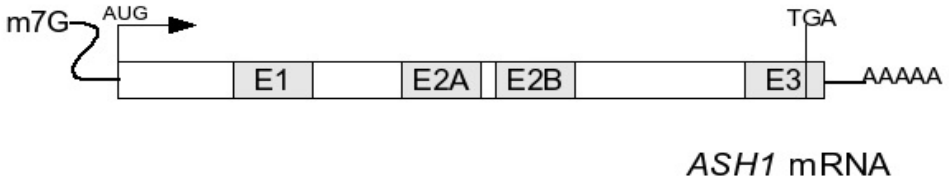


Fig. 1 – Schematic view of *ASH1* mRNA, showing the localization elements E1 (598-750), E2A(1044-1196), E2B(1175-1447) and E3(1752-1870).

1.9 ASH1 mRNA

Four zipcode elements (Fig. 1) have been described as essential for transport and for tight anchorage at the bud tip cortex, namely E1, E2A, E2B and E3 (Chartrand *et al.*, 1999; Chartrand *et al.*, 2002). Of these elements, three are located in the coding region and one, the E3, spans from the end of the open reading frame into the 3'UTR. Secondary structure prediction of these elements suggest that all *ASH1* zipcodes form extensive stem-loops and bulges (Chartrand *et al.*, 1999; Gonzalez *et al.*, 1999; Chartrand *et al.*, 2002; Olivier *et al.*, 2005; Jambhekar *et al.*, 2005). The disruption of these structures destroys the ability to direct mRNA localization and the ability to be bound by She2p (Olivier *et al.*, 2005). In addition, the integrity of the E3 element is essential for the binding of She2p (Boehl *et al.*, 2000; Long *et al.*, 2000). Although one element alone was shown to be sufficient for localization into the bud (Long *et al.*, 1997; Takizawa *et al.*, 1997; Bertrand *et al.*, 1998), all four have been proven to be essential for tight anchoring at the bud tip (Chartrand *et al.*, 2002). These secondary structured localization elements, not only serve as binding sites for trans-acting localization factors, such as She2p and Khd1p, but also seem to act as molecular obstacles, slowing down protein synthesis (Chartrand *et al.*, 2002). It has been suggested that this molecular

translation delay contributes to achieve a proper Ash1p asymmetry. Another contributing observation to strengthen the idea that translation plays a role in *ASH1* mRNA localization, was the fact that the presence of premature termination codons (PTC) prior to each of the zipcodes showed no decreased mRNA stability although showing a severe impairment in mRNA localization (Jaedicke A., PhD Thesis, 2004).

1.10 Translation Regulation

The local enrichment of an mRNA can lead to a higher concentration of the encoded protein at a specific location. Therefore, the cell has to have developped mechanisms by which the localizing mRNA is not translated before reaching its final destination. This forces a closer look at how translation of a localizing mRNP can be regulated. Not only can translation be regulated at the maturation of the ribosome, at the translation initiation step and at ribosomal subunit joining, but also, and most likely, translation can also be regulated by the removal of a translation repressor from the localizing mRNP.

The observation that the presence of premature stop codons prior to any of the zipcodes does not elicit NMD but severely impairs localization and achoring (Jaedicke, PhD Thesis 2004) seems to point to a role for the initial round of translation in the mRNA localization event. Furthermore, cycloheximide treatment of yeast cells, that blocks translation elongation leads to a loss of mRNA localization (Jaedicke, PhD Thesis 2004) and it has been shown that its own translation is required for proper anchoring of the localizing *ASH1* mRNA (Gonzalez *et al.*, 1999).

A recent example suggested that *ASH1* mRNA travels to the bud tip in a SHE-machinery dependent manner, in an mRNP and that upon reaching its destination Khd1p, a protein that binds the E1 element of *ASH1* mRNA, is phosphorylated by Yck1p, a kinase at the plasma membrane, and releases the *ASH1* mRNA translation block (Paquin *et al.*, 2007). Furthermore, Puf6p has also been shown to be an *ASH1* mRNA translational repressor (Gu *et al.*, 2004). Puf6p was shown to bind the E3 element of *ASH1* mRNA (Gu *et al.*, 2004) and repress its translation by interacting with Fun12p/eIF5B (Deng *et al.*, 2008). This interaction was abolished and *ASH1* mRNA was translated upon Puf6p phosphorylation by Yck2p (Deng *et al.*, 2008). This model of translational repression of the localizing mRNA and translation block release upon reaching the final destination is consistent with other observations for other mRNPs in higher eukaryotes, as already discussed.

1.11 The core localization machinery

The motor protein that mediates the active transport of the SHE mRNP is Myo4p (Jansen *et al.*, 1996, Muenchow *et al.*, 1999). Myo4p is a non-muscle myosin, that belongs to the class V myosins, where the only other member is a protein 57% identical to Myo4p, Myo2p (Haarer *et al.*, 1994). Although Myo2p is essential for growth (Johnston *et al.*, 1991), Myo4p is not (Haarer *et al.*, 1994). Both myosins localize to the bud tip during bud formation (Lillie and Brown, 1994; Schott *et al.*, 1999). Although it was previously assumed that Myo2p was only associated with organelle inheritance and Myo4p only with mRNA localization, recent publications show that Myo4p is also implicated in cortical ER inheritance (Estrada *et al.*, 2003; Schmid *et al.*, 2006).

An interaction partner of Myo4p is She3p, acting as an adaptor protein between the mRNA binding protein She2p and the motor (Takizawa and Vale, 2000). More detailed analysis (Boehl *et al.*, 2000; Long *et al.*, 2000) have shown that Myo4p interacts with NH2-terminus of She3p. This interaction is a direct physical link and is a tight and permanent association (Boehl *et al.*, 2000).
In addition, gel-shift assays have demonstrated that She2p binding to an *ASH1* zipcode element is specific and enhanced by She3p (Boehl *et al.*, 2000), providing evidence for a stabilization or even a cooperative binding to the mRNA.

She2p acts as a specific mRNA binding protein, that bridges *ASH1* mRNA to the She3p carboxy-terminus (Boehl *et al.*, 2000; Long *et al.*, 2000; Kwon and Schnapp, 2001). Although all four localization elements within *ASH1* mRNA are contacted and needed for efficient localization and transport (Boehl *et al.*, 2000; Chartrand *et al.*, 2002; Olivier *et al.*, 2005; Jambhekar *et al.*, 2005; Reviewed in Jambhekar and Derisi, 2007), the affinity of She2p to these elements has been proposed to be different (Long *et al.*, 2000; Niessing *et al.*, 2004). Nevertheless, a combination of zipcode-swapping and site directed mutagenesis have demonstrated that the zipcodes are redundant in function in regard to mRNA localization (Chartrand *et al.*, 2002).
Despite the fact that the She2p primary sequence does not contain a canonical mRNA binding site, information taken from the cristallographic structure of She2p has revealed a region that can be responsible for its mRNA binding ability (Niessing *et al.*, 2004). These regions include the COOH-terminus, the NH2-terminus and a positively-charged surface region, that could bind negatively charged nucleic acids (Niessing *et al.*, 2004). Although She2p is distributed uniformly throughout the cytoplasm (Jansen *et al.*, 1996), upon *ASH1* overexpression, She2p co-localizes together with *ASH1* mRNA at the tip of growing buds (Boehl *et al.*, 2000). Interestingly, upon mRNA export block, using a temperature sensitive *MEX67* allele, She2p can be trapped in the nucleus and is

excluded from the cytoplasmic fraction (Kruse *et al.*, 2002), indicating that it is a shuttling protein.

Since Myo4p, She3p and She2p all co-localize with trafficking *ASH1* mRNA containing particles (Takizawa and Vale, 2000; Irie *et al.*, 2002) and co-immunoprecipitate with *ASH1* mRNA (Muenchow *et al.*, 1999; Takizawa and Vale, 2000), the three proteins are thought to be the core locasome, the minimal SHE RNP (Darzacq *et al.*, 2003). Furthermore, She3p and Myo4p have been shown to co-localize with *ASH1* mRNA in the cytoplasm on string-like filamentous structures (Muenchow *et al.*, 1999), resembling transport intermediates. Other factors can be associated with this core locasome (reviewed in Paquin and Chartrand, 2007) either transiently, or associated but not required for transport.

1.12 Accessory factors

- *LOC1*

The strictly nuclear protein Loc1p was isolated by 3-hybrid screening due to its ability to bind to *ASH1* 3' UTR (Long *et al.*, 2001). In a *loc1* disruption, the asymmetric distribution of both *ASH1* mRNA and Ash1p is affected. However, Loc1p has been identified as part of the 66S-pre-rRNA complex and been shown to be involved in 25S rRNA processing (Harnpicharnchai *et al.*, 2001). Its function in assembly and export of 60S ribosomal subunit has been shown more recently (Urbinati *et al.*, 2006), Loc1p, due to its extremely high isoelectric point, binds unspecifically to double stranded RNA (Long *et al.*, 2001). In addition, a *loc1* disruption shows a severe slow growth phenotype at 30 degrees and an abnormal cell morphology (Long *et al.*, 2001), typical for genes involved in ribosome synthesis pathways. Therefore, the connection of Loc1p to mRNA localization seems to be rather indirect and a reflex of a defect in ribosome biogenesis and protein synthesis.

Recently however, it has been shown that Loc1p is instrumental in loading the *ASH1* mRNA onto to a specific non-canonical ribosome that includes a special combination of ribosomal protein phenocopies (Komili *et al.*, 2007). These non-canonical ribosomes are then localized to the bud tip, in a repressed state, taking with them *ASH1* mRNA and are translationally activated once reaching the target site.

▪*PUF6*

Another protein that has been shown to bind to the *ASH1* 3' UTR is Puf6p. This protein is a non canonical pumillio-related RNA binding protein and a Δ*puf6* has been shown to make *ASH1* mRNA translation faster and therefore Puf6p was proposed to be a translation repressor (Gu *et al.*, 2004).
The fact Puf6p could co-purify She2p seems to argue for a transient interaction with at least part of the locasome, namely She2p (Gu *et al.*, 2004). The question still remains whether this interaction with She2p takes place in the nucleus or whether it takes place in the cytoplasm.
Interestingly, Puf6p has also been implicated in rRNA processing and ribosome biogenesis (de Marchis *et al.*, 2005; Wade *et al.*, 2006; Fromont-Racine *et al.*, 2003) and so the translation repressor function could be indirect, due to incorrectly assembled ribosomes. Interestingly, Puf6p also co-purifies with pre-60S particles (Nissan *et al.*, 2002).
Recently, Puf6p was shown to repress *ASH1* mRNA translation by interacting with Fun12p/eIF5B. This interaction was abolished and *ASH1* mRNA was translated upon Puf6p phosphorylation by Yck2p (Deng *et al.*, 2008). In addition, Puf6p was also shown to lead to a 50% loss of localization to the incipient bud site of mRNAs encoding membrane associated polarization factors *CDC42* and *SEC4*, while not affecting localization of *SRO7*, a non membrane associated polarization factor and was proposed to play a role in the localization of mRNAs encoding the membrane-anchored small GTPases (Aronov *et al.*, 2007).

▪*SCP160*

Another accessory factor proposed to have a role in *ASH1* mRNA localization is *SCP160* (Irie *et al*., 2002), a member of the vigilin-like protein family (Lang and Fridovich-Keil, 2000; Baum *et al.*, 2004). This protein contains 14-KH domains, domains that are known to bind double stranded nucleic acids (Wintersberger *et al.*, 1995). It has also been shown to interact with membrane-bound polysomes (Frey *et al.*, 2001) and to be a component of RNPs (Lang and Fridovich-Keil, 2000). Scp160p could play a more general role, as it has been shown to interact with 69 mRNAs with diverse functions, among which *ASH1* mRNA was not found (Li *et al.*, 2003). A more general role for Scp160p functions seems to be supported by the observation that a *SCP160* disrupted cell has been shown to be sensitive to translation impairing drugs (Baum *et al.*, 2004).
A distinct function for Scp160p in mRNP formation, stability or maturation has not yet been found and has revealed difficult to analyse as a *SCP160* disruption leads to chromosomal instability and loss of ploidy control (Wintersberger *et al.*, 1995).

- *KHD1*

The KH-domain protein 1, Khdp1, has been reported to bind the *ASH1* N-element (Irie *et al.*, 2002), the region spanning the first 800 nucleotides of the coding sequence. This region, that comprises also the E1 element (Chartrand *et al.*, 1999) had been previously shown to be sufficient for targeting a reporter RNA to the bud tip (Gonzalez *et al.*, 1999). Interestingly, a deletion of *khd1* had only little effect on HO expression and no significant change on the frequency in mating type switching was observed in *khd1* disrupted cells. Although a genetic interaction between *MYO4* and *KHD1* has been shown no physical interaction has been shown.

It has been shown that a *KHD1* overexpression resulted in a decrease of *ASH1* mRNA localization efficiency and in a reduction of Ash1 protein levels (Irie *et al.*, 2002). It has been proposed that the anchorage deficiency observed for *khd1* disrupted cells is caused by the inhibition of translation of *ASH1* mRNA. Translation dependent *ASH1* mRNA anchorage to the bud tip has been observed earlier (Gonzalez *et al.*, 1999), which would fit nicely to the model proposed (Irie *et al.*, 2002).

Recently, this model has been confirmed. Not only has it been shown by immunoprecipitation that Khd1p binds to a part of eIF4Ep (Paquin *et al.*, 2007) and to eIF4G (Gavin et al., 2006), indicating that it might be connected to a eIF4E translation regulation event, but also the loss of Khd1p leads to an accumulation of *ASH1* mRNA in the heavier polysome fractions, indicating that it is Khd1p that somehow prevents the transition from the lighter polysome fractions to the heavier ones, thus blocking translation (Paquin *et al.*, 2007). Furthermore, the fact that Khd1p can be phosphorylated and thus release *ASH1* mRNA to be translated strengthens the translation repressor model (Paquin *et al.*, 2007).

Khd1p, similarly to She2p, is steady state localized in the cytoplasm but is a shuttling protein, and can be trapped in the nucleus in an mRNA dependent manner, when using a *MEX67* temperature sensitive allele (Du *et al.*, 2008). This fact, and localization data for Puf6p and Loc1p, points out that some localization factors have a transient nuclear localization, although being involved in a cytoplasmic process.

1.13 Aim of this work

The aim of this work is to elucidate the role of Khd1p in *ASH1* mRNP architecture and to elucidate its function in the *ASH1* mRNA localization process. In order to achieve it, a biochemical approach was designed that includes protein purification and interacting partner identification.

2. MATERIAL

2.1 PLASMIDS LIST:

Number	Construct
	PRS416-*XRS2*
1617	PRS416-*RAD52*
1620	PRS425-pADH1-YKU70
1621	PRS423-pADH1-YKU80
1063	pGAL-*ASH1*-MS2 on a *HIS3* marker
1213	PFA6a-natNT2
1214	PYM13 ProtA-TEV-CaBP, kanMX4
1407	YCplac133-*KHD1*
1417	p415-GAL1-*ASH1*-E1Stop (LEU2)
1438	PRS424-*TLC1*
1461	p413-GAL-HA6-*SHE3*-Cterm
1515	pJET-*KHD1*
1518	pGAL-*KHD1*-HA6
1525	pJET-*KHD1*-pointmutant1A-I59R
1526	pJET-*KHD1*-pointmutant1B-I68R
1527	pJET-*KHD1*-pointmutant2B-I183R
1528	pJET-*KHD1*-pointmutant3C-L284R
1529	pRS315-*KHD1*
1530	pRS315-*KHD1*-point mutant1A-I59R
1531	pRS315-*KHD1*-point mutant1B-I68R
1532	pRS315-*KHD1*-point mutant2B-I183R
1533	pRS315-*KHD1*-point mutant3C-L284R
1570	pJET-*SIR2*
1591	pRS424-*SIR2*
1599	pRS314-*SIR2*
1601	pRS314-*KHD1*
1602	pRS314-*KHD1*-pointmutant3C-L284R
1615	pRS424-*KHD1*
1616	pRS424-*KHD1*-pointmutant3C-L284R

Number	Construct
254	p415-*GAL1-ASH1* (LEU2)
276	pYM2 3xHA, S.p.HIS
277	pYM3 6xHA, K.l.TRP1
279	pYM5 3xMYC, S.p. HIS
280	pYM6 9xMyc, K.l. TRP1
285	pYM11 TEV-GST-7HIS, kanMX4
413	pRS315 (LEU2)
46	YEplac-*ASH1-Myc9*
700	pRS424
701	pRS314
741	pGPD-NLS-HA-MS2-RFP on a leu2 marker
88	YEplac-*ASH1*
909	SL plasmid-*KHD1*
287	YEplac181-ASH1-STOP-E1
113	p415-pGAL
135	pFA6-S.p.HIS3MX6
1618	pRS426-CLB1
1619	pRS426-CLB6

2.2 YEAST STRAINS:

Number	**Genotype**	**Origin**
JBL white...		Katja Straesser
RPY126	*Mat alpha, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1, SHE2::URA3*	Jansen *et al.*, 1996
RPY2000	*RPY585 scp160::TRP1 + 254*	Jaedicke, PhD Thesis
RPY2017	RPY2049 *puf6::kanMX4*	Euroscarf
RPY2049	BY4741 *Mat a, his3 delta 1, leu2 delta 0, met15 delta 0, ura3 delta 0*	Euroscarf
RPY2172	*Mat alpha, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1, SHE2-myc3 Eap1-HA6::K.l. TRP1 (Knop)*	Jaedicke, PhD Thesis
RPY2209	*RPY668 + 254*	This study
RPY2210	*RPY668 + 1417*	This study
RPY2211	*RPY671 + 254*	This study
RPY2212	*RPY671 + 1417*	This study
RPY2213	*RPY676 + 254*	This study
RPY2214	*RPY676 + 1417*	This study
RPY2215	*RPY585 + 1417*	This study
RPY2220	*RPY585 + 254*	This study
RPY2368	*MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ KHD1::HIS3MX6*	This study
RPY2448	*RPY2017 + 254*	This study
RPY2449	*RPY2017+ 1417*	This study
RPY2450	*RPY585 UPF1::TRP1*	This study
RPY2451	*RPY2450 + 254*	This study
RPY2452	*RPY2450 + 1417*	This study
RPY2460	*RPY2368 + 254*	This study
RPY2461	*RPY2368 + 1417*	This study
RPY2466	*RPY676 upf1::TRP1*	This study
RPY2467	*RPY2466 + 254*	This study
RPY2468	*RPY2466 + 1417*	This study
RPY2471	*RPY676 vts1::TRP1*	This study
RPY2482	*RPY2471 + 254*	This study

Number	**Genotype**	**Origin**
RPY2483	*RPY2471 + 1417*	This study
RPY2492	*RPY585 vts1::TRP1*	This study
RPY2493	*RPY2492 + 254*	This study
RPY2494	*RPY2492 + 1417*	This study
RPY2501	*RPY676 PUF3::TRP1*	This study
RPY2502	*RPY585 PUF3::TRP1*	This study
RPY2503	*RPY2502 + 254*	This study
RPY2506	*RPY2501 + 254*	This study
RPY2507	*RPY2501 + 1417*	This study
RPY2521	*RPY585 ccr4::TRP1*	This study
RPY2522	*RPY2521 + 254*	This study
RPY2523	*RPY2521 + 1417*	This study
RPY2525	*RPY676 ccr4::TRP1*	This study
RPY2526	*RPY2525+ 254*	This study
RPY2527	*RPY2525 + 1417*	This study
RPY2537	*Mat a, his3 delta 1, leu2 delta 0, met15 delta 0, ura3 delta 0, XRN1::KANMX*4	Roy Parker (euroscarf)
RPY2538	*Mat a, his3 delta 1, leu2 delta 0, met15 delta 0, ura3 delta 0, upf3::kanMX4*	Roy Parker (euroscarf)
RPY2539	*Mat a, his3 delta 1, leu2 delta 0, met15 delta 0, ura3 delta 0, upf1::kanMX4*	Roy Parker
RPY2541	*Mat a, his3 delta 1, leu2 delta 0, met15 delta 0, ura3 delta 0, upf2::kanMX4*	Roy Parker (Euroscarf)
RPY2544	*Mat alpha,, ura3-52, leu2-2 112, trp1-delta1, cup1::LEU2/PM, dcp1::URA3, lys2*	Roy Parker
RPY2544	*Mat alpha,, ura3-52, leu2-2 112, trp1-delta1, cup1::LEU2/PM, dcp1::URA3, lys2*	Roy Parker
RPY2591	*RPY2541 ash1::HIS3MX6*	This study
RPY2592	*RPY2591 + 254*	This study
RPY2593	*RPY2591 + 1417*	This study
RPY2595	*RPY2538 ash1::HIS3MX6*	This study
RPY2596	*RPY2595 + 254*	This study
RPY2597	*RPY2595 + 1417*	This study
RPY2599	*RPY2537 ash1::his3MX6*	This study
RPY2600	*RPY2599 + 254*	This study
RPY2601	*RPY2599 + 1417*	This study

Number	**Genotype**	**Origin**
RPY2603	*Mat alpha,, ura3-52, leu2-2 112, trp1-delta1, cup1::LEU2/PM, dcp1::URA3, lys2 she2::KANMX6*	This study
RPY2603	*Mat alpha,, ura3-52, leu2-2 112, trp1-delta1, cup1::LEU2/PM, dcp1::URA3, lys2 she2::KANMX6*	This study
RPY2608	*mat a, leu2-3,112, trp1, ura3-52, his4, cup1::LEU2/PGK1pG/MFA2pG DHH1-GFP-Neo she2::URA3*	This study
RPY2655	*mat a, leu2-3,112, trp1, ura3-52, his4, ade-SHE2-URA3 DCP1-GFP-Neo*	This study
RPY2665	*MAT??, ade???, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ SHE2::URA3 KHD1::HIS3MX6*	This study
RPY2666	*MAT??, ade???, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ ASH1::URA3 KHD1::HIS3MX6*	This study
RPY2667	*RPY2665+ 254*	This study
RPY2668	*RPY2665 + 1417*	This study
RPY2670	*RPY2666+ 254*	This study
RPY2671	*RPY2666 + 1417*	This study
RPY2687	*mat ?, leu2-3,112, trp1, ura3-52, his4, ade+ DHH1-GFP-Neo*	This study
RPY2688	*mat ?, leu2-3,112, trp1, ura3-52, his4, ade+ DCP1-GFP-Neo*	This study
RPY2689	*RPY2688 + 741*	This study
RPY2690	*RPY2689 + 1063*	This study
RPY2691	*RPY2687 + 741*	This study
RPY2692	*RPY2691 + 1063*	This study
RPY2693	*RPY2655 + 741*	This study
RPY2694	*RPY2693 + 1063*	This study
RPY2695	*RPY2608 + 741*	This study
RPY2696	*RPY2608 + 741 + 1063*	This study
RPY2698	*Mat a, his3 delta 1, leu2 delta 0, met15 delta 0, ura3 delta 0 PUF6::KANMX4 SHE2::URA3*	This study

Number	**Genotype**	**Origin**
RPY2911	*MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ KHD1-TAP::KANMX6*	This study
RPY2913	*Mat a, his3 delta 1, leu2 delta 0, met15 delta 0, ura3 delta 0, puf6::kanMX4 khd1::his3*	This study
RPY2915	*UCC506* *mat a ade2-101 his3-delta200 leu2-delta1 lys2-801 trp1-delta1 ura3-52* *URA3::TEL VR*	Xavier Marsellach *et al.*, 2006
RPY2915	UCC506 mat a ade2-101 his3-delta200 leu2-delta1 lys2-801 trp1-delta1 ura3-52 URA3::TEL VR	Gottschling *et al.*, 1990
RPY2920	RPY2915 *khd1::S.p.HIS3*	This study
RPY2922	RPY2915 *rpd3::LEU2*	Marsellach *et al.*, 2006
RPY2923	RPY2915 *rif1::TRP1*	Marsellach *et al.*, 2006
RPY2924	*RPY2915 RIF2::LEU2*	Marsellach *et al.*, 2006
RPY2925	*RPY2915 RIF1::TRP1 RIF2::LEU2*	Marsellach *et al.*, 2006
RPY2926	RPY2923 *khd1::S.p.HIS3*	This study
RPY2927	*RPY2924 KHD1::S.p.HIS3MX6*	This study
RPY2929	*RPY2925 RIF1::TRP1 RIF2::LEU2 KHD1::HIS3*	This study
RPY2933	*MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+ KHD1-GST::KANMX6*	This study
RPY2961	*ucc506 RIF1::TRP1 KHD1::HIS3MX6 PBP2::ClonNATNT2*	This study
RPY2962	*RPY2927 PBP2::ClonNATNT2*	This study
RPY2963	*RPY2923 PBP2::ClonNATNT2*	This study
RPY2972	*RPY2920 PBP2::ClonNATNT2*	This study
RPY2988	*RPY2923 SIR3::ClonNATNT2*	This study
RPY2989	*RPY2926 SIR3::ClonNATNT2*	This study
RPY2990	*RPY2927 SIR3::ClonNATNT2*	This study
RPY2991	*RPY2920 SIR3::NAT2*	This study
RPY2993	*RPY2915 SIR4::S.pombe HIS3*	This study
RPY2994	*RPY2923 SIR4::ClonNATNT2*	This study
RPY3033	*SIR4::S.pombe HIS3 diploid*	Heidi Feldmann
RPY3036	*RAD52::KANMX4*	Euroscarf
RPY3037	*XRS2::KANMX4*	Euroscarf

Number	**Genotype**	**Origin**
RPY3038	*RAD52::KANMX4 KHD1::S.pombeHIS3*	This study
RPY3039	*XRS2::KANMX4 KHD1::S. pombe HIS3*	This study
RPY3040	*EAP1-HA6::TRP1 SHE2-MYC3 KHD1-TAP::KANMX6*	This study
RPY3041	*SIR4::S.pombe HIS3 KHD1::ClonNATNT2*	This study
RPY3046	*XRS2::KANMX4 KHD1::S.p.HIS3*	This study
RPY3047	*rad52::KANMX4 KHD1::S.p.HIS3*	This study
RPY3091	*rad52::KANMX4*	Euroscarf
RPY3120	*xrs2::KANMX4*	Euroscarf
RPY3135	RPY358 *Sir2p-Myc9::K.l.TRP1*	This study
RPY3154	RPY2922 *khd1::S.p.HIS3*	This study
RPY3154	RPY2922 *khd1::S.p.HIS3*	This study
RPY3155	*RPY2922 pbp2::S.p.HIS3*	This study
RPY3159	*RPY2915 yku70::S.p.HIS3*	This study
RPY3160	RPY2923 *rad27::S.pHIS3*	This study
RPY3167	RPY3160 *khd1::ClonNATNT2*	This study
RPY3172	RPY2922 *sir2::ClonNATNT2*	This study
RPY3174	*RPY2922 rad27::ClonNATNT2*	This study
RPY3176	*RPY2915 rad27::S.p.HIS3*	This study
RPY3177	*RPY2920 rad27::ClonNATNT2*	This study
RPY3182	RPY2915 *sir2::ClonNATNT2*	This study
RPY3186	RPY3135 *khd1::S.p.HIS3*	This study
RPY3187	*RPY3159 khd1::ClonNATNT2*	This study
RPY3219	RPY2915 *pbp2::ClonNATNT2*	This study
RPY3245	RPY3135 *pbp2::ClonNATNT2*	This study
RPY3286	*RPY3177 + 1529*	This study
RPY3288	RPY2920 + *1529*	This study
RPY3290	*RPY3187 + 1529*	This study
RPY3299	*RPY3177 + 1532*	This study
RPY3300	*RPY3177 + 1533*	This study
RPY3301	*RPY3187 + 1532*	This study
RPY3302	*RPY3187 + 1533*	This study
RPY3303	RPY2920 + *1532*	This study
RPY3304	RPY2920 + *1533*	This study
RPY3313	*RPY3159 + 297*	This study

Number	**Genotype**	**Origin**
RPY3314	*RPY3187 + 297*	This study
RPY3317	*RPY3177 + 413*	This study
RPY3317	RPY2920 + *297*	This study
RPY3318	*RPY3176 + 413*	This study
RPY3323	RPY2915 + *1438*	This study
RPY3324	RPY2920 + *1438*	This study
RPY3325	*RPY3159 + 1438*	This study
RPY3326	*RPY3187 + 1438*	This study
RPY3327	*RPY3176 + 1438*	This study
RPY3328	*RPY3177 + 1438*	This study
RPY3333	*RPY359 SIR3-Myc9::K.l.TRP1*	This study
RPY3341	*RPY3159 sir2-myc9::TRP1*	This study
RPY3342	*RPY3159 sir3-myc9::TRP1*	This study
RPY3343	RPY2922 + *1438*	This study
RPY3344	RPY3154 + *1438*	This study
RPY3348	RPY2915 *sas2::S.p.HIS3*	This study
RPY3353	RPY2915 *vts1::ClonNATNT2*	This study
RPY3359	RPY2922 *sas2::S.p.HIS3*	This study
RPY3394	*RPY612 Vts1-Myc9::K.l.TRP1*	This study
RPY3396	*YKU70::URA3 yku70::leu2*	Heidi Feldmann
RPY3404	*YKU70::URA3 KHD1::S.p.HIS3*	This study
RPY3407	RPY2922 *vts1::ClonNATNT2*	This study
RPY3411	RPY2915 + *1570*	This study
RPY3413	RPY2920 + *1570*	This study
RPY3415	RPY3154 + *1570*	This study
RPY3416	RPY2922 + *1570*	This study
RPY3422	RPY2915 + *700*	This study
RPY3425	RPY2920 + *700*	This study
RPY3428	RPY2922 + *700*	This study
RPY3431	RPY3154 + *700*	This study
RPY3443	RPY3154 + *1599*	This study
RPY3444	RPY2922 + *1599*	This study
RPY3445	RPY2920 + *1599*	This study
RPY3446	RPY2915 + *1599*	This study
RPY3447	RPY3407 + *1438*	This study

Number	Genotype	Origin
RPY3448	RPY3353 + *1438*	This study
RPY358	MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+	
RPY359	*MATalpha, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+*	
RPY585	*Mat a, trp1-1, leu2-3, his3-11, ura3, ade2-1, HO-ADE2, HO-CAN1. ASH1::S.pombe HIS3*	Jaedicke, PhD Thesis
RPY612	*Mat a, ade2, his3, leu2, trp1, ura3, mex67:HIS3 (pUN100-LEU2-mex67-5)*	Segref *et al.*, 1999
RPY668	*RPY585 she3::URA3*	Jaedicke, PhD Thesis
RPY671	*RPY585 myo4::URA3*	Jaedicke, PhD Thesis
RPY673	*RPY585 she4::URA3*	Jaedicke, PhD Thesis
RPY676	*RPY585 SHE2::URA3*	Jaedicke, PhD Thesis

2.3 OLIGONUCLEOTIDES LIST

Name	Oligo Sequence	Number
PUF3_KO_F	CGCATTTAAATTTCTTCTGAATAACGCAATATTGC GGGTATAACTGTGCGGTATTTCACACCG	2167
PUF3_KO_R	AAATAGTAAAAAGTGAAAGGAGAACGATGATAAC ACTAAAGATTGTACTGAGAGTGCAC	2168
natNT2 fw	AATCGGACGACGAATCGGACG	2330
KHD1kor	ATAGTCTCGATGATATTGCTATTG	1884
VTS1_KO_F	GAAAAACTGTTCATATAAAGTAATTGTCAGCAAA GAAATCCTGTGCGGTATTTCACACCG	2046
VTS1_KO_R	CTTTATGCAACGTCAAGACAATCAACTTTATTATG CCAGATAGATTGTACTGAGAGTGCAC	2047
Vts1_KOFO RKnop	GAAAAACTGTTCATATAAAGTAATTGTCAGCAAA GAAATCCGTACGCTGCAGGTCGAC	2700
VTS1_KORE VKNOP	CTTTATGCAACGTCAAGACAATCAACTTTATTATG CCAGATATCGATGAATTCGAGCTC	2701
pbp2-ko-his-ss	GCCACATCCACTCTAACAATTTTAATCGTCCAGCG CGGCAGCTGAAGCTTCGTACGC	1260
pbp2-ko-his-ass	GAGGGGCGACCTTCTTTTACGTTCAGCATTTGATC GCATAGGCCACTAGTGGATCTG	1261

Name	Oligo Sequence	Number
aKHD1-KO-HIS	GTTTTGTCTGTGTGGGACGTGCGCACGCACACGTATATAGCATAGGCCACTAGTGGATCTG	1293
sKHD1-KO-HIS	CGGGTAACTTAGAGACAGCATTAGTATATATACCAGCCCAGCTGAAGCTTCGTACGC	1292
pbp2-ko-select-ass	GTCTTCTCTCCGAAGAGCC	1265
SIR2_KNOPKOF	GGGCGTGTATGTCGTTACATCAGATGAACATCCCAAAACCCTCCGTACGCTGCAGGTCGAC	2741
SIR2_KNOPKOR	GTAAATTGATATTAATTTGGCACTTTTAAATTATTAAATTGCCTTCTACAATCGATGAATTCGAGCTC	2742
SIR2_cloningF	CCGATCGGAAGCTCTAATTTG	3048
Sir2_cloningR	CAACGCTGGACCACGACATG	3059
KHD1_KNOP_F	AAGAAGAACCTCAAGAGAATCATGATAACAAAGAGGAGCAGTCGCGTACGCTGCAGGTCGAC	2115
KHD1_KNOP_R	TTTGTTTTGTCTGTGTGGGACGTGCGCACGCACACGTATATAATCGATGAATTCGAGCTC	2116
She2_seqfor_P_300	CTTATAGAATGGTTCTTCGTGCATGCC	1813
She2_seqrev_UTR_250	CGGAGGAGACTACACCCTCCC	1815
Khd1_for	CAGTTTGCCAATATAAGCGC	1751
Khd1_rev	AATCTAGAGGAAACGCCAATAGTCTCGA	1752
KH1.1F	CATTGAAAGAGGCTGCCAAGAGGATTGGCACTAAGGGCTCCAC	2803
KH1.1R	GTGGAGCCCTTAGTGCCAATCCTCTTGGCAGCCTCTTTCAATG	2804
KH1.2F	GAGCTGCAAACGCCGTCAAGAGAGGTATTTCTGAAAAGGTGCC	2805
KH1.2R	GGCACCTTTTCAGAAATACCTCTCTTGACGGCGTTTGCAGCTC	2806
KH2.1F	CCAATTCCCATATCTCATCGCGTATCGGGAAAGCAGGCGCCAC	2807
KH2.1R	GTGGCGCCTGCTTTCCCGATACGCGATGAGATATGGGAATTGG	2808
KH2.2F	CAATAAGCACGGCGTTAAGAGGGTGGCTTCCAAGGACTTCTTAC	2809
KH2.2R	GTAAGAAGTCCTTGGAAGCCACCCTCTTAACGCCGTGCTTATTG	2810

Name	Oligo Sequence	Number
KHL-RF	GTGGCTTCCAAGGACTTCCGACCTGCTAGCGACGAGAGAATTATC	2811
KHL-RR	GATAATTCTCTCGTCGCTAGCAGGTCGGAAGTCCTTGGAAGCCAC	2812
KH3.1F	CCAGAACTGTATGTAGGCGCCAGGATTGGCCGTGGAATGAACAG	2813
KH3.1R	CTGTTCATTCCACGGCCAATCCTGGCGCCTACATACAGTTCTGG	2814
KH3.2F	GAAAACTTTCACAAAAACCAATAGGGTCGTGGAAAGGAAGGATGACGATG	2815
KH3.2R	CATCGTCATCCTTCCTTTCCACGACCCTATTGGTTTTTGTGAAAGTTTTC	2816
VTS1_KNOPTAGF	CAAAGAACGTGATTTAATTGATAGATCTGCTACGCTGCAGGTCGAC	
VTS1_KNOPTAGR	GCAACGTCAAGACAATCAACTTTATTATCGGAGATAATCGATGAATTCGAGCTC	
RIF1_KNOPF	TATTACTCAAACAGGGATAATGATATGAATTGACGTACGCTGCAGGTCGAC	3052
RIF1_KNOPR	TTTATTGCCATTTTGATCTATTCTACATACTAAATCGATGAATTCGAGCTCG	3053
RIF2_KNOPF	CTTCCACTTAAGTTAACTCGAAAAGTACATGATAGACGTACGCTGCAGGTCGAC	3054
RIF2_KNOPR	GTATTGTTCGAACTCTTTCAAAAGACCTTGGTAATATCGATGAATTCGAGCTCG	3055
SIR3_KNOPF	GAATTCAAAAATATGGACTGCATTCGTACGCTGCAGGTCGAC	2934
SIR3_KNOPR	GGAAGTGAAAATGAATGTTGGTGGATCGATGAATTCGAGCTCG	2935
PDA1_F	CTGCCAATGCTTGCTGCTT	321
PDA1_R	TCCCTAGAGGCAAAACCTTG	322
CCR4_KO_F	AGGGAACTCCGACTGACGTTATCCCTGCAAACTACCGCTACTGTGCGGTATTTCACACCG	2171
CCR4_KO_FR	TACAGAGAGGAGGGAGGGAGTGGGATGAAAGTGTGCGGTAGATTGTACTGAGAGTGCAC	2172
CCR4_KO_XEKF	CGACCCTTCTTTACTAGGC	2173
CCR4_KO_XEKR	CCGTGCCTGAGGGAGTG	2174
PUF3_KO_XEKF	GAACTCGCATCCATAGTTTC	2169

Name	Oligo Sequence	Number
PUF3_KO_XEKR	CATCTTGTTGGTTAGGAAGC	2170
vts1rsal1clon	AAAAGTCGACCTTGTACCATTCATTGTATAAAC	2121
UPF1_KO_xekf	TTCCGGTTCTCACACTCC	2050
UPF1_KO_R	AATATACTTTTTATATTACATCAATCATTGTCATTATCAAAGATTGTACTGAGAGTGCAC	2053
UPF1_KO_F	GCCAAGTTTAACATTTTATTTTAACAGGGTTCACCGAACTGTGCGGTATTTCACACCG	2052
UPF1_KO_xekr	TTGCCATTGATCAGTATCCC	2051
ASH1-Xho(+2300)	CTACTCGAGTACTAGACATAG	141
ASH1-BH1(-1)	AAGCGGATCCATGTCAAGCTTATAC	142
SAS2_KOF	TATTTTCTAGTTGCTTTTTGTTTTCACTCGCAAAAAAACGTACGCTGCAGGTCGAC	2801
SAS2_KOR	GAAATACATATGCCATTAAGTTACATCCTGAATAGATTCATCGATGAATTCGAGCTCG	2802
rad27_KOF	AAAGAAATAGGAAACGGACACCGGAAGAAAAAATCGTACGCTGCAGGTCGAC	2743
rad27_KOR	GGACCAAAAGAAGAAAGTGGAAAAAGAACCCCATCGATGAATTCGAGCTCG	2744
RAD27_rev	GAGAAACCCACTTTCTTGGAG	3060
TLC1_probF	GATGCTTGTGTGTGCGCAA	2564
TLC1_probR	GTCACCTTAAACAGTGTCAG	2565
RAP1_probF	CACTGGTGCTACTGCTGC	2560
RAP1_probR	CACCGTTTGCTCTAATCAGACGC	2561
SIR3_probF	GATGCATAAGATGGGAGTTGA	2562
SIR3_probR	TCGGCCGTCAACGAGTTC	2563
SIR2_probF	GATGAACCTTCACATAAGAAG	2948
SIR2_probR	ATGGAGGCCTTTCCGACA	2949
1800_ecoRI	CAGCCGAATTCATGTCACAGTTCTTCGAAGC	

2.4 General Laboratory Material

2.4.1 Laboratory equipment and disposables

Standard Laboratory equipment and disposables have been used. All solutions have been autoclaved or sterilized by filtration.

Standards	
Chemicals	Merck, Fluka, Serva, Sigma, ICN
Enzymes	NEB, Roche, Biomol, Axon, MBI Fermentas
Oligos	Thermo Scientific, MWG.
Kits	
Plasmid Miniprep	Metabion, Macherey-Nagel
Plasmid Midiprep	Qiagen
Gel Extraction/PCR Purification	Qiagen, Metabion
Colloidal Blue Stain	Invitrogen
QuickChange Site Directed Mutagenesis Kit	Stratagene
Prime-It II Random Primer Labelling	Stratagene
ECL Detection	Applied Biochem
RNAse Away	Molecular Bioproducts
Quick Ligation Kit	NEB
pJET Cloning Kit	Fermentas
TOPO-TA Cloning Kit	Stratagene
Special equipment	
1.Hardware	
Criterion eletrophoresis system	Bio-RAD
Ika vibrax VXR	NeoLab
Beadmill	
2.Disposables	
glass beads, 0.45-0.5mm	NeoLab
Positive TM membrane	Qbiogene
Hybond-P	Pharmacia

Hyperfilm ECL	Pharmacia
NytranN	Schleicher&Schuell
GB 003/GB 004 Gel-Blotting Paper	Schleicher&Schuell
Micro Bio-spin 30 Chromatography Columns	BIO-RAD
Mobicol, 35 ⌈m pore filters	MOBITEC

3.Reagents

Poly-L-Lysine	Sigma
Triton X-100	Gerbu
Tween 20	Sigma
IGEPAL CA-630, equals to NP40	ICN
Trichloroacetic acid, TCA	Sigma
Salmon Sperm DNA	Roche
RQ1 DNAse	Promega
Oxalyticase	Enzogenetics
IgG Sepharose	Pharmacia
Calmodulin Affinity Resin	Stratagene
Complete EDTA-Free Protease Inhibitor Cocktail	Roche
Diethylpyrocarbonate, DEPC	Roth
diamidino-2-phenylindol dihydrochloride	Roth
zymolase 20T/100T	ICN
Cycloheximide	Sigma
Paromomycin	Sigma
Hygromycin B	Sigma
Geneticin	Sigma
Hydroxyurea	Applichem
MethylMethanoSulphonate	Sigma
5' Fluorotic Acid	Apollo Scientific
RNAseA	Roche
DNAse I	Sigma
Taq Polymerase	Axon
VENT Polymerase	NEB

4. Antibodies

Primaries

rabbit anti protein A	Sigma
Peroxidase anti-peroxidase	Sigma
3F10, rat anti HA	Roche
9E10, mouse anti Myc	Roche
rabbit anti Sec61	M. Seedorf, ZMBH, Heidelberg
Mab414, mouse anti actin	Chemicon
rabbit anti Histone H4	BioLegend
rabbit anti Histone H4 Lys16	Upstate
rabbit anti Histone H4 Lys12	Upstate
rabbit anti Histone H4 Lys8	Upstate
rabbit anti Histone H3	Upstate
rabbit anti Histone H3K79Me3	Upstate
rabbit anti Histone H3K4Me3	Upstate
rabbit anti Histone H3K36Me3	Upstate
mouse anti Rpl13	M. Seedorf, ZMBH, Heidelberg
mouse anti Rps3	M. Seedorf, ZMBH, Heidelberg

Secondaries

rabbit anti rat, peroxidase conjugated	DIANOVA
sheep anti mouse, peroxidase conjugated	DIANOVA
donkey anti rabbit, peroxidase conjugated	DIANOVA
rabbit anti mouse, Alexa 488	Molecular Probes

3. METHODS

3.1 Bacteria

3.1.1 Preparation of *E. coli* competent cells.

E. coli strain: TOP10
hint: it is better to scale up the volume to get more aliquots (4fold)
1. inoculate 3ml LB-media with one colony from a fresh plate, incubate ON at 37°C on a wheel
2. the next evening take 500µl of this culture and inoculate 20 ml LB-Media in an Erlenmeyer flask, incubate ON at 37°C (shaking)
3. with this ON-culture inoculate 50 ml LB-Media the next morning with a dilution of 1:100; let cells grow at 37°C to an OD 600 0,7-0,8 (shaking)
4. cool down culture on ice for 15 min, centrifuge for 15 min at 5000 rpm in a GSA rotor
5. cool pellet on ice, resuspend with the half of the culture volume with cold 0,1M CaCl2-solution, transfer to SS34 tubes
6. incubate 30min on ice
7. pellet cells at 5000 rpm for 15 min at 4°C
8. resuspend the cellpellet with 1/20 volume of your culture volume in 0,1 M CaCl2/10% Glycerol solution, make 100µl aliquots
9.store aliquots at -80°C

3.1.2 *E. coli* transformation

quick method (high efficiency 5min transformation of E. coli)
(Nucleic Acids Research, 1996, Vol.24, No.3)

1. thaw an aliquot of competent cells slowly on ice
2. 1-10ng of DNA or 5-10µl ligationmix (which have to be transformed) have to be cooled on ice in an Eppi
3. add 50-100µl of the competent cells and incubate at least 5 min on ice
4. plate on a warm LB-Amp-plate

3.1.3 Preparation of Electro-Competent cells.

Preparation of electrocompetent cells (DH5alpha)

1. inoculate1Liter LB-Media with 10 ml of a fresh ON-culture
2. incubate the culture on a shaker up to a cell density of O.D.600= 0,5-1,0. Measure during your incubation time (takes about 2-3 hours)
3. if the cells are dense enough, incubate the culture for 15-30 min on ice, then centrifuge at 4°C with a COLD rotor for 15 min at 4000 x gmax.

4. discard SUP, resuspend pellet with 1l cold water, centrifuge like in step 3
5. discard SUP, resuspend pellet in 0,5l cold water, centrifuge like in step 3
6. discard SUP carefully (pellet is not very stable), resuspend pellet in 20 ml cold 10% Glycerol, centrifuge like in step 3
7. discard SUP, resuspend pellet in 10% glycerol up to a final volume of 2-3 ml
8. make aliquots of 100 µl, store at -80°C, durable for 6 months

3.1.4 Transformation of Electro-Competent cells.

1. prepare the cuvettes(they can be used more than one time, for re-using wash them with 0.1% SDS, then with water (3 times), after that with EtOH (2 times), then dry for 20 min, crosslink in the UV-Stratalinker
2. thaw the competent cells slowly at RT (2min), after that put them on ice
3. pipet 1-2µl ligation mix (or 1ng Plasmid-DNA) in an Eppi-tube and cool on ice, the clean and dry cuvettes also cool on ice. Add 50µl competent cells to the DNA, mix and transfer directly into the cuvette, the mix should be at the bottom of the cuvette, incubate for 1 min on ice
4. because you use the BIORAD cuvettes (0,2cm) chose the program EC2 (2,5 kV) , put the cuvette in the electroporator and pulse; if this was successful, no noise will be heard
5. add 1ml warm LB-Media (or SOC medium) to the transformationmix and transfer it with a pasteur-pipet into a fresh Eppi. incubate 30-45 min at 37 °C (shaking), plate 100µl and 900 µl on LB-Amp-plates

SOC-Medium:
1g Bactotryptone, 0,25g Yeast-extract, 166µl 3M NaCl, 125µl 1M Kcl, 44,21ml H2O, autoclave.
add:
500µl 1M MgSO4, 500µl 1M MgCl2, 4,5ml 4% Glucose

3.2 *Eukarya*

3.2.1 Transformation protocols

- **High efficiency Yeast transformation**

(adapted from Gietz and Schiestl, 1991)

-inoculate 50 ml YEPD medium with an over night culture so that the start-OD is 0.25 (check)
-let cells grow for 2 generations, if OD600 is 1 - START
-harvest the cells in a Falcon tube at 2500 rpm, 5 min
-discard SUP,resuspend the cells in 25 ml sterile water, centrifuge again
-discard the water, resuspend the cells in 1 ml 100mM LiAc (freshly diluted from stock) and transfer the suspension to an Eppi
-pellet the cells at top speed for 15 sec and remove the LiAc with a micropipette
-resuspend the cells to a final volume of 500 µl - about 400 µl of 100 mM LiAc+pellet
-boil a 1 ml sample of SS-DNA for 5 min, then quickly chill on ice
-vortex the cell suspension and pipette 50µl samples into labeled Eppis - do not forget one for the

negative control! Pellet the cells and remove the LiAc with a micropipette
-add the transformation mix in the following order:
-240µl PEG (50%w/v)
-36 µl 1M LiAc
-25 µl SS-DNA (2mg/ml)
-50 µl water and plasmid DNA (0,1-10 µg)
-vortex each tube vigorously until the cell pellet has ben completely mixed. Usually it takes about 1 min
-incubate 30 min at 30°C
-heat shock 20-25 min at 42°C (NOTE: for tranformation of pseudohyphal strains it is better to make just a 20 min heat shock, for TS mutants just a 15 min heat shock)
-centrifuge at 6000-8000 rpm for 15 sec and remove the transformation mix with a micropipette
-Pipette 1 ml of water into each tube and resuspend the pellet by pipetting it up and down (gently!!)
-plate 100 and 300 µl of the transformation mix onto selective plates (2 plates)

- **Yeast One-Step Transformation**

(modified from Chen *et al.*, *Curr Genet* 21:83-84, 1992)

1. Cells.
You should have a culture of at least 10exp8 cells/ ml. You need 1ml per transformation. They can be e. g. stationary phase cells from an YEPD culture (harvested by centrifugation in Eppendorf tubes). It works well with cells scraped off a fresh (1-2 days) plate and is reported to work (less well) even with cells from an old plate kept at 4 ºC.

2. Procedure.
a.) Thaw ssDNA ("helper DNA"), heat 5-10' at 95 C, then chill on ice.
b.) Pellet 1ml of culture by centrifugation in Eppendorf centrifuge, discard supernatant. Resuspend cells in 100 µl of ONE-STEP buffer, vortex heavily.
c.) Add 20µg ssDNA (10 µl of 2mg/ml) + 100ng - 500ng plasmid DNA to be transformed, vortex, incubate at 45 ºC for 30 min.
d.) Add 1 ml YEPD (or YEPGal), mix and spin 10 sec full speed. Discard supernatant.
e.) Resuspend cell pellet in 1000 µl YEPD (or YEPGal) and plate 100 µl directly on appropriate selective plates. Colonies appear after 2 days.

3. ONE-STEP buffer.
0.2 M LiAc, 40% PEG 3350 (is the same as old PEG 4000), 100 mM DTT. Dissolve LiAc and PEG, then add DTT, filter sterilize. Keep in 1 ml aliquots at -20 ºC.

3.2.2 Determining mRNA half-life:

Total mRNA extraction

(modified from Cross and Tinkelenberg, 1991)

- Start with cca 15 ml of YEPD cultures O.D. cca 0.7 - 0.8. (they can be obtained by diluting an overnight inoculum 0.25 ml into 20 and 4-5 h cultivation - as for spheroplasts).

- Spin cells down at 4 degrees 2000 rpm 2 min (Heraeus centrifuge), discard supernatant.
- Resuspend pellets into cca 1 ml of ice-cold TE, transfer into 2 ml safelock Eppendorf tubes. Spin for 10s in the cold and discard supernatant. You can freeze the pellet here (-20 ºC is O.K.).

To the pellet add:
1. cca 200 µl of glass beads
2. 400 µl of 50:49:1 mixture of phenol: chloroform: isoamylalcohol, equilibrated with TE
3. 500 µl of Cross RNA buffer 1

- Close the tubes. Shake vigorously on the Ika-Vibrax-VXR mixer in the coldroom for 10 min.
- Spin for 5 min in an angle microfuge and transfer the upper phase (carefully, w/o interphase!) into 1ml of precooled -20 degrees ethanol in 1.5 ml Eppendorf tubes. Mix and leave at -20 for 10 min.
- Spin in the angle microfuge in the coldroom for 5 min full speed. Dissolve sediment in 30-100µl Cross RNA buffer 2, by 10 min incubation at 65 degrees. Measure RNA concentration. Store in -20 freezer.

Cross RNA buffer1: 0.3 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 0.2 % SDS
Sterility and DEPC-treatment NOT essential since this is to be added to phenol. Keep at 4 degrees. Shake the buffer well before use and try to get as homogeneous suspension as possible.

Cross RNA buffer 2: 1x TE + 0.2 % SDS
Sterility ESSENTIAL. DEPC-treated water recommendable. Keep at room temp.

DOT BLOT

For dot blotting follow the instructions from the manufacturer - "Schleicher and Schuell Blotting Manual"

Sample preparation

- dissolve reasonable amount of RNA (10µg) in 10 µl H2O
- add 7ul 37% formaldehyde
  20 µl 100% formamide
  2 µl 20xSSC
- heat 15 minutes 68°C, cool on ice
- add 2 volumes (=78µl) of 20xSSC

Membrane preparation

- wet precut S+S Nytran for 10 minutes in H2O, then transfer for 10 minutes to 6xSSC

Blot assembly

- clean each component of the blotter that is going to be in direct contact with the sample with

‘RNase away’
- make sure that the plastic rings under each well of the top plate fit correctly
- place 1 sheet of GB003 blotting paper prewet in 6xSSC on filter support plate and roll out possible air blobs with a pipet
- cover with equilibrated membrane, roll out possible air blobs with a pipet and carefully assemble the clamps in antiparallel direction

Sample application

- connect blotter with water pump and apply vacuum to a flow rate of approximately 1ml per minute per well
- wash wells 2x500 µl 6xSSC
- apply sample
- wash 1x500 µl 6xSSC
- carefully open clamps in antiparallel direction and remove the membrane
- crosslink RNA to the membrane using twice the ‘autocrosslink program’ from the STRATALINKER.
- stain blot with methylene blue solution

Hybridization and washing

HYBRIDISATION

use 8.7ml of hybridization solution per 100cm2 of filter

Prehybridization:

1.4ml H2O (0.46 vols.)
14µl SpermDNA (10mg/ml) (0.0046vols.)
heat at 95°C for 7min
rapidly chill on ice
add 1.6ml Scp/Sarc/DS Mix (0.54vols.)

add the solution to filter in hybridisation-tube, wet the filter thoroughly by rolling the tube.
Incubate at 65°C for 1-2h

meanwhile prepare the hybridization-solution:
1.4ml H2O (0.46 vols.)
14µl SpermDNA (10mg/ml) (0.0046vols.)
heat at 95°C for 7min
rapidly chill on ice
add 1.6ml Scp/Sarc/DS Mix (0.54vols.)

Before adding your probe to the hybridisation-solution, heat your probe at 95°C for 5min.
Add the hybridisation-solution to your filter in the tube and incubate it overnight.

WASHING AND AUTORADIOGRAPHY

After removing the probe wash the filter 2x briefly with 2xSSC, 0.1% SDS at RT and then 2x 20 min in 0.5x SSC, 0.1% SDS at 43 degrees. Rinse in 3mM Tris-HCl pH 8 at RT, place your filter between 2 overhead-foils and autoradiograph with intensifying screen

Solutions:

10xMOPS: to 800 ml of DEPC-treated water add 48.1 g MOPS, adjust pH to 7 with NaOH (the "pH-strip" accuracy is sufficient) (that is app. 25.005 mL of 5 M stock), add 16.6 ml of DEPC-treated 3M Na acetate and 20.0 ml of DEPC-treated 0.5 M EDTA pH 8, adjust volume to 1l w. DEPC-treated water and filter. Keep at RT in a bottle wrapped in aluminium foil. The solution can turn yellow, but it does not matter.

1x MOPS: by dilution of 10x MOPS.

Loading buffer: 1mM EDTA pH8, 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol.

20x Scp:
2M NaCl, 0.6M Na2HPO4, 0.02M EDTA, ph 6.2 (with HCl), autclave

Scp/Sarc/DS:
Dissolve 20g of Dextran sulfate (500,000) in 60ml of 20x Scp, making up to 101ml with H2O (this will need gentle heating and stirring). Then make up to a final vol of 108ml with 7ml of 30% SLS.

Probe preparation

ASH1 probe
Template – plasmid RPP88
Expected product size - 1Kb

SIR2 probe
Oligo 1 - 2948
Oligo 2 - 2949
Template – Genomic DNA
Expected product size - 0,8kb

TLC1 probe
Oligo 1 - 2564
Oligo 2 - 2565
Template – Genomic DNA
Expected product size - 300pb

RAP1 probe
Oligo 1 - 2560
Oligo 2 - 2561
Template – Genomic DNA

Expected product size - 500pb

SIR3 probe
Oligo 1 - 2562
Oligo 2 - 2563
Template – Genomic DNA
Expected product size - 500pb

PDA1 probe
Oligo 1 - 321
Oligo 2 - 322
Template – Genomic DNA
Expected product size - 1,2kb

Detection

- Scan intensifying screen on Typhoon scanner.

3.2.3 Determining protein content

Very quick western extracts

- have ready 10 OD-units of your culture
- spin down cells 5 min at 4000 rpm
- heat 1x SDS-sample buffer at 80°C
- wash the cell pellet once with 1 ml cold water and transfer to an Eppi (WORK ON ICE!!!)
- spin down 1min at full speed
- discard Supernatant, ressuspend pellet in 200 µl 1xSDS sample buffer
- vortex 30 sec
- add 100µl glassbeads
- vibrax 1 min full speed
- 2 min at 95°C
- vibrax 1 min full speed
- 2 min at 95°C
- spin down 5 min @ full speed
- take SUP and load 15µl (= 0,75 OD-units) on a SDSgel

Whole cell extracts

- Start with 15-20 OD600 units of exponentially growing cells.
- Spin down cells (2minutes at 3000rpm in Heraeus). Resuspend cell pellet in 1ml H20 and transfer to Eppendorf tube. Spin down cells 10-15sec at full speed, discard supernatant. You may snap-freeze the pellet at this point in liquid N2 or on dry ice and store at -80 ºC.
- Work on ice.
- Resuspend the sediment in 2x its volume (i.e. 300-400 µl for 20 OD units) of the breaking buffer: 50 mM Tris pH 7.5, 1 mM EDTA, 20 mM DTT, 100X protease inhibitor cocktail (store the buffer

without inhibitors in aliquots at -20 ºC).
- Add a bit less than an equal volume of acid-washed glass beads (there should be some liquid above the beads) and mix on the IKA vibrax at 4 ºC for 2x 3-5 min with a 2 min break on ice.
- Take 10 µl of the extract for protein determination. Spin this aliquot (5 min max. speed), take 0.2 µl (i.e. 1 µl of 1:5 dilution) and 1 µl for the BioRad protein assay (protocol 15).
- Transfer 120µl of cell extract to new tube and add 40µl of 4xSDS loading buffer, boil for 5 min (or 95 ºC).
- Spin down, then take the supernatant. Load 70 µg (minigel) to 150 µg (large pocket gel) /lane. Or load 15 or 20µl, respectively, if you have not determined protein conc.
- Store the rest at -20 ºC.

3.2.4 Tandem Affinity Purification (TAP)

1. Yeast Culture
- inoculate 3 ml YPD with single clone from plate
- grow overnight at 30°C (wheel)
- dilute culture to OD600 = 0.2
- grow during the day at 30°C
- inoculate 2 l YPD (in 5 l flask) with 1 ml of culture
ON @ 30°C, 130 rpm to OD600 = 3.5 – 4.0
- harvest cells by centrifuging 5 min at 5000 rpm at 4°C
- resuspend P in 20 ml water, transfer to 50 ml Falcon
- spin 5 min at 4000 rpm at 4°C

2. Cell Lysis
- add to harvested cell pellet (about 20 ml)
- add 20 ml Lysis Buffer (LB) (prepare 500 ml)
  50 mM Tris Hcl pH7.5
  100 mM NaCl
  1.5 mM MgCl2
  0.15% NP-40
- add 40 ml acid-washed glass beads
- bead mill (default program, approx. 15 min)
- remove glass beads by filtering (50 ml syringe)
- spin 1 h at 20,000 g (13000 RPM, SS-34) at 4°C;
- remove fatty top layer with waterpump (important! fat clumps IgG beads)
- transfer lysate to 15 ml Falcon tube
+ glycerol (cf = 5%)
freeze lysate in liquid N2, store at -80°C

3. Purification
3.1.IgG beads
- wash 0.2 ml packed IgG beads (0.4 ml 50% slurry) with cold LB
- thaw cell lysate at 37°C (waterbath)
- put on ice
- remove aliquot T(otal)
- add beads to lysate
- incubate 1 h at 4°C (wheel)

- spin 3 min at 1800 rpm, at 4°C
- remove aliquot “FT”
- remove supernatant to Vf = 0.8 ml (waterpump)
- transfer beads to Mobicol with luerlock cap (closed at bottom)
- fix 20 ml syringe to Mobicol
- wash Mobicol with 10 ml LB + 0.5 mM DTT (gravity flow)
- close Mobicol

3.2.TEV cleavage
- add 150 µl LB + 0.5 mM DTT
- add 4 ul TEV (1 mg/ml)
- mix by inverting
- incubate 1 h at 16°C (wheel)

3.3.Calmodulin (CaM) beads
buffer preparation:
- 1 ml LB + 4 mM CaCl2 + 1 mM DTT
- 25 ml LB + 2 mM CaCl2 + 1 mM DTT

bead preparation: wash 250 µl packed beads 3 × with LB + 2 mM CaCl2 + 1 mM DTT (centrifuge always 2 min at 1800 rpm)
transfer beads to new Mobicol
- let excess buffer drop out
- close new mobicol
- prepare 150µl LB + 4 mM CaCl2 + 1 mM DTT

TEV Elution:
- Spin Mobicol 1 min at 2000 rpm, at 4°C (in 1,5 ml tube)
- add 150 µl of TEV eluate to second Mobicol, keep rest of TEV eluate at -20°C
- mix by inverting
- incubate for 1 h at 4°C (wheel)
- connect Mobicol to 10 ml syringe
- wash with 5 ml LB + 2 mM CaCl2

4. Elution
- close Mobicol
- add 600 µl Elution Buffer
  10 mM Tris/HCl pH 8.0
  5 mM EGTA
  (adjust pH to 8.0 !)
  and mix by inverting
- shake for 10 min at 37°C (600 rpm Eppendorf Thermomixer)
- To elute, spin 1 min at 2000 rpm in 1.5 ml tube
- add cold TCA (cf=10%)
- leave 15 min on ice
- spin 15 min at 13000 rpm, at 4°C
- remove Supernatant
- ressuspend pellet in 25-50 µl 1× SDS-LD
- neutralize with 1 M Tris Base

3.2.5 Determining Telomeric Silencing

- 10-fold serial dilutions from an overnight culture were plated out on YEPD plates, 5'FOA containing plates and SDC-URA and incubated at 30 and 37 degrees for 2-5 days.

λ**Telomeric Length analysis:**

Genomic Extraction

–Harvest 10ml of an overnight culture.
–Centrifuge for 5 minutes, 4000 RPM, at room temperature.
–Ressuspend pellet in 0,5ml H2O.
–Centrifuge at maximum speed for 5 seconds and remove supernatant.
–Vortex pellet.
–To the pellet add 200 µl Breaking Buffer, 200 µl Glass Beads, 200 µl Phenol-Chloroform-Isoamylic acid.
–Vortex hard for 3-4minutes.
–Add 400⌈l TE, vortex carefully.
–Remove aqueous phase into a new tube.
–Add 1ml 96% Ethanol.
–Centrifuge maximal speed for 3 minutes, at room temperature.
–Remove supernatant and dry pellet.
–Ressuspend in 50 µl H2O.

Restriction Digestion of the genomic DNA

- Digest extracted DNA overnight with *Xho* I and add RNAseA (µg/µl).

Southern Blot

- Load digested samples onto 0.8% agarose gel containing EtBr in the gel and in the running buffer (1XTAE). Run at no more than 100V. Takes between 2 and 5h. It is best to have 32P-endlabelled Lambda markers. If your DNA is a bit dilute, then you might consider digesting 10 µl. Take a picture of the gel.

<u>BLOTTING:</u>

- De-purinate by shaking in 0.5 liter 0.25 M HCl (1 to 50 dilution of conc HCl) for 20 min. The bromophenolblue dye should turn yellow.
- Rinse in H2O.
- Denature by shaking the gel(for 2 X 20 min) in 0.5 liter denaturing solution: 0.5M NaOH 1.5 M NaCl (20g NaOH and 87g of NaCl in 1 litre).
- Set up blotting with 5 sheets of Whatman 3MM folded around a glass plate sitting in a shallow

plastic container with a shallow reservoir of denaturing solution in the bottom.
- Place the gel upside down on the filter paper (avoiding air bubbles), surround the gel with parafilm so that there are no short circuits. Put a pre-wetted Hybond N+ filter on the gel (pre-wet in H2O followed by soaking in denaturant and blot excess liquid off) and then put a couple of sheets of semi-dry/prewetted and blotted dry whatman 3MM paper on the membrane filter. Remove all air bubbles by rolling a 25ml pipet over the sheets. Then put several stacks of hand towels on top and leave to blot for 2-3 hours. When unmounting the blot, note that the membrane should be marked with a pencil so that you know which side the DNA is on.
- Rinse the membrane in 2x SSC for 1 min, blot dry on filter paper (3MM). Cross link immediately after drying by exposing the DNA side of the filter in a Stratalinker (AUTO program).

Preparation of Probe

–Telomeric DNA probe
Amplified by PCR.
Template – TG-repeat-containing plasmid (Heidi Feldmann)

Hybridization and washing

- See Northern Blot.

Detection

- See Northern Blot.

3.2.6 FACS

–Harvest 2 OD600 units of logarithmically growing cells.
–Wash twice with 50mM Tris-HCl pH8.0
–Resuspend in 1ml 50mM Tris-HCl pH8.0, 70% EtOH.
–Incubate for at least 1 hour at room temperature or for up to several days at 4degrees.
–Spin down 100 µl of the suspension in a safelock tube, wash twice with 1ml 50mM Tris-HCl pH8.0 (spin at least 1 minute).
–Resuspend in 0,5ml 50mM Tris-HCl pH8.0 containing 2mg/ml RNAse A (pre activated for 15minutes at 100 degrees).
–Incubate at 50degrees for 2 hours in roll oven.
–Add 20 µl of 20 mg/ml Proteinase K stock solution (in H20) and incubate for 1 hour at 50 degrees.
–Spin down, wash with 1ml FACS buffer. Samples can be stored ON at 4degrees at this time.
–Resuspend cells in 500 µl FACS-PI and incubate for 1-2 hours in the dark at room temperature on a wheel.
–Prior to analysis, incubate for 10seconds in an ultrasonic bath.
–Prepare 1:4 dilution in 50mM Tris-HCl pH8.0 buffer.

FACS Buffer (100mL)
200mM TrisHCl pH7.5, 211mM NaCl, 78mM MgCl2

FACS-PI
180mM Tris-HCl pH7.5, 190mM NaCl, 70mM MgCl2, 50 µg/ml propidium iodide.

3.2.7 Immunofluorescence

Cell preparation
- Grow overnight culture.
- Dilute to OD600=0.3 and let grow until OD600=1
- Split culture into two.
  - To one culture add 10% final volume of Formaldehyde and incubate 1h at 30 degrees. Thereafter on ice.
  - The other culture place to 37 degrees for 2hours, in a shaking waterbath.

- After the 2 hours at 37 degrees, add 10% final volume of formaldehyde. Keep for 15 minutes more at 37 degrees and change to 30 degrees for 45 minutes.
- Spin both cultures for 3minutes at 3000RPM.
- Remove supernatant and ressupend pellet in spheroblasting buffer.
- Wash 3 times.
- Add spheroblasting buffer + beta-mercaptoetanol + zymolase 100T and incubate for 45 minutes at 30 degrees.
- Wash once with spheroblasting buffer
- Ressuspend in 250 µl, aliquot and freeze.

Slide preparation and protocol
- coat slide wells with 10ul Poly-L-Lysine (1:5) during 5 minutes, soak off.
- Add 10 µl H2O for 5 minutes and let dry.
- Wash wells with PBS+0,1% BSA for 5 minutes.
- Add 10 µl first antibody containing solution, anti-myc 9E10, 1:300 diluted in PBS+0,1% BSA. Incubate for two hours in a wet chamber.
- Wash three times with PBS/BSA/TritonX 0,1%, each 1 minute.
- Add 10ul of secondary antibody solution, Alexa488-coupled goat anti-mouse, for 1 hour in a wet chamber and kept in the dark.
- Wash three times with PBS/BSA/TritonX 0,1%, each 1 minute.
- Add 10 µl Hoechst for 15minutes and suck off.
- Wash once with 1xPBS.
- Mount with 80% Glycerol in DEPC.

3.2.8 UltraViolet CrossLinked Immuno-Precipitation (CLIP)

Day 0
- grow overnight the cultures
Day 1
- harvest, crosslink, lyse, IP, label, SDS-PAGE

- blot overnight
Day 2
- expose

Controls and the expected result when compared to protein of interest:

- no tagged protein: no labelling
- tagged protein + no crosslinking: absence or reduce labelling
- treatment with different RNase concentrations: change in the mobility of the RNA-protein complex expected, shifted up when lower amount or no RNase is used
- the band obtained by autoradiography should NOT fit EXACTLY with the band seen in Western Blot from the very same membrane. The reason is that not all protein gets crosslinked and only the crosslinked ones would give a shift. This shifted band would not be visible in Western blot.

Protocol:

Sample preparation (volumes for 200 ml culture)

- grow o/n. Dilute to OD 600/ml = 0.15 with fresh media. Grow until OD 600/ml = 0.5. Use 200 ml culture/testing condition.
- Pellet cells at 4°C and wash once with cold 1x PBS
- Resuspend in 10 ml (final volume) of cold 1x PBS and plate them into a precooled 10 cm Petri dish. place the Petri dish in an ice-water bath
- Crosslink in UV-crosslinker (Spectrolinker XL-1500 from Spectronics Corporation) 2x at 4500x100 microJ per square cm at 15 cm distance from the lamps on an ice-water bath. Shake vigorously the petri dish between crosslinking cycles.
- Recover all cells from the Petri dish with the help of cold 1x PBS. Pellet the cells at 4°C.
- Resuspend the cells in 400 microliter of 1x PXL buffer (cells can be stored at -80°C at this point)
- Add protease inhibitors while thawing the extract
- Add 250 microliter glass-beads. Lyse cells in vibrax for 3 cycles of 3 min on and 3 min off at 4°C.
- Spin cells at 3600 rpm in microfuge (1200 xg aprox.) for 3 min at 4°C. Take sup.
- Optional: Add 400 microliter of 1xPXL buffer + Protease inhibitors. Vibrax 1 x 3 min on. Spin in microfuge as before. Pool both sups.
- Add 8 microliter RNasIn (Promega) and 8 microliter RQ1 DNase (Promega). Incubate at 37°C for 15 min in a Thermomixer at 1000 rpm
- Dilute T1 RNase (Roche, 100 U per microliter) 1:40 in 1x PXL buffer. Add 12 microliter and incubate at 37°C for 10 min in a Thermomixer at 1000 rpm.
- Spin at 90000 rpm (360000xg aprox.) for 25 min and 4°C in RP120AT rotor in Polycarbonate tubes. Take sup (see note 6)
- Perform IP

IP (for Khd1-TAP)

- Add 30 microliter of equilibrated IgG-Sepharose and resuspend to 1:1 in 1x PXL buffer. Incubate for another 1 h at 4°C in a rotating wheel.
- Wash 2x with 1x PXL buffer and 3x with 1x PNK+ buffer
- Label RNA fragments

Labelling

- Resuspend beads in 100 microliter of 1x PNK+ buffer (remove 10 microliter for checking IP) and add 5 microliter of gamma-32P-ATP (Amersham) and 5 microliter of PNK enzyme RNase free.
- Incubate in Thermomixer at 37°C and 1000 rpm for 20 min
- Finish reaction by adding 10 microliter of 1 mM ATP. Let the reaction go another 5 min. Take 2 microlier and count them with Cherenkov program in scintillation counter (TOTAL)
- Wash 4x with 1xPNK+ buffer
- Prepare for SDS-PAGE

SDS-PAGE (Bis/Tris gel)

- Resuspend beads in 30 microliter of 1x PNK+ buffer and 30 microliter of Novex loading buffer.
- Incubate at 70°C fro 10 min at 1000 rpm. Take 2 microliter and count them in scintillation counter with cherenkov program (IP)
- Isolate beads and take sup for loading. count beads in scintillation counter with cherenkov program (Beads)
- load 2 wells per tube
- run gel following manufacturer conditions in 1xMOPS/SDS buffer (200V for about 1h)

Gel transfer

- Transfer to S&S BA-85 nitrocellulose using a wet transfer apparatus following manufacturer instructions in 1x Novex transfer buffer + 20% methanol (p.a.)
- Blot overnight at 30V and 90 mA in a Bio-Rad Mini Trans-Blot wet transfer unit without cooling unit and stirring the buffer
- After transfer, rinse the membrane in 1x PBS, and gently blot on Kimwipes. Wrap membrane in plastic wrap
- Expose to film with 2 intensifying screens at -80°C. Signal varies from experiment to experiment. I use to expose 3 films simultaneously and develop them at different exposition times

Buffer composition for the CLIP assay

20x PBS (treat with DEPC)
+ 2.74 M NaCl, (160 g/l)
+ 53.6 mM KCl, (4 g/l)
+ 202.9 mM Na2HPO4, (36 g of Na2HPO4xH2O per liter)
+ 29.4 mM KH2PO4 (4.8 g/l)

1x PXL (prepare with DEPC treated water), for 50 ml
+ 1x PBS (without Mg++ or Ca++) , 2.5 ml 20xPBS
+ 0.1% SDS, 250 microliter 20%SDS
+ 0.5% deoxycholate, 2.5 ml 10%DOC
+ 0.5% NP-40 2.5 ml 10%NP40
+ DEPC water, 42.25 ml

1x PNK+ or PNK (prepare with DEPC treated water), for 50 ml
+ 50 mM Tris-HCl pH 7.4, 2.5 ml 1M Tris
+ 10 mM MgCl2, 500 microliter 1M MgCl2
+ 0.5% NP-40, 2.5 ml 10% NP40
+ DEPC water, 44.5 ml

1x PNK + EGTA (prepare with DEPC treated water)
+ 50 mM Tris-HCl pH 7.4
+ 20 mM EGTA
+ 0.5% NP-40

20x MOPS/SDS running buffer (from Hahn's lab methods http://fhcrc.org/science/labs/hahn/)
+ 1 M MOPS (195.2 g/l)
+ 1 M Tris base (121.1 g/l)
+ 69.3 mM SDS (20 g/l)
+ 20.5 mM EDTA free acid (6 g/l)
make it with ultrapure water. 1x buffer should be pH 7.7 (do not adjust with acid or base)

20x NuPAGE transfer buffer (from Hahn's lab methods http://fhcrc.org/science/labs/hahn/), for 250 ml
+ 500 mM Bicine (81.6 g/l), 20.4 g
+ 500 mM Bis-Tris (104.64 g/l), 26.2 g
+ 20.5 mM EDTA free acid (6 g/l), 1.5 g
make it with ultrapure water. 1x buffer should be pH 7.2 (do not adjust with acid or base)

1x PK buffer (prepare with DEPC treated water)
+ 100 mM Tris-HCl pH 7.5
+ 50 mM NaCl
+ 10 mM EDTA

1x PK/7M urea buffer (this buffer must be fresh), for 10 ml
+ 100 mM Tris-HCl pH 7.5, 1 ml of 1 M
+ 50 mM NaCl, 250 microliter of 2 M
+ 10 mM EDTA, 200 microliter of 0.5 M
+ 7 M Urea, 4.2 g
+ DEPC-water up to 10 ml

10XTBE
+ 890 mM Tris, (108 g/l)
+ Boric acid, (54.1 g/l)
+ 20 mM EDTA, 40 ml 0.5 M

20% PAGE/UREA (it has to pre-run for at least 15 min)
+ 12.6 g urea
+15 ml acrylamide (40%, 19:1)
+3 ml 10XTBE
+2.5 ml DEPC-water
+300 microliter 10% APS (in DEPC-water)
+11 microliter TEMED

10% PAGE/UREA (it has to pre-run for at least 15 min)
+ 12.6 g urea
+ 7.5 ml acrylamide (40%, 19:1)
+ 3ml 10xTBE
+ 10 ml DEPC-water
+ 300 microliter 10% APS in DEPC-water
+ 11 microliter TEMED

Nucleic acid elution buffer, for 9 ml
+ 1 M NaOAc pH 5.2, 3 ml 3 M
+ 1 mM EDTA, 18 microliter 0.5 M
+ DEPC-water, 6 ml

3.2.9 Flotation Assay

Spheroblast preparation
–Chill cultures on ice
–Harvest 400 OD units of an early logarithmic culture (0,5-1,0), spin 10minutes at 6000xG
–Wash cells once, spin 5 minutes at 2000xG
–Resuspend cells in 5ml SB-buffer under gentle shaking
–Spheroblast cells for 1h at 37 degrees in SB-buffer under gentle shaking
–Pellet Spheroblasts trhough a 8ml sorbitol cushion at 600g for 12 minutes

SB buffer
1,4M sorbitol, 50mM KP pH7,5
add freshly before use:
10mM NaN3, 0,4% beta-MeEtOH, 2mg/ml Zymolyase 20T

Sorbitol cushion
1,7M Sorbitol, 50mM KP pH7,5

–lyse spheroblasts im 4ml Lysis buffer
20mM Hepes
140mM KAc
1mM MgAc
1mM EDTA
+Protease inhibitors

–stroke 22 times, up and down, in a douncer.
–Spin 5x at 450xG to remove cell debris.
–Layer Sucrose gradient: 4 layers, 3ml each.
Upper layer - 0%
second layer - 40%
sample loading layer - 50%
lowest - 70%

-Spin 8,75 hours in a SW40 rotor, at 4degrees
-Take aliquots from each layer, interfaces and resuspended pellet
-precipitate with 15% TCA.

3.2.10 Yeast Colony PCR

-suspend small amount of cells in 100 µl 0,02M NaOH.
-Add glass beads
-5minutes at 100 degrees, 1400RPM
-spin at full speed, 15 seconds
-use 5 µl as template for PCR

3.2.11 Immunoprecipitation

Day1
-Innoculate yeast strains and grow them overnight.
-Block coupled beads:
25 µl beads per 15OD culture.
Wash 3 x 10minutes with RNA buffer.

Day2
-dilute culture to 100 ml OD600=0,3, grow at 30 degrees to OD600=0,8-1,0.
-Harvest 15 OD600: 3 minutes at 3600RPM at 4 degrees and discard supernatant.
-Resuspend pellet in 1 ml cold H2O, transfer to 1,5ml tube.
-Spin at maximum speed for 15 seconds and discard supernatant.
-Add to pellet 200 µl breaking buffer and ressuspend.
-Add 200 µl glass beads
-break cells 4x 3 minutes with vibrax with a 1 minute break in between.
-Add 100 µl breaking buffer.
-Centrifuge 2 minutes at 3000RPM at 4 degrees.
-Transfer 250 µl into a new tube, keep a 30 µl aliquot (Total).

-Remove blocking solution from blocked beads (MPC)
-resuspend beads in 500 µl breaking buffer.
-Transfer aliquots in needed number of tubes.
-Add cell extract
-incubate 2hours at 4degrees (wheel)
-place tubes into MPC
-discard supernatant, but take an aliquot for western blot
-wash twice with 1ml wash buffer
-suspend in 350 µl wash buffer
-place tubes in MPC, discard supernatant
-resuspend pellet in 30 µl 1xSDS-loading buffer
-place 5minutes at 95 degrees
-place in MPC and transfer sup into a new tube.

Covalent Coupling of mouse anti-myc 9E11 to Dynabeads Protein G

- use 1ml antibody per 100 µl packed beads
- wash 500 µl beads twice with 1ml 0,1 M sodium phosphate pH7.0, use MPC for washing
- thaw 15ml aliquot bybridoma supernatant anti-myc 9E11
- add 12 µl 1M Tris-HCl pH7.5
- distribute beads to 4 1,5ml tubes (125 µl each).
- Add 1,25 ml hybridoma supernatant
- 40 minutesat room temperature with gentle rotation (wheel)
- wash twice in 1ml 0,1 M Na-Phosphate buffer pH7.0
- wash twice in 1ml 0,2 M Triethanolamine pH7,2
- pipet all beads into one 2ml tube
- add 1ml 20mM DMP in 0,2 M triethanolamin (5,4mg in 1ml, prepare freshly).
- Incubate for 30minutes at room temperature with gentle rotation.
- Add 1ml 50mM Tris-HCl pH7,5
- incubate 15 minutes at room temperature with gentle rotation.
- Wash 3 times in 1ml 1xPBS/0,1% Tween
- wash beads in MPC, twice with 1ml 1xTBS
- wash beads once with 1ml 0,1M glycine/HCl pH2,5
- wash beads once with 1ml 0,1 M Tris/HCl pH8,8
- wash twice with 1ml 1xTBS
- take up beads in 1xTBS pH7,4 (50% slurry, 500 µl)
- store beads at 4 degrees.

Breaking buffer
50mM Hepes/KOH pH7,3, 20mM KOAc, 2mM EDTA pH8,0, 0,1% (w/V) Triton X-100, 5% (w/V) Glycerol +1x Protease inhibitor Mix

wash buffer
50mM Hepes/KOH pH7,3, 50mM KOAc, 2mM Mg(OAc)2, 0,1% (w/V) Triton X-100, 5% (w/V) Glycerol

RNA buffer
50mM Hepes/KOH pH7,3, 20mM KOAc, 0,1% (w/V) Triton X-100, 5% (w/V) Glycerol, 0,1mg/ml tRNA

3.2.12 Point mutant generation

Site directed mutagenesis kit
2 µl plasmid DNA (1:10)
0,625 µl Oligo 1 (10pmol)
0,625 µl Oligo 2 (10pmol)
2,5 µl buffer
0,5 µl Super Taq
18,125 µl H2O

- Add 1 µl DpnI and digest for 1hour at 37 degrees.
–Add Top10 cells, heat shock at 42 degrees, for 30 seconds and incubate in preheated LB for 1hour shaking at 37 degrees.

3.2.13 Polysome profiling analysis

Prepare buffers:
- 10xPolysome Buffer
    200mM Hepes/KOH pH7.5
    750mM KCl
    25 mM MgCl2
    10 mM EGTA
–60% Sucrose in DEPC (pH7,5) filtrated
–10mg/ml Cycloheximide in water (freshly prepared)

- Grow overnight culture.
–Dilute to OD600=0,1 and let grow until OD600=0,5
–Add 0,1mg/ml cycloheximide to 50ml of cells (500 µl of 10mg/ml stock)
–Incubate 10 min at 30 degrees
–Harvest cells by centrifugation: 3minutes, 3000RPM at 4 degrees
–Prepare 1 eppendorf tube per strain with 50% glass beads
–Remove all traces of supernatant
–Add 20 µl 1xPolysome buffer (plus 0,1mg/ml Cycloheximide, plus 1mM DTT) per OD 600 cells (500µl for OD0,5)
–Lyse cells by vortexing in the cold room (250⌈l beads, 500 µl 1x Polysome buffer) for 5minutes.
–Spin 5minutes 13000RPM at 4 degrees.
–Take 10 µl of supernatant, dilute in 990 µl H2O and measure A260.
–Load 250 µg RNA onto the gradient (1 OD 260=40µg/ml RNA)
–Spin 2 hours, 38000 RPM, SW40 rotor.
–Collect 500µl fractions.

TCA precipitation
–Provide 1 ml TCA premix (10-15% TCA: 37,5ml H2O + 10 ml TCA) in tube on ice.
–Vortex sample.
–Take out 250 µl (enough for 2 blots) and add to TCA premix
–Vortex
–Incubate ON on ice in the cold room
–Spin 30 minutes maximum speed, at 4 degrees.
–Take off supernatant
–Add 500 µl ice cold aceton
–Spin 10 minutes, maximum speed at 4 degrees (small white pellet)
–Take off supernatant
–take off remaining supernatant with yellow tip.
–Add 20 µl 1x sample buffer Premix (1 ml 1 x Sample Buffer + 50 µl Tris base)
–Vortex

-Let stand for 1 hour
-Vortex sample
-Boil for 1 minute
-Vortex sample
-Boil for 1 minute
-centrifuge 1 minute, 13000RPM
-Load gel.

Preparation of gels:
-prepare 12 ml (enough for 2 gradients) a 10% and a 50% sucrose solution both with 1mM DTT and cycloheximide 0,1mg/ml.
-prepare 12 ml (enough for two gradients) a 10% and a 50% sucrose solution both with 1mM DTT and cycloheximide 0,1mg/ml and 0,01M EDTA.
-Pour 6ml of the 50% solution and then 6ml of the 10% solution, onto the same SW40 rotor tube. Cover with parafilm and lay gently down, allowing the fractions to mix. Let lie for 2hours. After that put them upright in the coldroom for 2 hours or until the cells are ready.

4. RESULTS

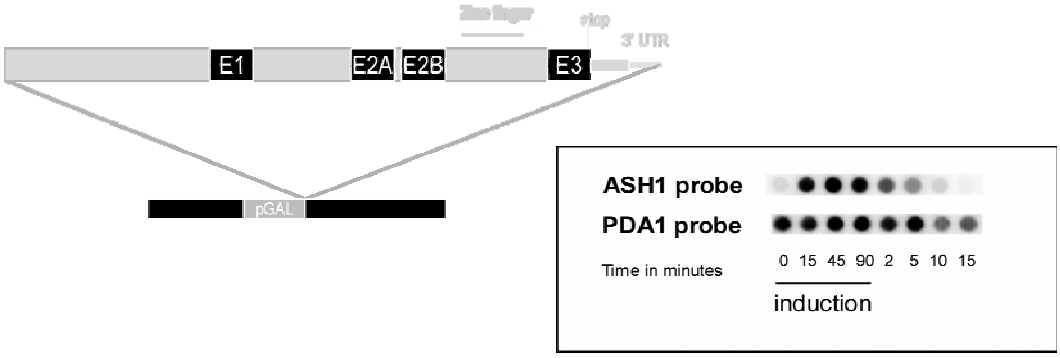
4.1 Relationship of ASH1 mRNA localization and nonsense mediated decay (NMD)?

As the *ASH1* mRNA localization process is a temporally well defined event and it has been shown that a premature stop codon (PTC) causes a loss of anchoring at the bud tip, while still bud localized (Jaedicke, PhD Thesis) we wanted to investigate whether there was a link between NMD and mRNA localization factors (RLFs).

To first address this issue we decided to use a galactose driven *ASH1* system (Fig. 2) to measure the half-life of this mRNA.

Fig. 2 - Schematic view of the *ASH1* construct used to determine *ASH1* mRNA half-life.
Another construct containing a PTC before the E1 element was also used in the same setup. The *GAL* promoter was induced and *ASH1* mRNA was transcribed from both constructs, the wildtype containing one and the PTC containing one. Half-life was determined by analysing the rate of decay after promoter repression.

Figure



	WT (n=3)	**E1-Stop (n=3)**
wt	9,9	8,66
Δ*ccr4*	7,8	10,47
Δ*ccr4* Δ*she2*	7,6	7,62
Δ*dcp1*	10,1	11,55
Δ*upf1*	7,69	9,43
Δ*upf1* Δ*she2*	7,96	10,12
Δ*vts1*	9,57	**9,72**

Δ*she2* Δ*vts1*	7,7	**12,1**
Δ*she2*	8,66	9,32
Δ*khd1*	**9,9**	**10,7**
Δ*khd1* Δ*she2*	**11,55**	**17,33**
Δ*puf6*	**11,55**	**11,55**
Δ*puf6* Δ*she2*	**16,08**	**14,84**
Δ*scp160*	20,10	20,04

Table 1 – Comparison of influences of different mutants on the half-life of *ASH1* mRNA, both wildtype and *ASH1-Stop-E1*.

These results, presented in Table 1, led us to the surprising finding that the disruption of the major *ASH1* mRNA binding protein, *SHE2,* has no influence on its own in *ASH1* mRNA half-life. Furthermore, factors involved in the NMD pathway, such as *CCR4*, *UPF1* and *DCP1*, also do not show any change in *ASH1* mRNA half-life, not even in combination with Δ*she2.* Interestingly, no measurable effect was observed for mRNA nonsense mediated decay mutants, indicating that there is no nonsense mediated decay for *ASH1* mRNA. *VTS1*, the yeast Smaug homolog and a partner to *CCR4-NOT* complex, when in combination with a Δ*she2* showed a 30% increase in *ASH1-E1-Stop* mRNA as compared to the wildtype, or even to Δ*she2*.

On the other hand, two other *ASH1* mRNA binding proteins, shown to be translation repressors, encoded by *PUF6* and *KHD1* do cause a change in *ASH1* mRNA half-life when in combination with Δ*SHE2*. Δ*puf6* Δ*she2* shows a 40% half-life increase for the wildtype and 30% increase for *ASH1-Stop-E1* when compared to Δ*puf6* alone and Δ*khd1* Δ*she2* shows a 15% half-life increase for the wildtype *ASH1* and 60% increase for *ASH1-Stop-E1* when compared to Δ*khd1*. In addition, another gene involved in translation regulation, *SCP160*, showed a 2x fold increase in *ASH1* mRNA half-life, for both constructs tested.

We found that *Δshe2 Δkhd1* and *Δshe2 Δpuf6* did show a cumulative effect on *ASH1* mRNA half-life. These findings were not so surprising, since these proteins are mRNA binding proteins and are active in localization of *ASH1* mRNA to the daughter cell. Although mRNA localization *per se* is not an essential process we wanted to test for cell viability and temperature sensitivity, in order to try to identify other processes that might be impaired.

4.2 Relationship between RNA localization factors and cell viability?

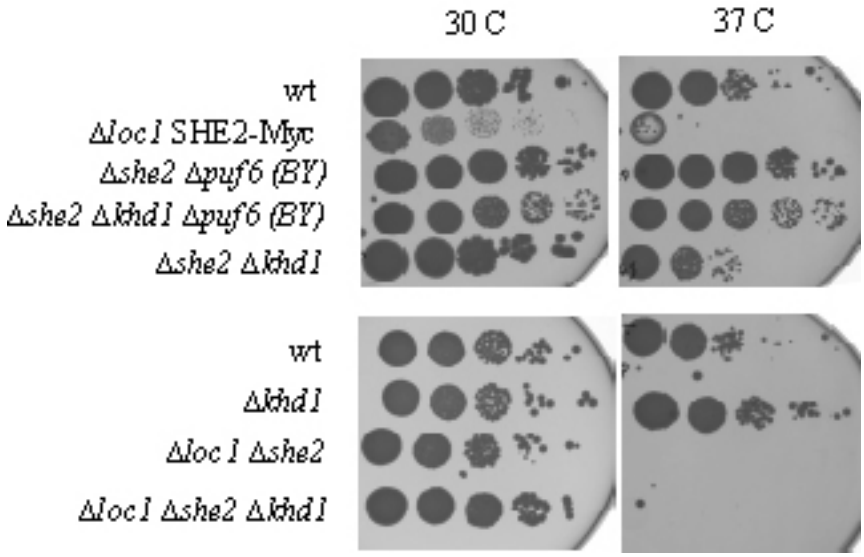


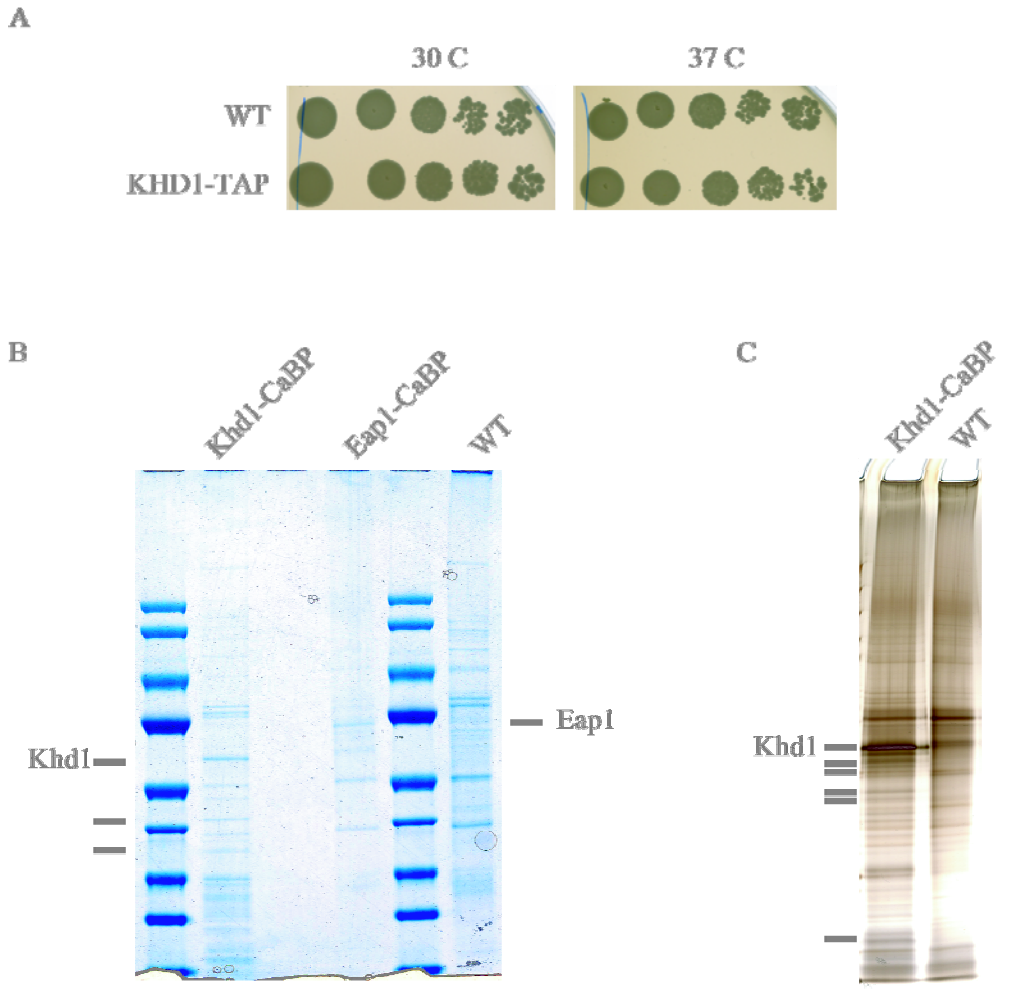
Fig. 3 - Temperature sensitivity of mRNA localization factors. 10-fold serial dilutions from an overnight culture were plated in YEPD media and then incubated at 30 and at 37 degrees.

The results (Fig. 3) show that there is a temperature sensitive growth defect for Δ*loc1 Δshe2*, that does not grow at 37ºC. Surprisingly, this double disruption grows better at 30ºC than a Δ*loc1* She2-myc3. In addition, a *Δshe2 Δkhd1* shows a synthetic growth defect at 37ºC.

4.3 What is Khd1p connection to ASH1 mRNA localization process?

Khd1p, has been shown to be localized with the *ASH1* mRNA to the bud tip (Irie *et al*. 2002). These results made us interested in addressing the questions: i) is Khd1p a part of the locasome? ii) Does it travel with the *ASH1* mRNP together? Is Khd1p involved in *ASH1* mRNP formation?

In order to achieve this, we set out to identify interacting protein partners of Khd1p and therefore a Khd1p-Tandem Affinity purification was established.



It has been observed, in our lab and others, that the addition of an epitope to the COOH-end of a determined gene can lead to a non functional or growth defect/growth impaired phenotype. To answer this question, drop tests were performed (Fig. 4A). No detrimental effect was found for a Khd1p-TAP tag.

Fig. 4 – Can Khd1p be purified with a tandem affinity purification approach?
A – Drop test for growth viability of a Khd1-TAP.
B – Coomassie stained gel of TEV cleaved eluates.
C – Silver stained gel of Calmodulin binding eluates.

4.4 Which co-purifying proteins can be found with this approach?

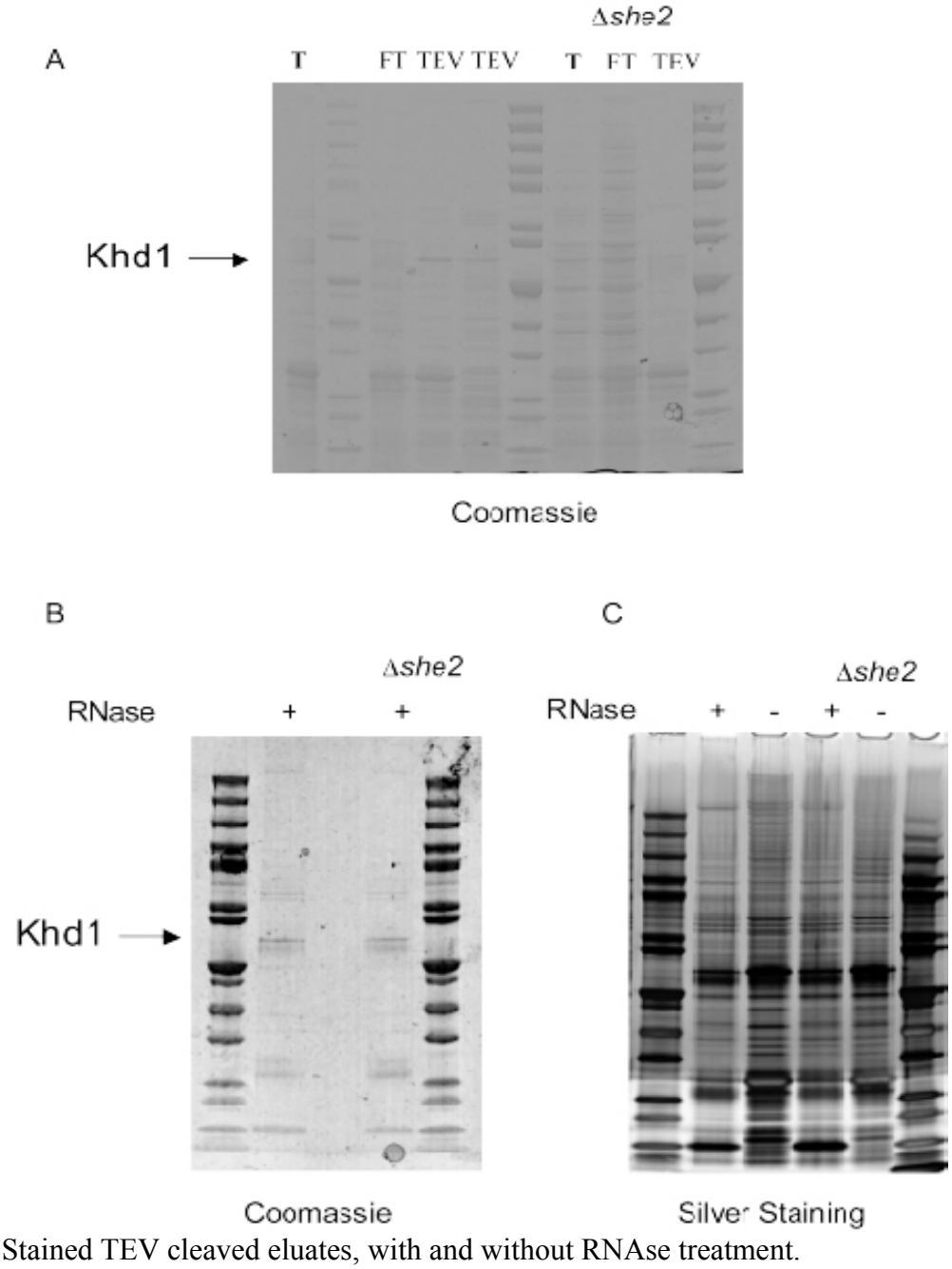
When the Khd1p purification was performed using a 100.000G 1hour centrifugation step only the bait was found on a coomassie stained gel, after the calmodulin binding step (data not shown). As Khd1p may play a role in the nucleus (Denisenko *et al.*, 2002; Du *et al.*, 2008), the centrifugation speed was reduced to 10.000g. Again, this approach did not yield after the calmodulin binding step, apart from the bait, coomassie detectable bands that were different from a mock purification. On the other hand, when a silver staining was performed a few co-purifying bands could be found on the

same calmodulin eluates (Fig, 4C). Furthermore, the TEV cleaved eluates after this slower centrifugation step showed additional coomassie co-purifiers, as expected. (Fig. 4B)

4.5 Is Khd1p a part of a RNA dependent protein complex?

How could Gavin and co-workers (Gavin *et al.*, 2002; Gavin *et al.*, 2006) find so many other co-purifying proteins? Could we be facing mRNA mediated interactions? Is She2p tethering any Khd1p binding partners?

Fig. 5 - Khd1p tandem affinity purification.
A – Coomassie Stained Khd1p-Tandem affinity purification.
B – Coomassie Stained TEV cleaved eluates after RNAse treatment.



C – Silver Stained TEV cleaved eluates, with and without RNAse treatment.

We observed that the RNAse treatment showed no change on the coomassie stained protein pattern (Fig. 5B). The RNAse treatment led to changes in the co-purifying protein pattern in the silver stained TEV-cleaved eluates (Fig. 5C).

4.6 Is Khd1p a part of the locasome? Can it co-purify other members of the ASH1 mRNP?

As can be seen above, a Khd1p tandem affinity purification did not yield coomassie stainable co-purifying bands and did not allow us to conclude whether Khd1p was a part of the locasome. We decided to tackle this question directly, by looking for the other members of the locasome in the eluates and trying to see whether some of the proteins that were missing in the silver stain after RNAse treatment corresponded to any of the locasome members.

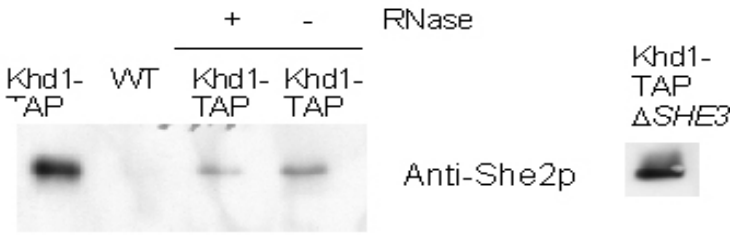


Fig. 6 - Can Khd1p co-purify She2p?
Western blot analysis of TEV cleaved eluates, probed with a Anti-She2p antibody.
Lane 1 – Khd1p-TAP TEV cleaved eluate; Lane 2 – Untagged strain TEV cleaved eluate; Lane 3 – Khd1p-TAP TEV Cleaved Eluate, after 20minutes RNAseA, at 16°C; Lane 4 - Khd1p-TAP TEV Cleaved Eluate, after 20minutes at 16°C. Lane 5 – Khd1p-TAP Δ*she3* TEV cleaved eluate.

She2p can be co-purified when using Khd1p as bait (Fig. 6). Surprisingly, this purification is independent of RNA, as a 20min 1mg/ml RNAse A treatment does not abolish this co-purification.

4.6.1 Does it also co-purify other members of the locasome?

She3p is the adaptor protein between She2p and Myo4p, the motor that transports the cargo. So we wanted to know whether She3p and Khd1p can also be co-purified.
She3p-myc6 can also be co-purified with Khd1p (Fig. 7). Interestingly this purification is also independent of She2p and of RNA. These findings indicate a direct interaction of Khd1p with She3p, thus suggesting that Khd1p is itself, and not only She2p, a cargo of the SHE machinery.

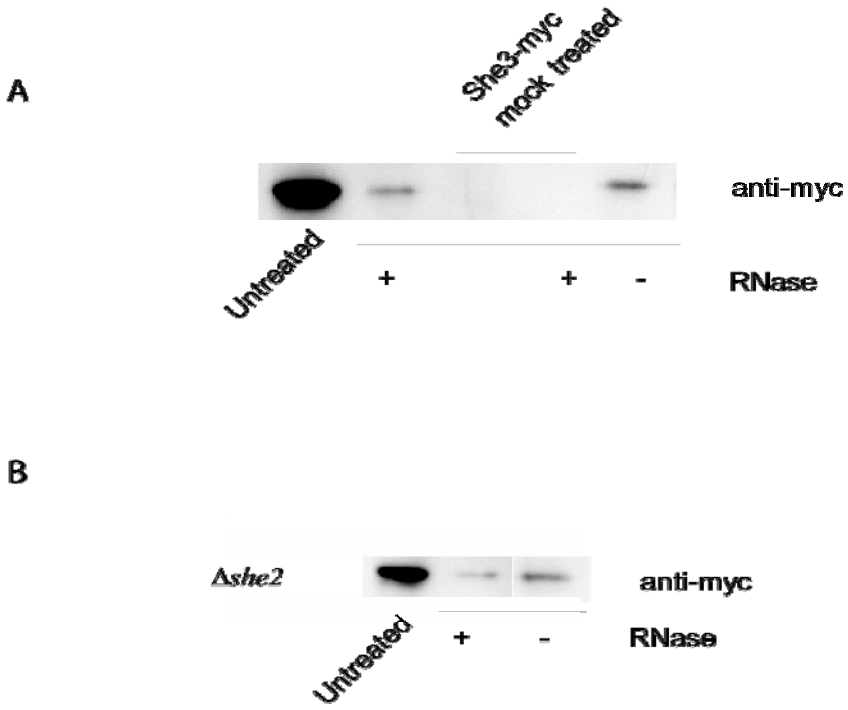


Fig. 7 - Khd1p co-purifies She3p-Myc6?
A - Western blot against She3p-Myc6. Lane 1 – Khd1p-TAP She3-Myc6 TEV cleaved eluate; Lane 2 – Khd1p-TAP She3-Myc6 TEV cleaved eluate, after 20minutes RNAse A treatment at 16°C; Lane 3 – untagged Khd1p She3p-Myc6 TEV cleaved eluate; Lane 4 – untagged Khd1p She3p-Myc6 TEV cleaved eluate, after 20 minutes RNAse A treatment at 16°C; Lane 5 – Khd1p-TAP She3-Myc6 TEV cleaved eluate after 20minutes at 16°C.
B - Western blot against She3p-Myc6. Lane 1 – Khd1p-TAP Δ*she2* She3-Myc6 TEV cleaved eluate; Lane 2 – Khd1p-TAP Δ*she2* She3-Myc6 TEV cleaved eluate, after 20minutes RNAse A treatment at 16°C; Lane 3 – Khd1p-TAP Δ*she2* She3p-Myc6 TEV cleaved eluate, after 20minutes at 16°C.

4.6.2 Is there a direct interaction between She3p and Khd1p?

The next question was to map the interaction between Khd1p and She3p. For this we performed a Khd1p tandem affinity purification again, but this time looking for co-purified HA-tagged She3-COOH.

Fig. 8 - Can Khd1p bind to the C-terminal end of She3p? Does it bind to the N-terminal end of She3p? Western Blot against HA-tagged She3p-COOH.
Lane 1 – Total, Khd1p-TAP induced pGAL-HA3-She3-COOH.
Lane 2 – TEV cleaved eluate, Khd1p-TAP induced pGAL-HA3-She3-COOH.

HA-She3p-COOH was not co-purified with Khd1p in this approach (Fig. 8). Does it not bind to the COOH-end of She3p? Is this end not stable enough?

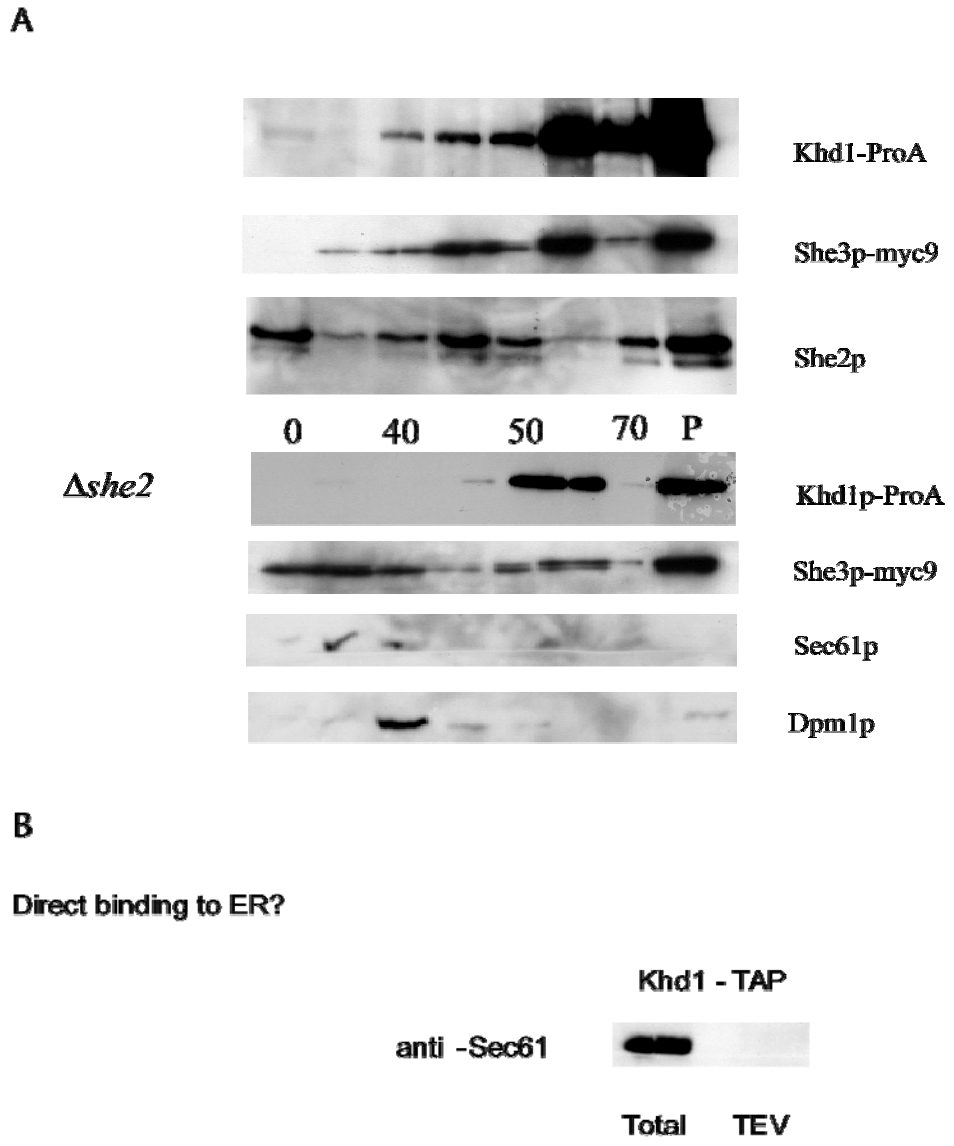
What about the NH2-end of She3p?

A Khd1p tandem affinity purification with a genomically Myc-tagged-She3p-NH2end was performed and analysed by western blot for Myc signal. A myc signal was observed even on the Khd1p-untagged strain, making this approach inconclusive (data not shown).

4.7 Khd1p, another mRNA binding protein associated to the Endoplasmic Reticulum (ER)?

Schmid and coworkers (2006) have shown that She2p binds to the ER and that it floats on a sucrose sedimentation gradient. As Khd1p can co-purify She2p the question arose whether Khd1p could also interact with the ER-associated pool of She2p. ER flotation assays were performed (Fig. 9A) and analysed for flotation of Khd1p.

Fig. 9 - Khd1p association to ER?
A – 4-step gradient sedimentation profiling. Lane 1 - 0%, Lane 3 - 40%, Lane 5 - 50% and Lane 7 - 70% sucrose layers.
B – Western Blot against Sec61p, on Khd1p-TAP Total and TEV cleaved eluate.



Khd1p partly mimicks the distribution observed for She3p-Myc6 in the lower fractions 40% to Pellet fractions. On the other hand, Khd1p floats to the top of a 4-step gradient profile as described

for She2p (Schmid *et al.*, 2006) and surprisingly this happens also in the absence of She2p – fraction 0-40% (Fig. 9A), indicating that She2p is not required for Khd1p-ER association.

4.7.1 Does Khd1p-affinity purification also yield ER-bound proteins?

After having shown that Khd1p floats with the ER membranes, the question arose whether Khd1p binds directly to the ER and whether Khd1p could co-purify ER proteins, such as Sec61p. After a Khd1p tandem affinity purification we observed that it is not the case (Fig. 9B).

4.8 Khd1p proposed to be a translation repressor. Does it show translation inhibitor sensitivity?

Khd1p has been putatively identified as a translation repressor and shown to be accumulating with a U1A-mRNP at the budtip. (Irie *et al.*, 2002). Recently it has been shown that indeed it has a translation repressor function (Paquin *et al.*, 2007).

Khd1p contains 3-KH domains. Scp160p is also a 14-KH domain containing protein and *Δscp160* has been shown to be sensitive to translation inhibitors (Baum *et al.*, 2004). To map any other possible roles for Khd1p in translation regulation, *Δkhd1* cells were tested with different translation inhibitor drugs for differential sensitivity.

Paramomycin is a drug that inhibits protein synthesis by binding to 16S ribosomal RNA (Vicens and Westhof, 2001).

Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms, produced by the bacterium *Streptomyces griseus*. Cycloheximide exerts its effect by interfering with the translocation step in protein synthesis (movement of two tRNA molecules and mRNA in relation to the ribosome) thus blocking translational elongation.

Geneticin (G418) is an aminoglycoside antibiotic similar in structure to gentamicin B1. It is produced by *Micromonospora rhodorangea*. G418 blocks polypeptide synthesis by inhibiting the elongation step in both prokaryotic and eukaryotic cells.

Hygromycin B is an aminocyclitol antibiotic with broad spectrum activity against prokaryotes and eukaryotes. Hygromycin B strongly inhibits protein synthesis through a dual effect on mRNA translation (Cabanas *et al.*, 1978; Lacal and Carrasco, 1983). Like other aminoglycoside antibiotics, hygromycin B induces misreading of aminoacyl-tRNA by distorting the ribosomal A site (decoding center) (Cabanas *et al.*, 1978; Davies and Davis, 1968; Moazed and Noller, 1987; Spahn and Prescott, 1986). Hygromycin B also affects the ribosomal translocation process (Cabanas *et al.*,

1978; Gonzalez *et al.*, 1978; Hausner *et al.*, 1988). In the presence of the antibiotic, mRNA is often mistranslocated, being moved more or less than the three necessary bases.

Anisomycin is an antibiotic produced by *Streptomyces griseolus* which inhibits protein synthesis. Partial inhibition of DNA synthesis occurs at anisomycin concentrations that effect 95% inhibition of protein synthesis (Grollman, 1967). Anisomycin interferes with protein and DNA synthesis by inhibiting peptidyl transferase or the 80S ribosome system.

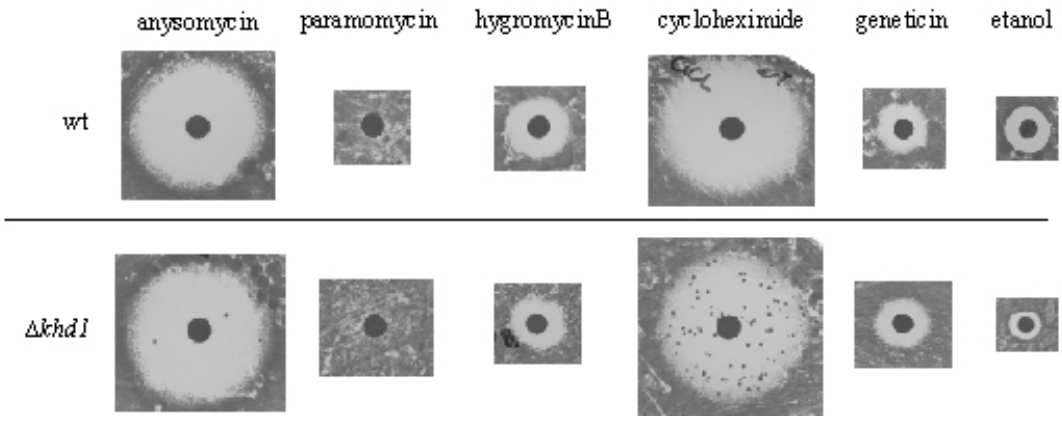


Fig. 10 – Drug sensitivity assay.
An exponentially growing culture was plated on to YEPD plates, were filters containing anysomycin, paromomycin, hygromycin B, cycloheximide, geneticin and 100% ethanol were placed. Plates were incubated for 18 hours and the halos of inhibition were measured.

We observed that Δ*khd1* does not show any difference in regard to translation inhibitor sensitivity, indicating that it is not directly involved in translation regulation.

4.9 Is Khd1p associated with polysomes?

Polysomes, or multiple ribosomes, form around actively translated mRNAs. In order to confirm the hypothesis that Khd1p is a translation repressor, one should be able to see that Khd1p affects ribosome loading onto an mRNA and one should not find Khd1p associated to heavy polysomes as they should dislodge the repressor from an actively translated mRNA.

Therefore, in a polysome profile one should encounter *ASH1* mRNA in the heavy polysome fractions but no Khd1p.

To test this idea polysome profiles were analysed.

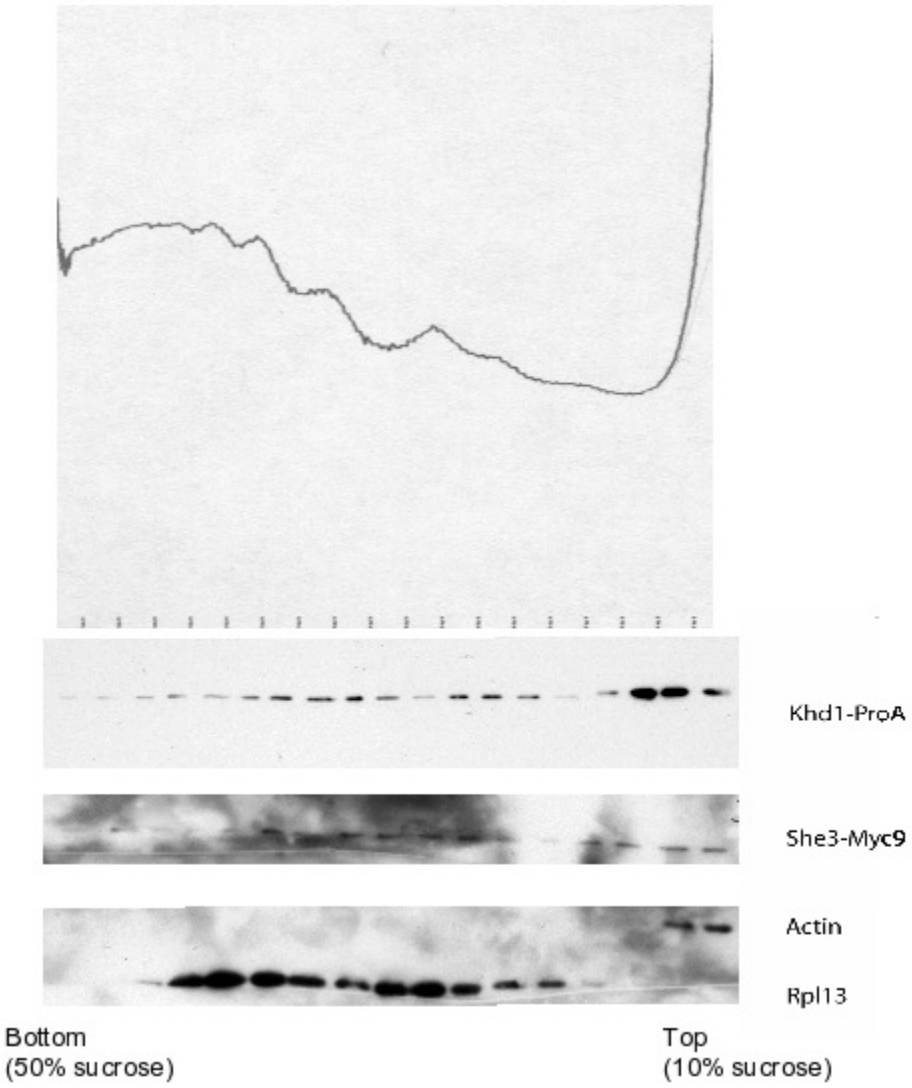


Fig. 11 – Is Khd1p associated to polysomes?
Polysome profile showing Khd1p-ProA, She3p-myc9, Rpl13p fractionation along the gradient.

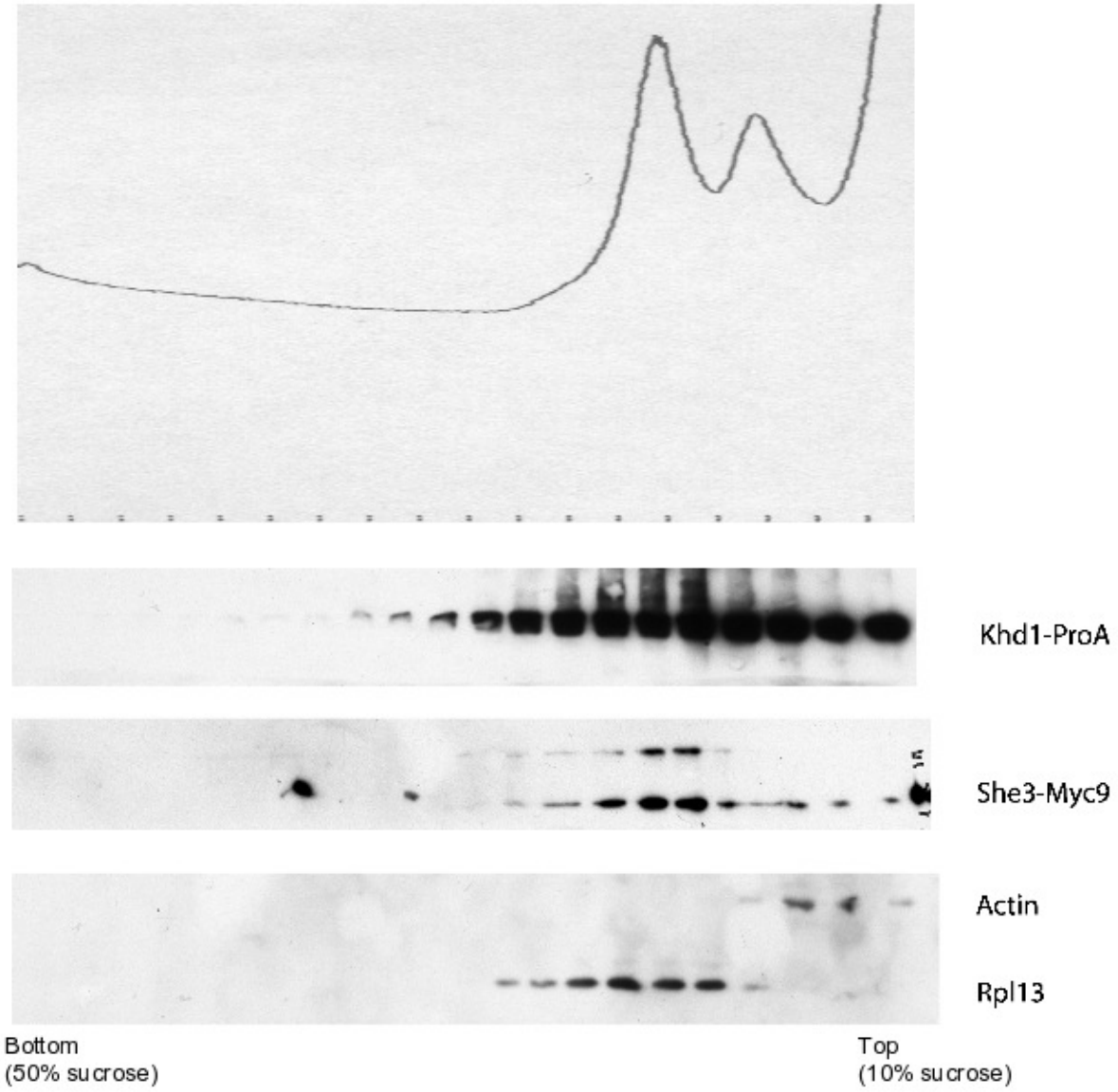


Fig. 12 – Does Khd1p co-migrate with the ribosomes upon EDTA treatment?
Khd1-ProA She3p-myc9 after EDTA treatment.

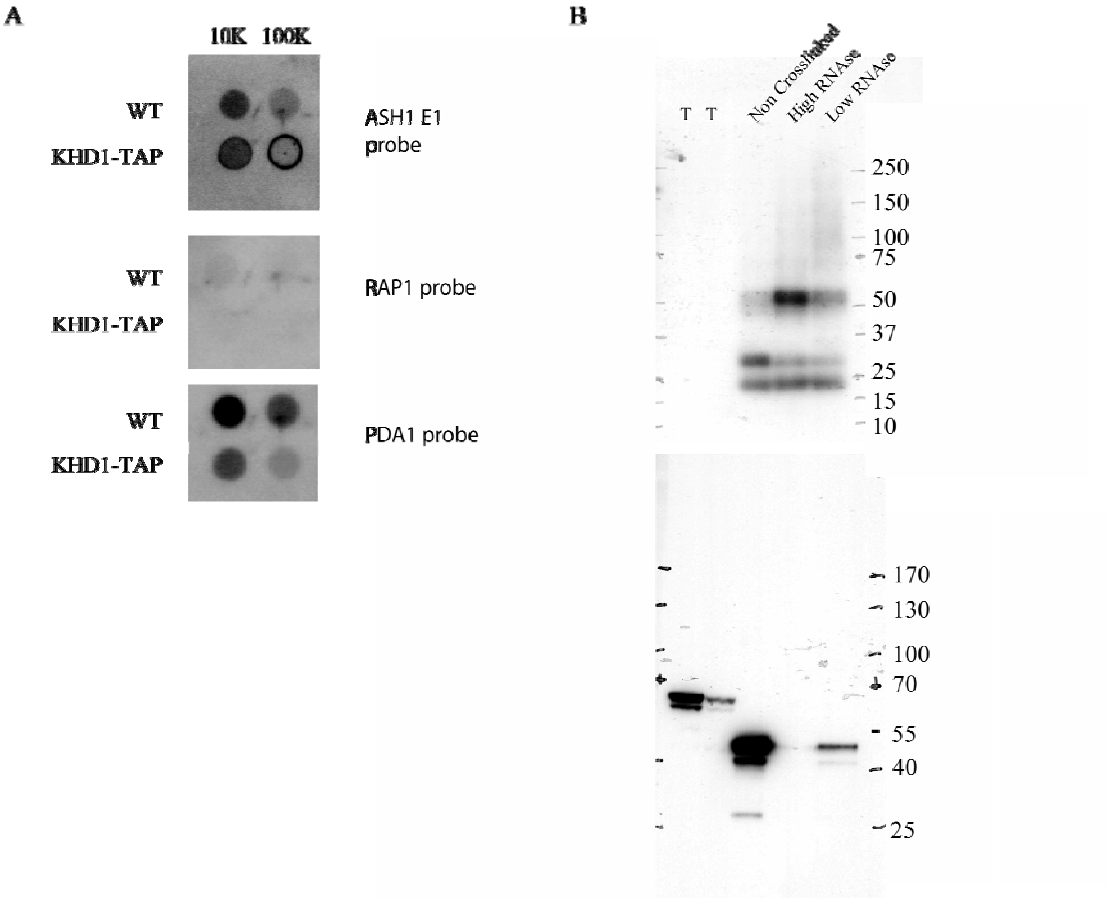
As can be seen above (Figure 11) Khd1p-myc9 associates with the polysomes, with 80S ribosomes and with 40S ribosomes. Upon EDTA treatment (Figure 12), that disrupts ribosome subunit association, Khd1p-ProA partially mimicks the distribution of the ribosomal protein Rpl3p. She3p-Myc9 mimicks Rpl3p distribution perfectly.

4.10 Khd1p, an mRNA binding protein?

Can we validate the purification from the TAP procedure for a stable mRNA-protein association? If so, to which other mRNAs, apart from *E1-ASH1* mRNA element, does Khd1p bind? Could it be that messages that encode for the telomeric remodelling complex are bound by Khd1p and therefore show this striking effect on telomeric length?

An approach to determine if any of the mRNAs involved in this process is also bound by Khd1p was followed.

Fig. 13 - RNA binding?
A – Northern Dot blot.
B – CLIP assay. Khd1p-CaBP-TEV-ProA
Top – Autoradiograph; Lane 1 – Total, Lane 2 – Total, Lane 3 – Non crosslinked, Lane 4 – High RNase, Lane 5 – Low RNase. Bottom – Western blot, anti-CaBP.



We observed that after a 10.000G centrifugation step 3x more *ASH1* E1-element mRNA could be detected than after a 100.000G centrifugation step (Fig. 13A). *ASH1* mRNA can be effectively purified bound to Khd1p, using the standard TAP protocol. However, other mRNAs were not enriched when analysed using this approach. We observed that *RAP1*, *SIR2*, *SIR3* and *TLC1* were not enriched, indicating that either these mRNAs are not bound by Khd1-CaBP-TEV-ProA (Fig. 13A) or the regions that the probes were directed against are not bound by this protein.

4.11 Ultraviolet CrossLinked ImmunoPrecipitation (CLIP)

Still, the question remained. To which other mRNAs does Khd1p bind to?

In order to follow this idea, a CLIP (Ule *et al.*, 2003; 2005) assay, based on the tandem affinity purification approach, was used.

We observed that even after RNAse treatment radioactively labeled protein still remained, indicating that Khd1p binds to DNA. In addition, upon treatment with less amounts of RNAse, the radioactively labeled protein is more intense and also larger in size, indicating that indeed the labeled material is in part RNA (Fig. 13B).

5. DISCUSSION

5.1 Is there a relationship between ASH1 mRNA localization and nonsense mediated decay (NMD)?

The *ASH1* mRNA localization process happens quite fast in the cell, since *ASH1* mRNA is transcribed in early anaphase and localized in late anaphase, thus making a very short time window for the localization event to take place.

The previous observation that a premature stop codon introduced before any of the zipcode elements of *ASH1* mRNA did not elicit nonsense mediated decay but led to a severe localization defect (5% tightly anchored, 95% mislocalized for *ASH1-E1-Stop* compared to 85% tightly anchored to 15% mislocalized for the wildtype *ASH1* – Jaedicke A., PhD Thesis, 2004), originated two hypothesis (Jaedicke A., PhD Thesis, 2004). *i*, The premature stop codons were not available to the surveillance machinery due to the mRNP architecture, where She2p, which binds to these localization elements, could mask these stop codons; *ii*, the premature stop containing transcripts, *i.e.* shorter transcripts, did not associate with sufficient ribosomes to be effectively anchored. The ribosome involvement in anchoring hypothesis is supported by the fact that cycloheximide treatment, that blocks ribosomal elongation, decreases *ASH1* mRNA tight anchoring (Jaedicke A., PhD Thesis, 2004) and also by the observation that Ash1p-COOH is required for active *ASH1* mRNA retention at the bud tip (Gonzalez *et al*., 1999). Further experiments from A. Jaedicke on this issue, showed that disruption of *SHE2* did not change *ASH1* mRNA stability, indicating that it is not the footprint of She2p that prevents premature stop recognition.

In order to establish whether nonsense mediated decay really occurs for these PTC containing *ASH1* transcripts the half-lives of two different transcripts were analysed (Fig. 2). The results presented in Table 1 show that there is no difference between the two transcripts analysed in most of the mutants tested. Surprisingly, mutations in genes involved in NMD such as *UPF1*, *DCP1*, *CCR4* (reviewed in Collier and Parker, 2004 and in Macquat, 2004) showed no effect on the half-lives measured for the two mRNA constructs, indicating that, indeed, classical NMD pathways do not affect *ASH1* mRNA stability. As most mRNAs in yeast, *ASH1* is intronless. In humans it has been described that intronless mRNAs do not undergo NMD (Brocke *et al.*, 2002). This observation is not likely to apply to yeast, since most of the mRNAs in this organism are intronless and a PTC containing mRNA could prove severely detrimental for the organisms viability. Furthermore, if this would be true, one would not expect any differences in the measured half-lives. The only observed differences were for mutations on *SCP160, PUF6* and *KHD1* (*PUF6* and *KHD1* in combination

with Δ*she2*). Δ*SCP160* causes a 100% increase in mRNA half-life as compared to wildtype but no difference between PTC-containing *ASH1* and wildtype *ASH1* was observed, indicating that it is a more general mRNA decay defect. The fact that *SCP160* has been implicated in ribosomal architecture in combination with Asc1p (Baum *et al.*, 2004) and bound to the polysomes in an mRNA dependent manner (Frey *et al.*, 2001) seems to point to a general defect instead of a *ASH1* specific defect. *LOC1*, another protein involved in RNA biogenesis and mRNA localization (Long *et al.*, 2001; Horsey *et al*., 2004; Komili *et al.*, 2007) was not tested, although in light of the presented results it should be looked into.

SHE2 plays apparently no role alone. However, when in combination with *PUF6* and with *KHD1* contributes to a significant increase in *ASH1* mRNA half-life. In combination with Δ*PUF6,* Δ*SHE2* increases the half-life of both transcripts analysed, 40% and 30%, an indication that this double mutant combination still allows PTC-containing-*ASH1* mRNA to evade NMD or that the rate by which the PTC-containing-*ASH1* mRNA is destroyed is undistinguishable from the rate with which the wildtype is translated into protein. As Δ*SHE2* causes a loss of *ASH1* mRNA localization and Δ*PUF6* contributes to the same process (Gu *et al*., 2004; Deng *et al.*, 2008) it is possible that this loss of efficient mRNA localization leads to an increase of mRNA half-life simply by the loss of spatial regulation or due to the misincorporation of these mRNAs into the mRNP or loss of another unknown factor targeted to the mRNP by Puf6p. In addition, the same could be true for Δ*KHD1* Δ*SHE2*. The half-life increase for PTC-containing *ASH1* mRNA observed for this double mutant was 60%, whereas for the wildtype *ASH1* almost no change was observed. This observation seems to point towards a role for *KHD1* in the recognition of this PTC-containing *ASH1* mRNA, although this seems unlikely as no connection between *KHD1* and NMD pathways has been until now elucidated.

These results support the hypothesis that the loss of anchoring at the bud tip and the unchanged *ASH1* mRNA stability between PTC containing transcripts and wildtype transcripts, observed by A. Jaedicke is directly linked to ribosomal function and that ribosomes are an important part of the localizing mRNP. They do not, however, allow us to conclude if it is a matter of transcript ribosomal loading. It is conceivable that a slower progressing ribosome, either due to a ribosome biogenesis problem (*PUF6* (Fromont-Racine *et al.*, 2003)) or due to the loss of regulation exerted by a translation repressor [*PUF6* (Gu *et al.*, 2004; Deng *et al.*, 2008), *KHD1* (Irie *et al.,* 2002; Paquin *et al.*, 2007)] can cause an extended mRNA half-life for the ribosome-bound transcripts. Loss of a translation repressor should speed up translation and thus making the mRNA half-life shorter, which is not the case (Table 1). It is conceivable that these translation repressors are also involved in the targeting of other factors such as RNA helicases to the mRNP. When the translation

repressors are absent the localizing mRNA might require more time to unwind and be translated, masking the effect caused by the loss of a translation repressor.

Although a shorter transcript should be translated faster than a full length one, this was not the case, even for the wildtype cells analysed (Table 1), indicating that there is a missing link in the regulation of these mRNAs. Another possibility is that the processes involved, localizing mRNA, translation and NMD are temporally coupled and their independent analysis therefore very difficult.

Although being a very atractive hypothesis, the data presented do not allow a conclusion to whether this nonNMD effect is a localized mRNA specific effect or not, as no other PTC containing transcripts were analysed.

Another aspect that cannot be ruled out is the possibility that *PUF6*, *KHD1* and *SCP160* contribute to the processivity and efficiency of the normal NMD process and so therefore the effect observed would be a reflex of this loss of processivity of the NMD machinery.

Recently, an NMD-related pathway was uncovered, the No-Go pathway (Doma *et al.*, 2007). This pathway is based on the ribosome being a central part and a key player to the onset of NMD. It includes two proteins, Dom34p and Hbs1p, similar to release factors eRF1 and eRF3. Dom34p and Hbs1p recognize a stalled ribosome due to a PTC and perform an endonucleolytic cleavage, thus eliminating the defective mRNA. This process can have a slower dynamics than the translation events and so lead to a higher half-life of a PTC-containing mRNA. It would be interesting to see if the disruption of *PUF6*, *KHD1*, *SCP160* or even *LOC1* affect this No-Go pathway. Furthermore, a study of epistasis between members of this No-Go pathway and *ASH1* mRNA localization factors could lead to a better understanding of the missing effects on PTC-containing *ASH1* mRNA. If this is the case, the fact that a localized mRNA does not suffer classical NMD can have biological implications and a more general model can be devised, one that includes the ribosome and the stalled-ribosome recognizing proteins, Dom34p and Hbs1p.

5.2 Relationship between RNA localization factors and cell viability?

As *ASH1* mRNA localization is not an essential process *per se* in yeast, mRNA localization factors disruption are unlikely to be lethal. However, the mRNA localization process is not for *ASH1* mRNA alone as other mRNAs are localized with the help of the same machinery (Shepard *et al.*, 2003; Irie, personnal communication). The fact that some RLFs overlap in their binding abilities (e.g. She2p, Khd1p and Puf6p) could prevent the detection of a lethal phenotype, related to the mRNA localization process. Although it is true that both Δ*she3* and Δ*myo4* are not lethal, alone or even together, it is possible that the mRNA localization events can still take place, although at a

later stage, based on microtubule-directed movements or even on ER inheritance mechanisms (Schmid *et al.*, 2006).

The analysis of several RLF disruptions (Fig. 3) showed that Δ*loc1* Δ*she2* is extremely temperature sensitive, since it does not survive to 37°C growth conditions, increasing even the phenotype observed for Δ*loc1 she2-myc3*. An additional Δ*khd1* produced no change in the behaviour of the Δ*loc1* Δ*she2* double mutant. On the other hand, a Δ*khd1* Δ*she2* showed a temperature sensitive phenotype, as it grows slower at 37°C than the single deletions. It would be interesting to see what effect an additional Δ*puf6 w*ould cause to this double mutant in a W303 background. It would provide a better understanding as to whether this temperature sensitivity is background specific, since a Δ*khd1* Δ*she2* Δ*puf6* (BY background) is not temperature sensitive. In addition it would give insights as to whether a Δ*puf6* reverts or even increases the temperature sensitivity of Δ*khd1* Δ*she2*, arguing for an effect over an essential process, affected by these mRNA localization factors.

5.3 What is Khd1p connection to ASH1 mRNA localization process?

Khd1p binds to and co-localizes with *ASH1* mRNA at the bud tip (Irie *et al.*, 2002). Khd1p has been proposed to be a translation repressor (Irie *et al.*, 2002; Paquin *et al.*, 2007) and these observations arose our interest in trying to determine whether Khd1p travels together with the mRNP and trying to fit Khd1p into the mRNP architecture.

As the results from a Khd1p-tandem affinity purification yielded no coomassie stainable bands (Fig. 4-5), other than Khd1p itself, an alternative approach to detect specific mRNP components was taken. This approach led to the confirmation that Khd1p can co-purify She2p (Fig. 6), indicating that at some point in time and space they are in the same *ASH1* mRNP particle. The fact that this interaction is RNA independent points to either a direct binding or binding via a common partner. The most likely partner would be She3p. Khd1p can also co-purify She3p (Fig. 7). This interaction also occurs in the absence of mRNA. In addition, the fact that Khd1p can co-purify She3p without She2p (Fig. 7), hints to a direct binding of Khd1p to She3p. These results suggest that Khd1p is a novel cargo of She3p.

Although Khd1p can pull down She3p from yeast lysates, the opposite could not be verified (Fig. 8), as Khd1p binds to every kind of protein immobilization matrix used. Further in-vitro experiments are required to prove that Khd1p and She3p interact directly. Unfortunately, the fact that a full length-She3p or a COOH-terminal-She3p cannot be stably expressed in *E. coli* causes a technical setback to the development of this strategy. Nevertheless, the results presented in figures 6-7 show that there are Khd1p-She2-She3p protein complexes and that also Khd1p-She3p protein

complexes exist. The localization destination of these complexes is an interesting question. The Khd1p-She2p-She3p complex should be localized to the bud tip (Irie *et al.*, 2002). The fact that Khd1p has been proposed to bind over 300 mRNAs (K. Irie, personal communication), can potentially increase the number of localized mRNAs to the bud tip. It is not yet clear how many of these mRNAs are localized to the bud tip, or even whether they are targeted there by Khd1p. It is also unclear whether these over 300 bound mRNAs are just bound by Khd1p as part of its translation repressor function and would therefore have no spatial correlation or even be localized in a SHE-dependent manner.

The localization of the Khd1p-She3p complex is however a very interesting question that needs to be addressed. It can be hypothesized that the Khd1p-She3p mRNPs are a part of an entirely autonomous system from She2p-She3p-Khd1p mRNPs and do not localize especifically to the bud tip, but to other parts of the cell, acting in a totally different process, e.g. ER inheritance, delivering membrane proteins encoding mRNAs to the daughter cell. To test this hypothesis, She3p and Khd1p double live imaging, in a *she2* disrupted strain, could provide insights as to whether these two partners are in the same particle, whether they still localize to the bud tip and could provide insights about its function according to its localization.

The fact that Khd1p is a conserved protein, homologous to hnRNP K, both in sequence and in function (Denisenko *et al.*, 2002), could improve our understanding how hnRNP K works and validate yeast as a model organism for studies on this protein.

It would be interesting to see whether Puf6p, that has the same translation repressor assigned function as Khd1p, is also associated to other components of the locasome, like She3p, and not only to She2p (Gu *et al.*, 2004). Furthermore, recently it has been shown that Puf6p is also a shuttling protein (Gu *et al.*, 2004; Du *et al.*, 2008) and that it is also phosphorylated by Yck2p at the bud tip, thus releasing *ASH1* mRNA and eliciting *ASH1* mRNA translation (Deng *et al.*, 2008). It would be extremely interesting to see if both translation repressors are present at the same time, in the same mRNP particles, associated to *ASH1* mRNA, thus exerting a double translational control. In addition, the possibility that Puf6p can bind other mRNAs other than *ASH1* mRNA should be addressed and detect if Puf6p binds to the same set of mRNAs as She2p or even Khd1p. Furthermore, the possible non overlap of binding targets between these three proteins could indeed bring mRNA localization in yeast a step forward, by increasing the known number of mRNAs that are bound and localized in yeast to closer to the recently published data on *Drosophila* (Lecuyer *et al.*, 2007).

In addition, a cryptic Scp160p binding site in *ASH1* mRNA 3'UTR (APyrUGA tandem repeat in position 80 after the stop codon), determined by homology from the binding affinity of Vigilin from

X. laevis (Cuningham *et al.*, 2000) should be confirmed. It could help explain the data obtained for *ASH1* mRNA half-life in *SCP160* disrupted cells.

5.4 Khd1p, another mRNA binding protein associated to the Endoplasmic Reticulum (ER)?

It has been found that locasome components She3p and Myo4p are required for proper ER inheritance to the daughter cell (Estrada *et al.*, 2003). Furthermore, Jaedicke and Schmid have shown that She2p, the major RNA binding protein, also associates with ER (Schmid *et al.*, 2006). In fact, they also elegantly showed that the *ASH1* mRNP was located at the tip of an ER tubule and were also able to confirm biochemically the interaction between the locasome and the ER, most strikingly the association between She2p and ER. In the absence of *she2*, ER is still inherited as expected, but the *ASH1* mRNP is no longer located at the tip of the migrating ER tubule, but at the nuclear periphery.

Therefore, the question arose whether Khd1p, as a potential member of the locasome, is also associated to the ER.

Results presented in section 4.5 have shown that Khd1p is a binding partner for She3p, independently of She2p. In addition, Khd1p has been shown also to float with ER membranes (Fig. 9A), even in the absence of She2p. Therefore, one can also imagine that at the tip of the migrating ER tubule She3p-Khd1p protein complexes can exist, since the association between She3p and Khd1p does not require She2p. This hypothesis is strengthened by the fact that one can float Khd1p with ER membranes in the absence of She2p. This observation points to either a direct binding of Khd1p to ER or to an ER associated factor, such as She3p, as shown in figures 6 and 7, since She3p has been shown to be involved in ER inheritance (Estrada *et al.*, 2003). The fact that Khd1p distribution partially mimicks She3p distribution in this 4 step gradient (Fig. 9A), strengthens this hypothesis. It is conceivable that the *ASH1* mRNP detected by Jaedicke and Schmid, associated to the tip of the migrating ER tubule, also contains Khd1p, since She2p-She3p-Khd1p-Myo4p should be a part of this *ASH1* mRNP. As Khd1p binds over 300 mRNAs (Irie K, personnal communication) it is possible that part of these mRNAs are localized. In this case, a fraction of these could be localized along with the *ASH1* mRNP and others could be localized using the ER inheritance machinery.

The finding that She3p-Khd1p interaction is independent of She2p is quite striking and can have implications in mRNA localization models. It would be extremely interesting to see whether She3p is indeed at the tip of the migrating ER tubule and whether one can also find Khd1p there. This

would indicate that Khd1p is indeed a She3p cargo and thus mimicking She2p, although binding a different set of mRNAs, but also with some common ones (e.g. *ASH1*). The possibility that Khd1p might not be at the tip of a migrating tubule would indicate that these Khd1p containing particles would be the She3p-Khd1p particles refered previously, observation that would also confirm Khd1p association to the ER.

Although Khd1p does not co-purify Sec61p, a bona-fide ER marker (Fig. 9B), one cannot rule out that Khd1p binds directly to the ER. In addition, the translation repressor function of Khd1p could also be aiding in this ER floatation assay, ribosomes bound to both ER and translationally repressed mRNA would float. Additional experiments addressing the issue of direct binding of Khd1p to the ER, independently of ribosomes would help in answering these questions and in determining whether She3p is the sole bridge between Khd1p and the ER. Instrumental in answering this question should be the Khd1-L284R-point mutant that corresponds to Ile304Asn from FMR-1 (Siomi *et al.*, 1994). This Ile304Asn mutant, a point mutant in a KH domain in FMR-1, leads to loss of FMR-1 polysome association (Laggerbauer *et al.*, 2001).

5.5 A new model for ASH1 mRNP architecture

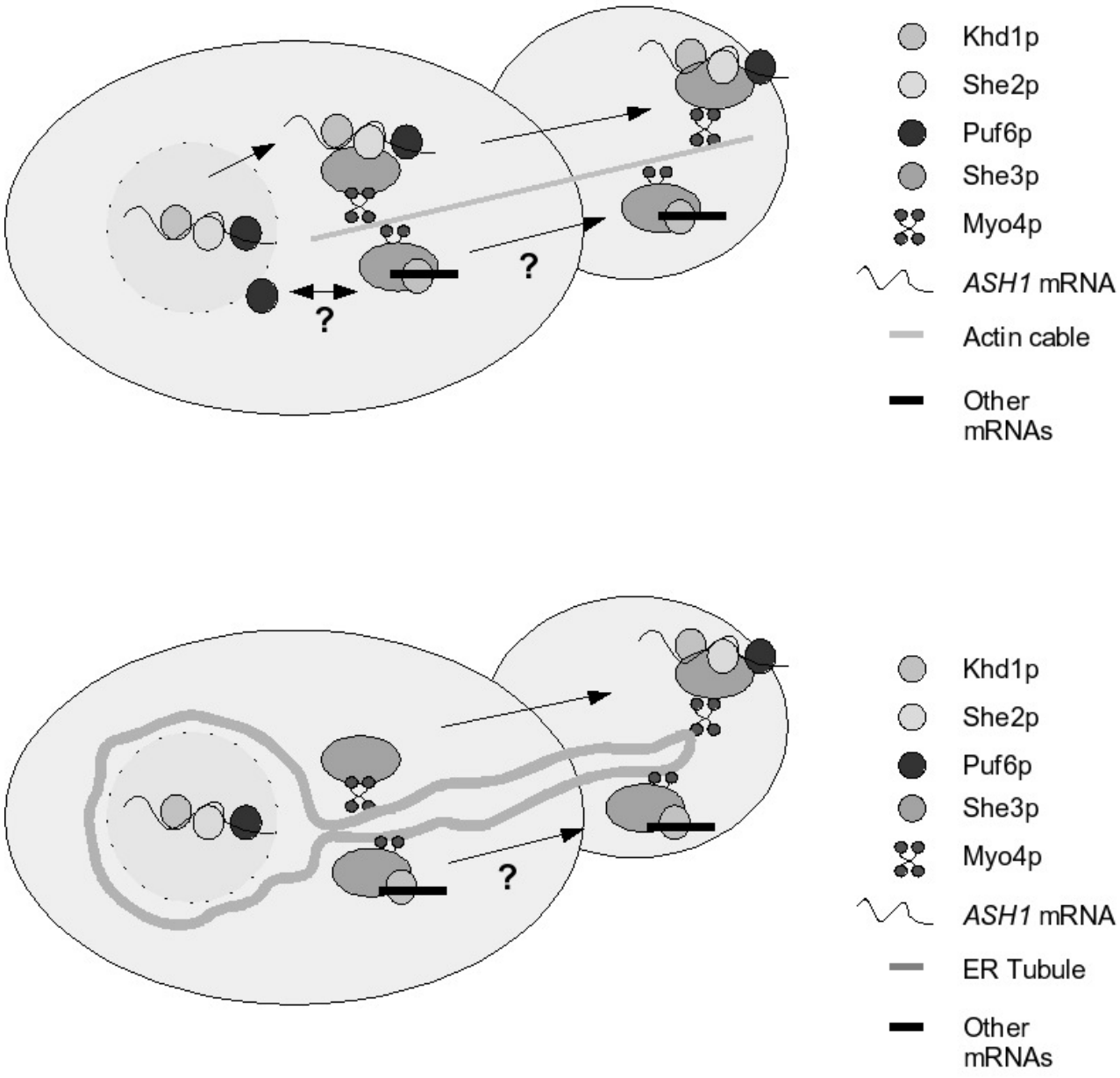


Fig. 14 - Putative Models for Khd1p function in mRNA localization.

Results presented in sections 4.6-4.9 indicate that a revision of the *ASH1* mRNP architecture model is needed. The results presented also indicate the existence of She3p-Khd1p protein complexes, where no She2p exists and that might also be localized in a She-dependent manner. This could increase the number of bud tip localizing mRNAs significantly. A new model that includes Khd1p directly bound to She3p and to the mRNA is proposed in Figure 14. It also includes a derivation of the Schmid *et al.*, (2006) model to include Khd1p associated to ER and associated to She3p, independently of She2p.

5.6 Khd1p has been proposed to be a translation repressor.

5.6.1 Does it show translation inhibitor sensitivity?

Previous contributions on Khd1p function had assigned a translation repressor function to Khd1p (Irie *et al.*, 2002; Paquin *et al.*, 2007). The fact that this function was assigned only in regard to an effect on a single transcript left space to try and generalize the translation repressor function. Genes that tamper with ribosome biogenesis can also be falsely interpreted as translational repressors, due to changes in processivity, as discussed above.

Drugs that can hamper the translation process *per se* are conventionally used to address roles of genes that are thought to be related to the ribosome biogenesis and ribosomal function (e.g. *SCP160* in Baum *et al*., 2004; *CTK1* in Roether *et al.*, 2007). In this way, when a general translation defect exists then one can easily observe at which stage this defect takes place, whether it is at rRNA processing or other ribosome maturation steps, whether it is at subunit joining or at elongation. For Khd1p, no inhibitor of translation gave significantly different results from those observed for wildtype (Fig. 10). This would mean that Khd1p has no effect on the ribosome *per se*, does not affect rRNA processing or any other ribosome maturation process, but can be a translation repressor as proposed.

5.6.2 Is Khd1p associated with polysomes?

As a translation repressor generally prevents the translation of an mRNA, it also prevents the assembly or the advance of the ribosome. In fact, when a ribosome cannot advance, no more ribosomes can be loaded, due to the stalling of this large multiprotein complex and so in order to measure the effectiveness of a translation repressor one looks at the presence of the translation repressed mRNA at the polysomes. Polysomes are chains of actively translating ribosomes. During the course of this work it was shown that in the presence of Khd1p, *ASH1* mRNA is not co-fractionating with heavy and thus highly active polysomes (Paquin *et al.*, 2007). Results presented in figures 11 and 12, show that Khd1p is present in the light polysomes and is absent from the heavier polysomes, supporting the proposed translation repressor model. Upon disruption of the ribosomes, Khd1p migrates to the even lighter fractions, although one should expect more Khd1p associated to the 40S subunit if it is indeed an mRNA translation repressor effect. On the other hand, if it is a eIF4E-eIF4G related event, so a cap-binding complex event, such as in the case of Cup in *Drosophila* (Wilhelm *et al.*, 2000; Wilhelm *et al.*, 2003), or Maskin (Cao and Richter, 2002), one should expect that Khd1p only binds to one of them, either to eIF4E (specially to eIF4E) or to eIF4G and not to both. In this respect, Gavin and Paquin are in contradiction, since Gavin

(Gavin *et al.*, 2002; Gavin *et al.*, 2006) finds Khd1p with both and Paquin (Paquin *et al.*, 2007) only with eIF4E.

Although no experiments were performed in regard to eIF4Ep and eIF4Gp association to Khd1p, the observation that Khd1p is not mostly associated to the 40S subunit after EDTA treatment, but is still present in the heavier fractions, creates a difficult problem to solve.

The observation that Khd1p is only present in the lighter polysomes might be an indication that these are membrane-bound polysomes and therefore, would point to an ER structure organization, specially if one considers that membrane bound polysomes cannot conceptually be loaded indefinitely onto an mRNA. Although it might be true that Khd1p is a translation repressor, there is still much confusion in regard to the mechanism by which it really functions, since the proposed mechanism involving eIF4Ep and Yck1p cannot entirely be fitted on to the data.

5.7 Khd1p, an mRNA binding protein?

Khd1p has been shown to bind *ASH1* E1 element (Irie *et al.*, 2002) and to localize at the bud tip. The fact that this was the only available target for Khd1p led us to try to investigate whether other mRNAs are also targeted. We were specially interested in analysing whether Khd1p would bind to mRNAs that encode for proteins members of the telomere maintenance pathway, since this finding could connect the Denisenko and Bomstzky (2002) findings and Khd1p translation repressor function (Irie *et al.*, 2002; Paquin *et al.*, 2007). After validation of the experimental approach (Fig. 13A), involving RNA purification and northern analysis we observed that the *SIR2, SIR3, RAP1, TLC1* probes used yielded no significant enriched signal, as compared to a mock purification, indicating that either they are not bound by Khd1p-CaBP-TEV-ProA or that the probes designed do not span over the bound region. The use of other probes could provide additional information that could allow us to find out if indeed Khd1p binds any of these messages.

5.8 Ultraviolet CrossLinked ImmunoPrecipitation (CLIP)

Although the direct and defined binding approach did not yield any relevant results, we still wanted to know which other mRNAs, other than *ASH1*, are bound by Khd1p. For that purpose we used the CLIP assay. This assay, developed by the Darnell laboratory, to study protein-RNA interactions is a powerful tool to analyse bulk RNA binding to a purified protein complex. In fact, this approach has been used successfully to elucidate mRNA binding partners for Nova-1 (Ule *et al.*, 2003; Ule *et al.*, 2005). With Khd1p, we were able to show that indeed it binds RNA (Fig. 13B), in the sense that when a low amount of RNAse was used to treat the purified complex, the gel retardation assay

showed a band shift, indicating a heavier complex and that when more RNAse was used this band shift was reduced.

In addition, the results observed for the non-crosslinked lane where there is also a faint signal would argue that this signal is due to bound DNA, as it is more stable than RNA. In fact, the observation that Khd1p binds to the subtelomeric DNA region (Denisenko *et al.*, 2002) is a strong point in favour of this hypothesis. Even so, we still faced two problems: i) the fact that the purified protein is not so abundant, as seen by the coomassie gel in Figures 4 and 5, ii) the fact that Khd1p contains 3-KH domains that can also bind DNA (Denisenko *et al*., 2002) as DNA contamination of the sample can be a problem for the subsequent steps of identifying the mRNA targets bound by Khd1p.

In conclusion,

- Khd1p seems to be a novel cargo for She3p.
- Khd1p floats with ER membranes and does so independently of She2p.
- *KHD1* disruption is insensitive to translation impairing drugs.
- Khd1p is associated with lighter polysomes.
- Khd1p can bind both DNA and RNA, although until now no other mRNA other that *ASH1* has been validated as a binding target.

PART II

Role of *KHD1* in telomeric gene transcriptional silencing and DNA repair

6. INTRODUCTION

6.1 Organization of genetic information - Chromosomes

The nuclear genome of eukaryotic organisms consists of linear chromosomes. These chromosomes, unlike the circular ones of most prokaryotic organisms, face a problem during replication. As conventional polymerases need RNA primers placed 5' of the DNA sequence to be replicated, the chromosome ends might not be fully replicated or are shortened at the end of each round of replication (Watson, 1972). Over the years, studies in a wide range of organisms have shown different approaches from cells to circumvent this problem. These mechanisms involve inverted-terminal repeats and a covalently bound protein, a cap, at the 5' end as seen for adenoviral replication (Challberg *et al.*,1980; Lichy *et al.,* 1981) and for *Streptomyces* (Bao and Cohen, 2001), hairpin structure at the chromosomal end, as observed for *Vaccinia* virus replication and for *Borrelia burgdorferi* (Baroudy *et al.*, 1983; Winters *et al.*, 1985).

Therefore, higher eukaryotes have to deal with a variety of problems that come with the linear nature of their genome. Namely, 1, how to replicate ends of linear DNA molecules without loss of genetic information and 2, how to prevent chromosome ends to be recognized as DNA double strand breaks that have to be repaired. A special enzyme has evolved and been kept throughout evolution to ensure proper replication of chromosome ends and a large protein network is required to protect the chromosomal ends from degradation and from being mistaken as DNA damage.

6.2 Replication of DNA ends

During DNA replication the two DNA strands of a chromosome are separated and serve as single stranded templates for newly synthesized DNA. A large protein complex, containing a DNA polymerase assembles at the replication fork and polymerizes complementary nucleotides in an unidirectional 5'-3' manner to synthesize the new DNA strands.

While one daughter strand, the leading strand, is continuously generated as the replication fork proceeds, the lagging strand is made discontinuously as short DNA fragments, the Okazaki fragments. This process requires RNA primers to initiate the DNA synthesis. These primers are later removed and the gaps filled in and the fragments are then joined by a DNA ligase. However, at the very 5' end of the newly synthesized DNA strand a gap remains caused by the removal of the original RNA primer. This shorter DNA strand would then be used as a template in the following

rounds of replication leading to an increasing shortening of the chromosomal end with every replication cycle. In most eukaryotes a telomere specific reverse transcriptase, telomerase, has been identified that maintains chromosome ends in a constant length range.

Telomerase recognizes the G-rich parental strand via its RNA subunit. Telomerase RNA forms base pairs with the G-rich single stranded overhang and a translocation step allows several rounds of extension of the single stranded DNA. Replication of the chromosome is then completed by DNA polymerase using the extended strand as template for lagging strand elongation. This replication mechanism leads to a simple, repetitive DNA sequence at the ends of eukaryotic linear chromosomes. The sequence and the length of these repeats varies greatly between different organisms, indicating that the telomeric RNA template is not identical among species and that different repeat lengths might be required to ensure end protection. A 3' single stranded overhang comprised of a few to several repeats of the telomerase synthesized strand has been shown to be present at telomeric DNA in ciliates, yeast and humans (Hemann and Greider, 1999).

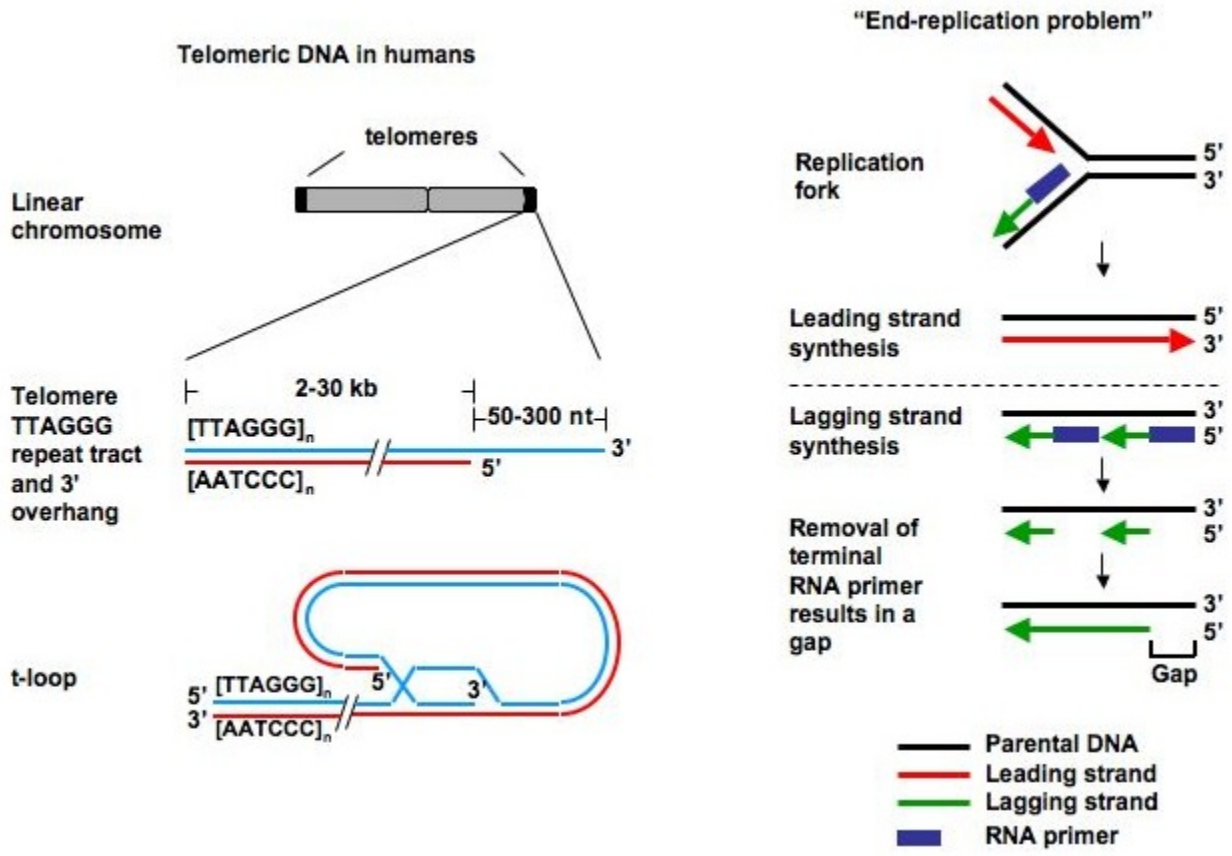


Fig. 15 – End Replication Problem (*Source:* Telomeres, CSHL 2005)

6.3 Telomeres, Senescence, aging and cancer

Early experiments have revealed that normal human fibroblasts show a limited dividing capacity and enter a non-dividing state after a defined number of passages (Hayflick and Moorehead, 1961). Only a very small portion of cells can escape this cell cycle arrest, termed c risis and become immortal, dividing indefinitely. This effect was restricted to somatic cells, whereas germline and tumor cells are immortal. Telomerase is active in germline cells and in 90% of all tumors. In contrast, most human somatic cells lack significant telomerase activity (Broccoli *et al.*, 1995; Counter *et al.*, 1994; Kim *et al.*, 1995; Shay *et al.*, 1993; Vaziri *et al.*, 1993) and telomeres shorten with every round of DNA replication (Allsopp *et al.*, 1992). Somatic cells that escape crisis stabilize telomere length and activate telomerase (Counter *et al.*, 1994), similar to germline cells. Therefore, telomerase is required for indefinite growth of immortal cells in culture. Ectopic expression of hTERT in human fibroblasts restores telomerase activity, stablilizes telomere length and induces indefinite growth (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998), providing strong evidence that the activation of telomerase is sufficient to induce immortalization.

In addition to the limited dividing potential of somatic cells, Hayflick perceived that fibroblast cultures derived from embryos divided more often than those derived from adults (Hayflick, 1965). The observation that the replicative capacity of a cell correlated with donor age, led to the proposal that the cellular dividing capacity correlates with the aging process. Hayflick's predictions have not been able to be thouroughly confirmed experimentally. First, in vivo a variety of somatic cells are quiescent and proliferate seldom if at all. Second, human fibroblasts taken from elderly individuals undergo an additional 20-50 doublings in culture (Dice *et al.*, 1993), representing 40-70% of their dividing capacity as defined by Hayflick. One possible explanation for this contradicting observation would be that most telomere-negative cells may never reach their Hayflick limit *in vivo*. Third, a mouse strain deleted for the RNA component of the telomerase showed no phenotype during the first 2-6 generations and age associated phenotypes, such as graying hair or hair loss and wrinkled skin did not occur earlier than in the control mice (Lee *et al.*, 1998). Only in later generations did the loss of telomerase lead to early-onset of gray hair and hair loss (Rudolph *et al.*, 1999) and germline mortality, thereby inducing sterility (Lee *et al.*, 1998). These observations argue against a model where organismal life span is a function of telomerase activity.

The possibility that enhanced telomere shortening might play a role in human ageing is supported by the analysis of telomeres from patients with premature ageing syndromes. Telomere length of fibroblasts taken from patients with Hutchinson-Gilford progeria, a premature ageing syndrome, was reduced as compared to age-matched control cells (Allsopp *et al.*, 1992). Werner's and ataxia telangiectasia patients, who experience premature ageing exhibit accelerated telomere shortening

(Kruk *et al.*, 1995; Smilenov *et al.*, 1997). In addition, the overexpression of telomerase could restore wildtype telomere length in Werner's syndrome cells and compensated for the early onset of replicative senescence (Wyllie *et al.*, 2000).

Tumors are highly proliferative cells that have escaped growth control or death control and keep dividing indefinitely. Maintaining telomere stability is required for long term proliferation of tumors (Shay and Wright, 1996; Wright and Shay, 2001) and telomerase activity has been detected in approximately 90% of all tumors. However, another mechanism to stabilize telomere length, alternative lengthening of telomeres (ALT), initially described in telomerase negative cells, has also been detected in tumor cells. ALT is independent of telomerase and relies on homologous recombination (Bryan *et al.*, 1995; Dunham *et al.*, 2000). Seemingly, cancer cells have circumvented the progressive telomere loss, present in normal somatic tissues, that is the limiting factor for the cellular replicative potential. Although reconstitution of telomerase has been shown to antagonize cellular senescence, it is not sufficient to induce tumor formation (Hooijberg *et al.*, 2000).

Tumor formation has been shown to be a multistep process in which telomere integrity plays an important role. Since telomerase is required for proliferation of most tumors, it is an attractive target for anti-cancer drug therapy. Interestingly, the inhibition of telomerase in tumor cancer cell lines by a hammerhead ribozyme can induce an immediate apoptotic signal without prior telomere shortening (Ludwig *et al.*, 2001), suggesting that telomerase is not only required for telomere length regulation but is also involved in the capping of telomeres (Blackburn, 2000).

6.4 Telomere structure and telomere associated proteins

Telomeres, the ends of linear chromosomes are physically indistinguishable from DNA double strand breaks. The genetic information of the budding yeast is packaged into 16 chromosomes bearing 32 telomeres in the haploid cell. Simple physical ends of DNA, such as those generated by DNA double stranded breaks (DSBs) or naked telomeres, are genetically unstable, mutagenic and sometimes oncogenic (de Lange, 2005). A single DSB leads to death in cells incapable of repairing these breaks (Resnick and Martin, 1976). Thus, sensing the natural end as a break would be deleterious for the cell and therefore telomeres must have properties that distinguish them from DSBs.

In mammalian cells, double stranded telomeric DNA is bound by two proteins, TRF1 and TRF2 (Chong *et al.*, 1995; Broccoli *et al.*, 1997). Overexpression of TRF1 or TRF2 has been shown to trigger telomeric shortening (Smogorzweska *et al.*, 2000; van Steensel and de Lange, 1997)

indicating that these proteins negatively regulate telomere length and might inhibit telomerase function when bound to telomeres. In mammals, a 75-300 nucleotide long single stranded (ss) overhang exists (Makarov *et al.*, 1997; Wright *et al.*, 1999). Upon overexpression of TRF2 lacking a basic domain this ss overhang at the telomere is lost (van Steensel *et al.*, 1998) and results in chromosome fusions and cell cycle arrest or apoptosis (van Steensel *et al.*, 1998; Karlseder *et al.*, 1999) without a detectable reduction of the telomere length (van Steensel *et al.*, 1998). These results indicate that TRF2 is not only involved in negative telomere length regulation but is also essential in telomeric capping.

Electron microscopy of telomeric DNA purified from human and mouse cells led to the discovery of large loops at chromosome ends in vivo, the t-loops. TRF2 is capable of promoting and stabilizing the formation of a t-loop structure, where the single stranded G-rich extension is buried into more proximal double stranded regions (Griffith *et al.*, 1999; Stansel *et al.*, 2001). Mutations, like the TRF2 basic domain truncation, that might prevent the stable formation of the t-loop could facilitate the opening up of the chromosome ends and also fusion events.

In *S. cerevisiae*, that has a very short telomere repeat tract, the presence of this t-loop seems to be unlikely. However, a number of observations suggest that this back-folding indeed occurs (Fig. 15). Budding yeast telomeres repress transcription of adjacent genes, a phenomenon called telomeric positioning effect (TPE) (Gottschling *et al.*, 1990). The placement of an enhancer 1-2Kb away from the gene at the telomere can be activated (de Bruin *et al.*, 2001). This result strongly supports the notion that the *S. cerevisiae* telomere folds back on itself into subtelomeric regions. The fact that Rap1p, repressor/activator protein-1, a protein that binds sequence-specific to telomeres, can co-immunoprecipitated with sub-telomeric chromation regions (Strahl-Bolsinger *et al.*, 1990) allows the prediction of a 3kb telomeric end in budding yeast.

6.5 Telomerase – completing the end

The telomerase specific DNA polymerase, telomerase, was identified in *Tetrahymena* using biochemical assays designed to test for an activity that incorporates radioactively labeled dGTP in chromosomal ends (Greider and Blackburn, 1985). This incorporation could be abolished by treatment of *Tetrahymena* extracts with RNAse, suggesting that the enzymatic activity was dependent on an RNA subunit as proposed for reverse transcriptases (Greider and Blackburn, 1987). The enzymatic activity was purified, yielding also a 160bp RNA fragment (Greider and Blackburn, 1989). The purified 160bp RNA fragment, the telomerase RNA, has diverged significantly through evolution. It varies from 159 nucleotides in *Tetrahymena*, to 450 nucleotides

in mammals (Blasco *et al.*, 1995; Feng *et al.*, 1995) to up to 1,3kb in *Saccharomyces cereviseae* (Singer and Gottschling, 1994). Strikingly, the secondary structure of this RNA seems to have been conserved through evolution (Chen *et al.*, 2000; Lingner *et al.*, 1994; Romero and Blackburn, 1991). A pseudoknot structure has been proposed for ciliate and vertebrate telomerase RNA containing a single stranded template region (Chen *et al.*, 2000). Several other conserved domains, helices and stem loops, are present that might play in function or stability of the telomerase RNA or might be required for the interaction with the protein subunit of the telomerase (Sperger and Cech, 2001).

The catalytic protein component, telomerase reverse transcriptase (TERT) was first identified in *Euplotes aediculatus* (Lingner *et al.*, 1997b). The protein that was purified with telomerase activity contains a signature motif for reverse transcriptases. This protein showed homology to yeast protein Est2p. Mutations in *EST2* had been previously identified as leading to replicative senescence (Lingner *et al.*, 1997a) and homology searches have shown that this enzyme is phylogenetically conserved.

In budding yeast, the *TLC1* gene, encoding the telomerase RNA, was initially identified in a screen for genes that, when expressed in high amounts, would suppress telomeric silencing (Singer and Gottschling, 1994). A genetic screen designed to identify mutations that lead to a defect in telomerase activity, rendered five genes, as well as *TLC1* (Lendvay *et al.*, 1996). These mutations, *EST1-4* lead to *e*ver *s*horter *t*elomeres until senescence (Lendvay *et al.*, 1996). Although Est1p, Est3p and Est4p are required for *in vivo* telomerase activity, *in vitro*, as in higher eukaryotes, it can be limited to Est2p and *TLC1* (Lingner *et al.*, 1997). Est1p and Est4p have the properties of single strand telomere binding proteins (Virta-Pearlman *et al.*, 1996; Wang *et al.*, 2000), but genetic data argues for different roles for these two proteins *in vivo*. Est1p has been proposed to function in directing telomerase to the chromosomal terminus (Zhou *et al.*, 2000). In contrast, Est4p has been proposed to play a dual role while bound to the telomere: it protects the end of the chromosome (Garvik *et al.*, 1995), and regulates telomerase by mediating access of this enzyme to the terminus (Evans and Lundblad, 1999; Grandin *et al.*, 2000). *EST3* encodes a novel 20-kDa protein that has been shown to dimerize, bind RNA and DNA and contribute to the unwinding of the RNA-DNA heteroduplexes (Yang *et al.*, 2006; Sharanov *et al.*, 2006).

The purification of the telomerase reverse transcriptase from *Euplotes aediculatus* yielded also an uncharacterized protein (Lingner and Cech, 1996), indicating that additional proteins might contribute to telomerase function.

Little is known about the regulation of telomerase activity, especially how chromosome replication and telomere elongation are coupled. Recent studies from yeast and humans suggest that telomerase

does not act as a monomer but is present as a multimer *in vivo* (Prescott and Blackburn, 1997; Wenz *et al.*, 2001; Beattie *et al.*, 2001). It is feasible that telomerase is a holoenzyme and a number of other factors mediate its regulation and temporal cell cycle activity. Recently, and in accordance with this prediction, a study has shown that telomerase cell cycle regulation is dependent on Ku heterodimer (Fisher *et al.*, 2004).

6.6 Telomere Length regulation

Within a cell population, telomere length is maintained within a narrow size due to a balance between elongation and shortening, preserving telomere structure. In mammals, additional factors as tankyrase (Smith *et al.*, 1998), Ku (Hsu *et al.*, 1999), the Mre11-Rad50-Nbs1 complex (Zhu *et al.*, 2000), Tin2 (Kim *et al.*, 1999) and hRap1p (Li *et al.*, 2000) associate indirectly with telomeric DNA via TRF1 and TRF2. One single stranded binding protein, Pot1p, has been shown to bind to the terminal telomeric end (Baumann and Cech, 2001).

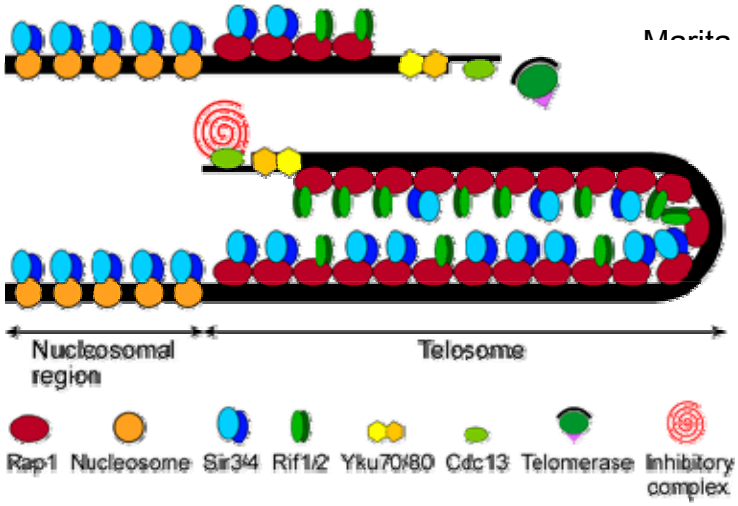


Fig. 16 - Schematic view on Telomere structure, showing the main proteins involved in telomere regulation.

An extremely complex and increasingly longer list of genes that influence telomere length regulation as been drawn from budding yeast (Fig. 16). Several proteins have been shown to be responsible for the regulation of telomerase function at yeast telomeres. A conditional loss of function mutation in Cdc13p/Est4p, a protein that binds ss telomeric DNA (Lin and Zakian, 1996) and is essencial for *in vivo* telomerase activity (Lendvay *et al.*, 1996), causes an increase in telomere length (Grandin *et al.*, 1997).

When shifted to restrictive temperature, this Cdc13-1p mutant accumulates unusually long telomeric single-stranded extensions of the 3' G-rich strand (Garvik *et al.*, 1995; Polotnianka *et al.*,

1998). A suppresor of this single-stranded phenotype, Stn1p, interacts with Cdc13p by two hybrid criteria (Grandin *et al.*, 1997). A third protein, Ten1p, has been shown to be a part of this complex (Grandin *et al.*, 2001). Conditional loss of function mutants in Stn1p or Ten1p lead to telomere elongation and the accumulation of ss telomeric DNA (Grandin *et al.*, 1997; Grandin *et al.*, 2001) as described for the *cdc13-1* mutation. Mutations in the catalytic subunit of polymerase ⟨, *POL1*, that disrupt its interaction with Cdc13p, cause elongation of the telomere (Qi and Zakian, 2000). Interestingly, mutations in Cdc13p have been described that lead to telomere elongation and others that lead to telomere shortening (Grandin *et al.*, 2000; Meier *et al.*, 2001). These observations provide strong evidence that the telomere regulation cannot be pinpointed to a single factor but has to be looked with all its interacting partners.

Yeast telomeric DNA is assembled into a nonnucleosomal chromatin structure, the telosome (Wright *et al.*, 1992). The major protein at the telomere, Rap1p, binds sequence-specifically to telomere repeats (Berman *et al.*, 1986; Conrad *et al.*, 1990; Wright *et al.*, 1992). Rap1p was originally identified as a transcriptional regulator that can play a role in either repression or activation (Kurtz and Shore, 1991; Shore and Nasmyth, 1987). The consensus sequence for Rap1p binding (Buchman *et al.*, 1988) is found approximately at every 35 bp in telomeric DNA (Wang and Zakian, 1990) and therefore multiple Rap1p proteins bind at telomeres (Gilson *et al.*, 1993). Yeast strains carrying a C-terminally truncated Rap1p have elongated telomere repeat tracts (Hardy *et al*., 1992). This C-terminal mutation has been described to prevent the interaction between Rap1p and one of the four Silent Information Regulation (SIR) proteins, Sir3p (Roy and Runge, 1999). SIR proteins interact with Rap1p at different sites in the genome such as the mating type loci, rDNA loci and at the telomeres (Gotta *et al.*, 1996). The *SIR* complex is essential for silencing in yeast and *sir2*, *sir3* or *sir4* mutants are no longer able to repress mating type loci *HML* and *HMR* (Ivy *et al.*, 1986) or telomere adjacent genes (Aparicio *et al.*, 1991). In addition, *sir4* mutants have a slight but stable reduction in telomere length (Palladino *et al.*, 1999).

Interestingly, certain components of the DNA damage response pathways, originally identified by their involvement in the repair of DNA double strand breaks (DSBs) are found at telomeres and are required for wildtype telomere length and function. The Ku heterodimer, essential for the repair of DNA damage by non-homologous end joining, localizes to telomeres in budding yeast (Martin *et al.*, 1999). Yeast Ku is required for the normal localization of telomeres at the nuclear periphery and has been shown to interact with proteins of the nuclear matrix (Galy *et al.*, 2000). A disruption on either *YKU70* or *YKU80* gene leads to a growth defect at elevated temperatures (Feldmann *et al.*, 1996; Feldmann and Winnacker, 1993) and to enhanced sensitivity to the DNA damaging agents bleomycin and methyl methanosulfonate (MMS) (Feldmann *et al.*, 1996; Mages *et al.*, 1996). Ku is

in addition required for the maintenance of wildtype telomere structure and length. Yeast strains deficient for *YKU* exhibit stable but shortened telomeres (Boulton and Jackson, 1998; Porter *et al.*, 1996), loss of subtelomeric silencing (Galy *et al.*, 2000; Gravel *et al.*, 1998; Mishra and Shore, 1999; Nugent *et al.*, 1998; Pryde and Louis, 1999) and a G-rich single stranded overhang is detectable in *yku* mutants over the entire cell cycle, not only just during S-phase. In *S. pombe* and mammals, loss of Ku results in telomere-telomere fusions (Baumann and Cech, 2000; Hsu *et al.*, 2000; Samper *et al.*, 2000).

Another complex that is required for the repair of DNA damage and also is needed for telomeric length regulation in yeast is Mre11p/Rad50p/Xrs2p (Nbs1p in mammals) complex (MRX complex). The localization of this complex to telomeres has been investigated in mammalian cells (Lombard and Guarente, 2000; Zhu *et al.*, 2000) and a deletion of the corresponding yeast genes leads to telomere shortening (Boulton and Jackson, 1998; Nugent *et al.*, 1998). Mutations on *TEL1* and *TEL2*, the first genes described that had a role in telomeric length regulation, lead to temperature-sensitivity and to short telomeres (Lustig and Petes, 1986). This progressive shortening event occurs with every generation until reaching a stable level, after approximately 150 generations. Double mutations with any component of the MRX complex and a *tel1* mutation causes no additional change in telomere shortening, suggesting that *tel1* and the MRX complex both act in the same pathway to regulate telomere length (Ritchie and Petes, 2000).

Rap1p is a key player at the telomere in that it binds the double stranded telomeric repeats and helps to recruit additional proteins. Rif1p and Rif2p, Rap1p interacting factor 1 and 2, are recruited to the telomeres by their interaction with Rap1p C-terminus. Once at the chromosomal ends, they negatively regulate telomere length (Wotton and Shore, 1997). Telomere length homoeostasis is thought to be achieved by a negative feedback circuit: the activity of the telomerase on a specific telmere creates more binding sites for Rap1p. This would lead to more Rap1p bound to the telomere, recruiting more Rif1p/Rif2p in the process. This accumulation of proteins would prevent this telomere from further being used as a substrate for telomerase (Smogorzewska and de Lange, 2004), achieving then a stable steady state.

Genes, other than those already mentioned, have also been shown to play some role in telomeric length regulation.

RAD27 encodes a multi-functional nuclease involved in processing Okazaki fragments during DNA replication, base excision repair (BER), and maintaining genome stability (Liu *et al.*, 2004). Its 5'-flap endonuclease activity is required to cleave the 5' flap from Okazaki fragments that is generated

during lagging strand synthesis and to remove the 5'-deoxyribosephosphate end that is formed at apurinic/apyrimidinic sites during BER (Kao Hi *et al.*, 2002; Rossi *et al.*, 2006; Wu *et al.*, 1999). *RAD27* has also been implicated in double-strand break repair via non-homologous end-joining. Rad27p is highly conserved in bacteria, other fungi, and mammals (Carr *et al.*, 1993; Reagan *et al.*, 1995; Bibikova *et al.*, 1998; Hansen *et al.*, 2000). It contains three highly conserved domains, two of which are conserved in prokaryotes (Lieber *et al.*, 1997). Because deletion of *RAD27* in *S. cerevisiae* leads to expansion of repetitive DNA and trinucleotide repeat instability, *RAD27* (known as *FEN1* in mammals and humans) has been implicated in the triplet repeat expansions that lead to Huntington disease and fragile X (Jonhson *et al.*, 1995; White *et al.*, 1999; Otto *et al.*, 2001).

A mutation in both *KHD1* and *PBP2*, genes that encode proteins with 3-KH (hnRNP K homology) domains, lead to an elongated telomere (Denisenko *et al.*, 2002). Although this elongated telomere has been proposed to be due to the loss of the Telomeric Rapid Deletion pathway function, dependent on the MRX complex, it still remains to be proven (Denisenko *et al.*, 2002). In addition, *KHD1* has been shown to cause a slower growth phenotype in conjuntion with a *RAD27* disruption (Pan *et al.*, 2006), evidencing a putative link between *KHD1* and *RAD27*.

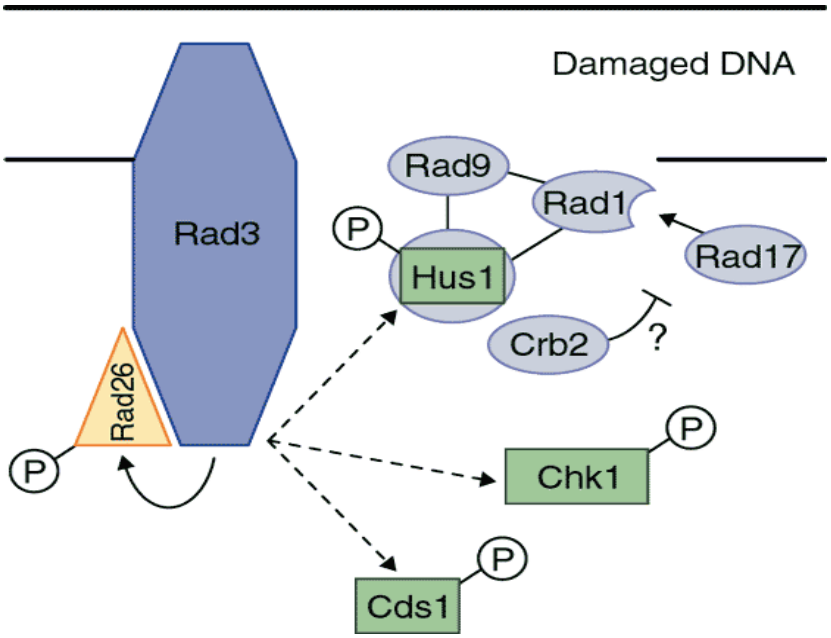
Telomere length regulation has been a proficuous field in findings. Recently, a checkpoint network of genes has been uncovered. Mutations in *DDC1* and *RAD53* of budding yeast (Longhese *et al.*, 2000), *rad1* and *rad3* of *S. pombe* (Dahlen *et al.*, 1998) as well as mutations in human ATM (Vaziri *et al,*, 1999) have been shown to induce telomere shortening and instability. The observation that a *tel1 mec1* double mutant, the budding yeast ATM homologs, shows replicative senescence (Ritchie *et al.*, 1999) supports these findings. A model for how a checkpoint gene might regulate telomere length was provided by the finding that the telomere shortening observed for *rad53* mutants could be restored by increased dNTP pools, indicating that limiting dNTPs may cause a defect in DNA replication at the telomere (Longhese *et al.*, 2000). On the other hand, *ddc1* mutants exhibit short telomeres independent from the intracellular dNTP level, suggesting that the *DDC1* checkpoint affects telomere ends by a different mechanism (Longhese *et al.*, 2000). The regulation of checkpoint genes and their cell-cycle coordination, are therefore, very important mechanisms in maintaining telomere length.

6.7 Telomere disfunction and the DNA damage checkpoint

The *RAD9* checkpoint in *S. cerevisiae* (Fig. 17) serves to arrest the cell cycle in G2 phase when a

DNA damage is present or when DNA replication is incomplete (Weinert and Hartwell, 1988; Weinert and Hartwell, 1993). The ends of linear chromosomes are capped and folded into a special structure and a large network of proteins is required to maintain telomere integrity. Analysis of the *cdc13-1* mutation, that affects telomere capping, provided evidence that a defect at telomeres can trigger a *RAD9* dependent cell cycle arrest. When *cdc13-1* cells were grown at a semipermissive temperature an increase in recombination events could be detected at telomeric regions but not at the centromere (Garvik *et al.*, 1995). In addition, an accumulation of ss DNA was detectable at elevated temperatures at the telomeres in *cdc13-1* mutant cells (Garvik *et al*., 1995), suggesting that the viable phenotype observed might be due to a loss of essential genetic information. The loss of Stn1p, an interactor of Cdc13p, also induces ss DNA formation at the telomeres and mutants show an activated *RAD9* checkpoint (Grandin *et al.*, 1997).

Fig. 17 - RAD9 checkpoint (Adapted from Mitchelson and Weinert, 1999).



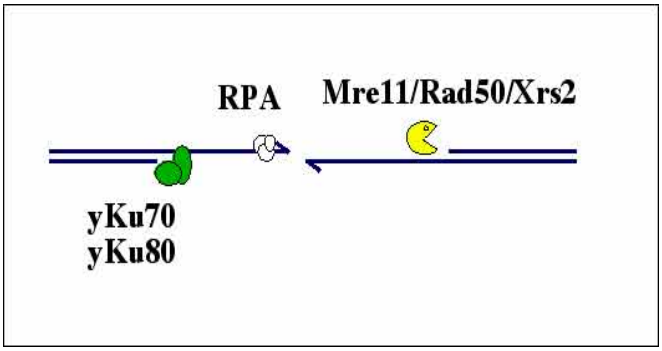


Fig. 18 - Recognition of the ssDNA stretch.

Single stranded DNA (Fig. 18) might be one lesion that activates the DNA damage checkpoint (Garvik *et al.*, 1995; Lydall and Weinert, 1995). This control mechanism also becomes active in *yku* mutants when they are shifted to elevated temperatures (Teo and Jackson, 2001). Overexpression of telomerase has been shown to suppress the temperature sensitivity (Nugent *et al.*, 1998). Although it can suppress the checkpoint activation, ssDNA is still present at the telomeres (Teo and Jackson, 2001). This suggests that the telomerase might cap the telomere in *yku* mutants and thus prevent it from being recognized as a DNA break.

In mammalian cells there is evidence that one critically short telomere is may be recognized as a DNA damage and as a consequence induce p53 cell cycle arrest to cause senescence (Burkhart *et al.*, 1999; Chin *et al.*, 1999). In addition, mutations on TRF2 cause the degradation of the ss DNA overhang and lead to the formation of dicentric chromosomes and eventually to senescence (van Steensel *et al.*, 1998) or to p53-mediated apoptosis (Karlseder *et al.*, 1999). Increasing evidence arises that telomere regulation machinery overlaps with the cellular DNA repair machinery and with the apoptotic pathway and that this interconnection is an essential process for proper cellular function.

As a safeguard against the ocurrence of DNA damage prokaryotic and eukaryotic cells have developed at least three different DNA repair mechanisms. In *S. cerevisiae* DNA double strand breaks are repaired by the activities of the *RAD52* epistasis group (Friedberg *et al.*, 1995) (Fig. 19) and this pathway seems to be evolutionarily conserved (Bezzubova *et al.*, 1993). Another mechanism of DNA DSB repair and recombinational events was uncovered (Gottlieb and Jackson *et al.*, 1993; Harley *et al.*, 1995) that involves a DNA-activated protein kinase and its regulatory subunit, the Ku heterodimer (Fig. 19), also known as non-homologous end joining (NHEJ). It has been shown that the Ku heterodimer can be coeluted with Rpb1p and Rad51p (Maldanado *et al.*, 1995) and that it also interacts with DNA polymerase ∑, a specific polymerase involved in DNA repair synthesis (Hwang *et al.*, 1995; Aboussekhra *et al.*, 1995).

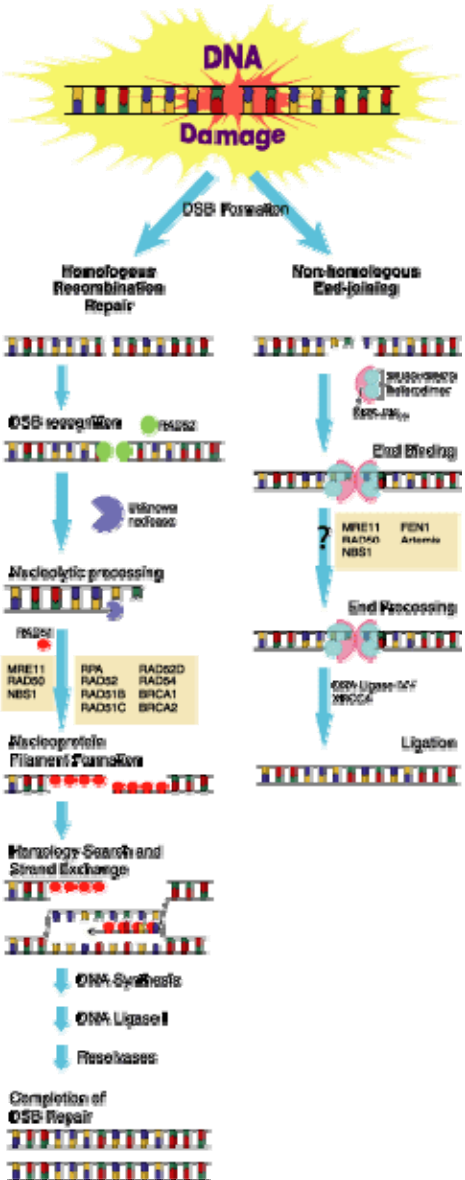


Fig. 19 - DNA Double Strand Break Repair Pathways (adapted from R&D Systems DNA Damage Response Minireview, catalog 2003)

6.8 Telomeres and Nonsense mediated decay

TLC1, the RNA subunit of the telomerase had until recently two possible models for its biogenesis. (Ferrezuelo *et al.*, 2002). i) the shuttling model, that the maturation of *TLC1* occurs in the cytoplasm and is imported back into the nucleus, in a Mtr10p dependent manner and ii) the processing enzime model, where the enzyme responsible for the maturation of the *TLC1* RNA would be imported in an Mtr10p dependent manner. Recently, it has been shown that *TLC1* shuttles into the cytoplasm (Gallardo *et al.*, 2008) and that this shuttling, dependent on Mtr10p, is essential for biogenesis and for telomerase function (Ferrezuelo *et al.*, 2002; Teixeira *et al.*, 2002) . The fact that telomerase includes an RNA intuitively hints that RNA turnover and surveillance mechanisms would be somehow related to telomerase function and maturation. The observation that *TLC1* is a polyadenylated RNA (Lowell and Pillus, 1998) supports the idea that it would follow a regular mRNA processing pathway. It was only very recently that a link between the surveillance NMD mechanism, namely *UPF1,* and *TLC1* and telomerase function was shown (Azzalin *et al.*, 2006; Azzalin *et al.*, 2007). *UPF1* seems to be essential for the cell to cope with basal transcription events that occur at the telomere. This basal transcription produces telomeric transcripts, partially complementary to *TLC1*. These incomplete transcripts, that could compete with *TLC1* for Est1-4p binding, would not elicit a functional telomerase after maturation and the cellular surveillance mechanism targets them for destruction at the nuclear exosome.

6.9 Aim of this work

The aim of this work is to elucidate the role of *KHD1* in telomeric gene transcriptional silencing and the mechanisms underlying its function. For this, a strategy designed to address the role of *KHD1* in this process and in connected processes such as DNA repair, was implemented.

7. RESULTS

Khd1p, also known as Hek2p, has already been shown to have a dual role in the cellular context. Firstly, Irie and coworkers showed that Khd1p accumulates at the bud tip, by using a reporter assay called the green mRNA technology. This assay is based on a fusion of a GFP to a U1A binding protein, that recognizes and attaches to specific U1 RNA loops fused to your mRNA of choice. They also showed that Khd1p binds the E1-element of *ASH1* mRNA and they propose a translation repressor activity for this protein (Irie *et al.*, 2002). Recently this translation repressor activity has been proven (Paquin *et al.*, 2007). Secondly, Khd1p has already been shown to bind to subtelomeric DNA and to play a role in telomeric positioning effect (Denisenko *et al.*, 2002). The work of this group also showed that a *HEK2* deletion leads to a longer telomere (in combination with a *PBP2* deletion, a gene similar to *KHD1* in the sense that it also encodes a 3 KH domain-protein) and probably due to a defect in the telomeric rapid deletion pathway.

7.1 Is KHD1 involved in Telomeric silencing?

How could Khd1p be involved in such different cellular processes? Telomeric positioning and length control occur in the nucleus and mRNA localization and translation regulation occurs in the cytoplasm. Can Khd1p shuttle between cellular compartments?

In the course of this work, Du and coworkers showed that indeed Khd1p shuttles between cytoplasm and nucleus (Du *et al.*, 2008)

With these results in mind we set out to determine if *KHD1* had indeed a role in telomeric silencing and telomeric length control. By using the telomeric silencing system (Fig. 20) developed in the Zakian laboratory (Gottschling *et al.*, 1990), the role of Khd1p was analysed in this pathway.

Fig. 20 - Schematic view of the gene transcriptional silencing assay

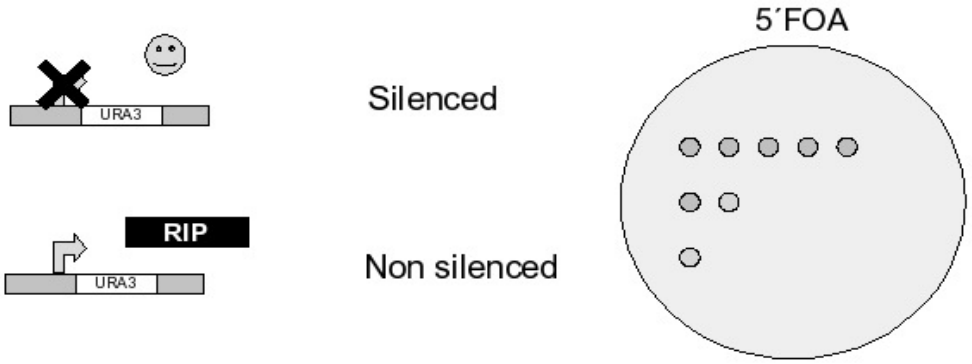
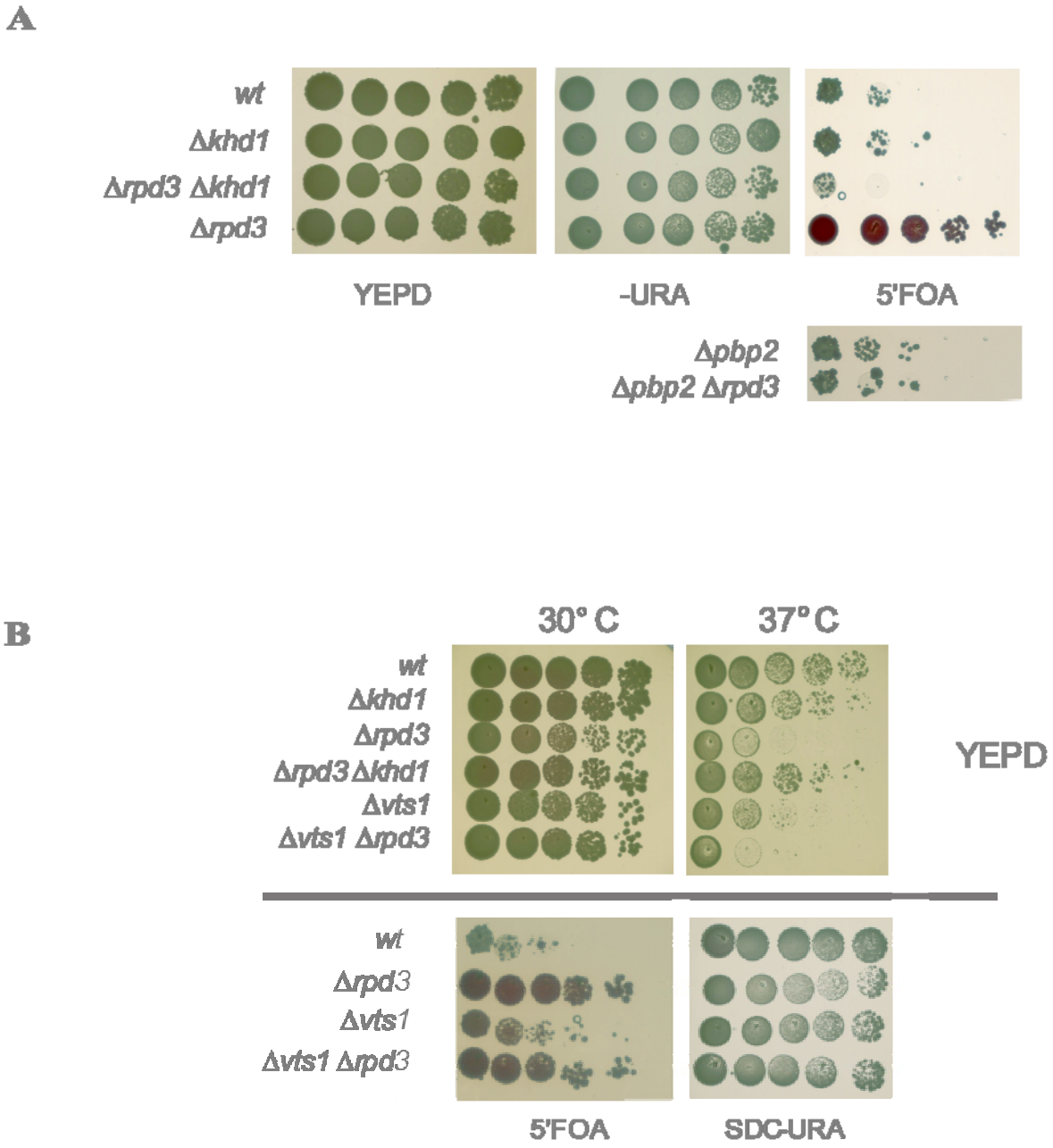


Fig. 21 - *KHD1* in telomeric silencing?
A - Serial dilutions growth tests in different media
B – Serial dilutions growth tests in different media and at different temperature



KHD1 has no significant effect on its own on telomeric silencing. In fact, the surprising result is observed in a combination with *Δrpd3*. *RPD3*, a deacetylase (Rundlett *et al.*, 1996), shows a silenced phenotype, as expected and when *KHD1* is disrupted it leads to a desilencing effect (Fig. 21A).

PBP2 is very similar to *KHD1*, it also has 3 KH domains and it has been shown also to contribute to telomeric silencing and to control telomeric length (Denisenko *et al*., 2002). Again, in our observations *Δpbp2* had no significant effect on silencing by itself. Interestingly it also reverted the silencing phenotype of *Δrpd3*, although to a lesser extent. To rule out a translation related effect, another translation repressor was analysed, *VTS1,* and we observed that it does not cause desilencing with *Δrpd3* (Fig. 21B). On the other hand, *Δrpd3* is slightly temperature sensitive and an additional *Δkhd1* rescues this phenotype, whereas an additional *Δvts1* increases this temperature sensitivity.

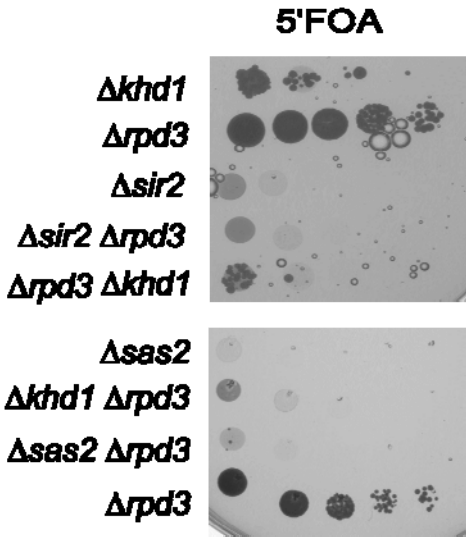


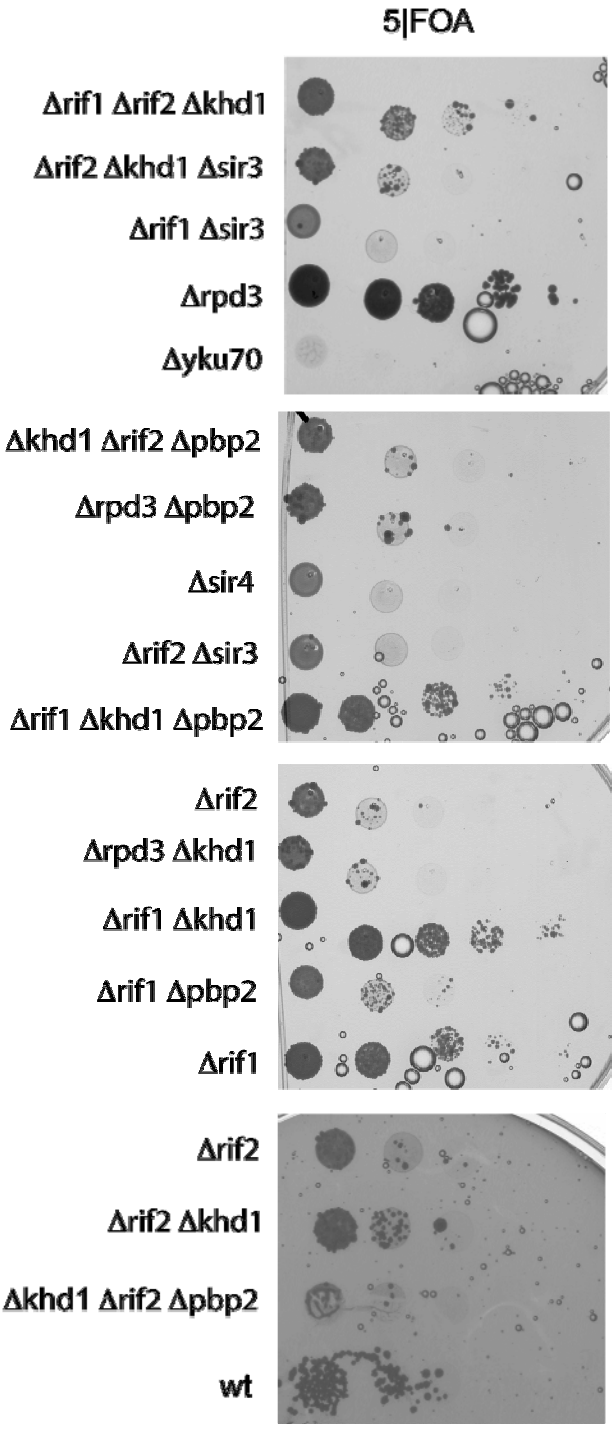
Fig. 22 - Influence of other chromatin remodellers in telomeric silencing?

In addition, we observed that other chromatin remodellers, as *SIR2*, a deacetylase (Aparicio *et al.*, 1991) or *SAS2* (Osada *et al.*, 2001), an acetyl-transferase, lead to a similar phenotype as a *Δrpd3 Δkhd1*. A *Δsir2 Δrpd3* and *Δsas2 Δrpd3* also show desilencing (Fig. 22), as observed for *Δkhd1 Δrpd3*.

7.2 KHD1 and other mutant combinations

We also analysed the effects *khd1* disruption has in combination with other mutants that have been shown to be involved in telomere gene transcriptional silencing.

Fig. 23 - *KHD1* and other genes involved in telomeric silencing.



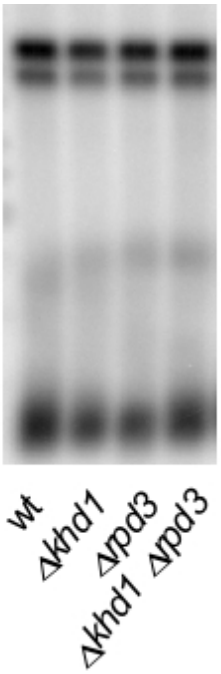
We observed (Fig. 23) that a *Δpbp2 Δrif1* combination leads to a loss of silencing, whereas the single *rif1* disruption is clearly silenced. Furthermore, we observed that a Δ*rif2* Δ*khd1* Δ*pbp2* is surprisingly desilenced as compared to a *rif2* disruption or even to Δ*rif2* Δ*khd1,* that is slightly better silenced than a *rif2* disruption.

We also observed that an additional *khd1* disruption to both *rif1* or *rif2* disruptions leads to better silencing.

As expected cells that do not possess an active *SIR* complex (meaning Δ*sir2,* Δ*sir3* or Δ*sir4*) are desilenced and do not grow.

7.3 Does Δkhd1 influence telomeric length? Does Δpbp2 act the same way?

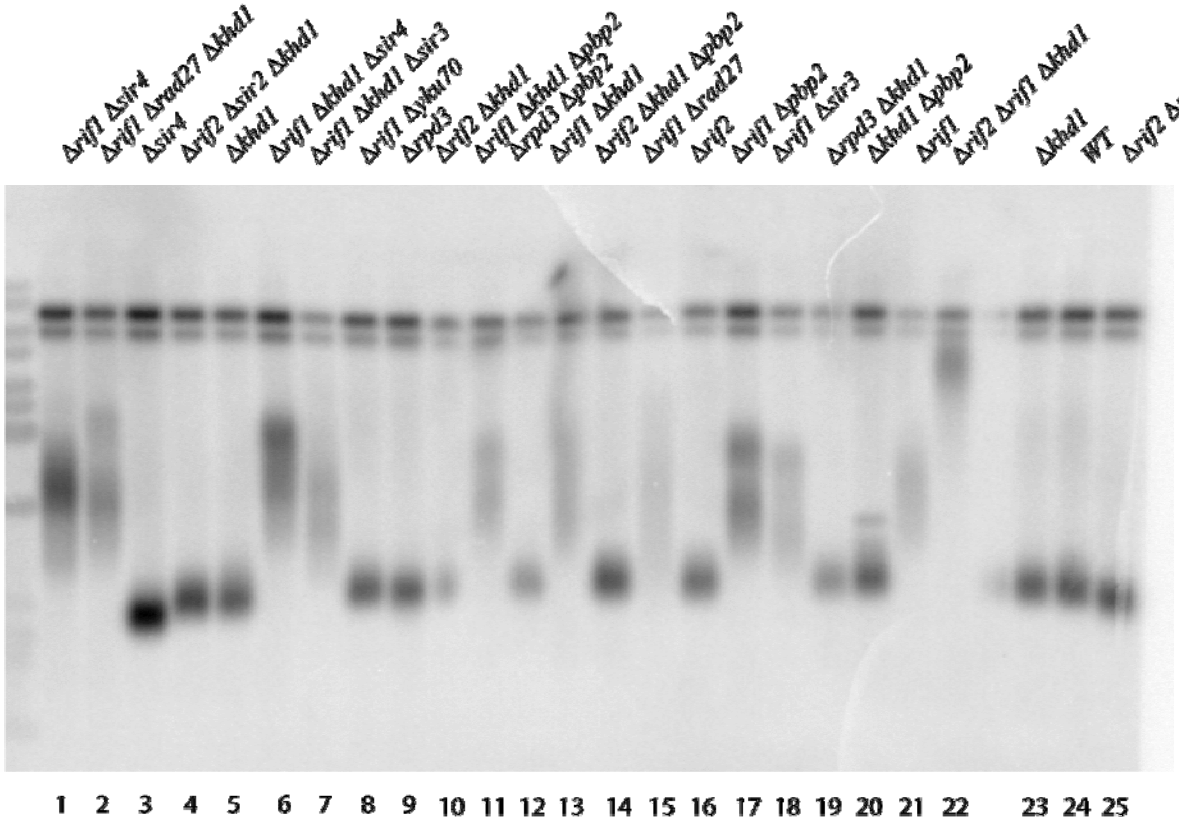
Fig. 24 - Telomeric length – Southern Blot probed with TG1-3 repeat probe.



KHD1 disruption does not change the length of the telomeric region on its own (Fig. 24). Not surprisingly it does not change telomeric length on *Δrpd3* (Fig. 24), suggesting that the observed loss of silencing is not related to a change in telomeric length.

7.4 Can the other phenotypes observed be explained by changes in telomeric length?

Fig. 25 - Telomeric Silencing – Southern Blot, probed with TG1-3 repeat probe.



We observed that an additional *Δkhd1* increases the telomeric length of a *Δrif1* (Lanes 13 and 21 – Fig. 25) fitting with a minor increase in the gene transcriptional silencing of *Δrif1*. Furthermore, we observed that an *Δkhd1* Δ*rif2* (Lanes 10 and 16) shows no change in telomeric length, although this cannot explain the change in telomeric gene transcriptional silencing observed (seen in Fig. 25). In addition, *Δpbp2* Δ*rif1* and Δ*rif1* (Lanes 17 and 21) show differences in telomeric length, but this cannot also explain the variation on gene transcriptional silencing observed (Fig. 25).

Δpbp2 Δrpd3 and *Δrpd3* (Lanes 9 and 12) show no telomeric difference indicating the changes observed for gene transcriptional silencing cannot be due to a change in telomeric length.

Although both Δ*khd1* and Δ*pbp2* contribute to *Δrif1* telomeric elongation, that is not the case for Δ*rif2* (Lanes 10, 14, 16).

Another surprising finding is that an aditional *khd1* disruption to a *Δrif1 Δrad27* leads to an even

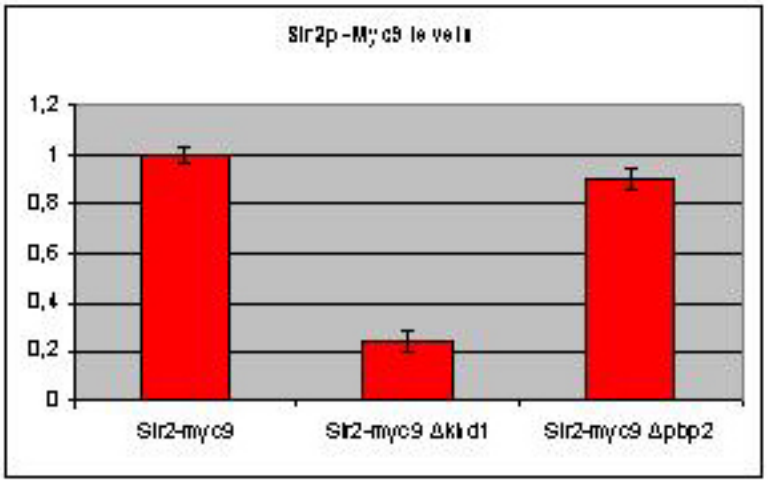
more elongated telomere (Lanes 2 and 15 – Fig. 25).

7.5 Loss of Silencing due to loss of SIR complex?

As shown on figure 22, mutations that affect Sir2p localization (e.g. *SAS2*) or even a complete disruption mimick the observed phenotype for *Δrpd3 Δkhd1*.

Could the desilencing effect observed for *Δrpd3 Δkhd1* be related to a loss of Sir2p or to its mislocalization?

Fig. 26 - Sir2p-Myc9 levels downregulated due to *Δkhd1*?
Myc9 signal quantification (n=5) by Western Blot, using ImageQuant 5.0.



A disruption of *khd1* leads to a severe downregulation of Sir2p-Myc9, by approximately 3/4, whereas this is not observed when *PBP2* is disrupted (Fig. 26), thus indicating that this is a specific effect of *KHD1*. This downregulation of Sir2p is at the post-transcriptional level, since mRNA levels did not differ (Fig. 27).

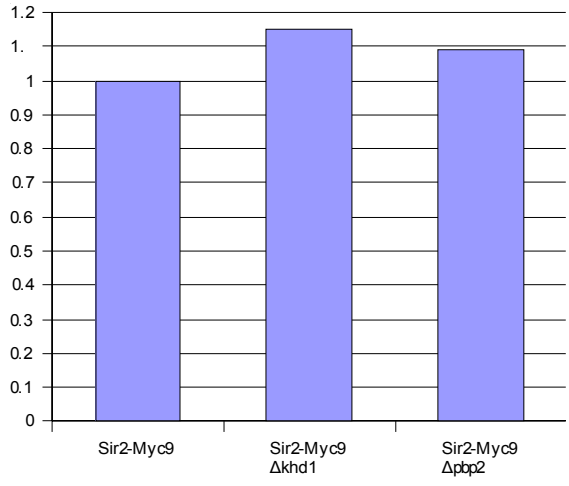
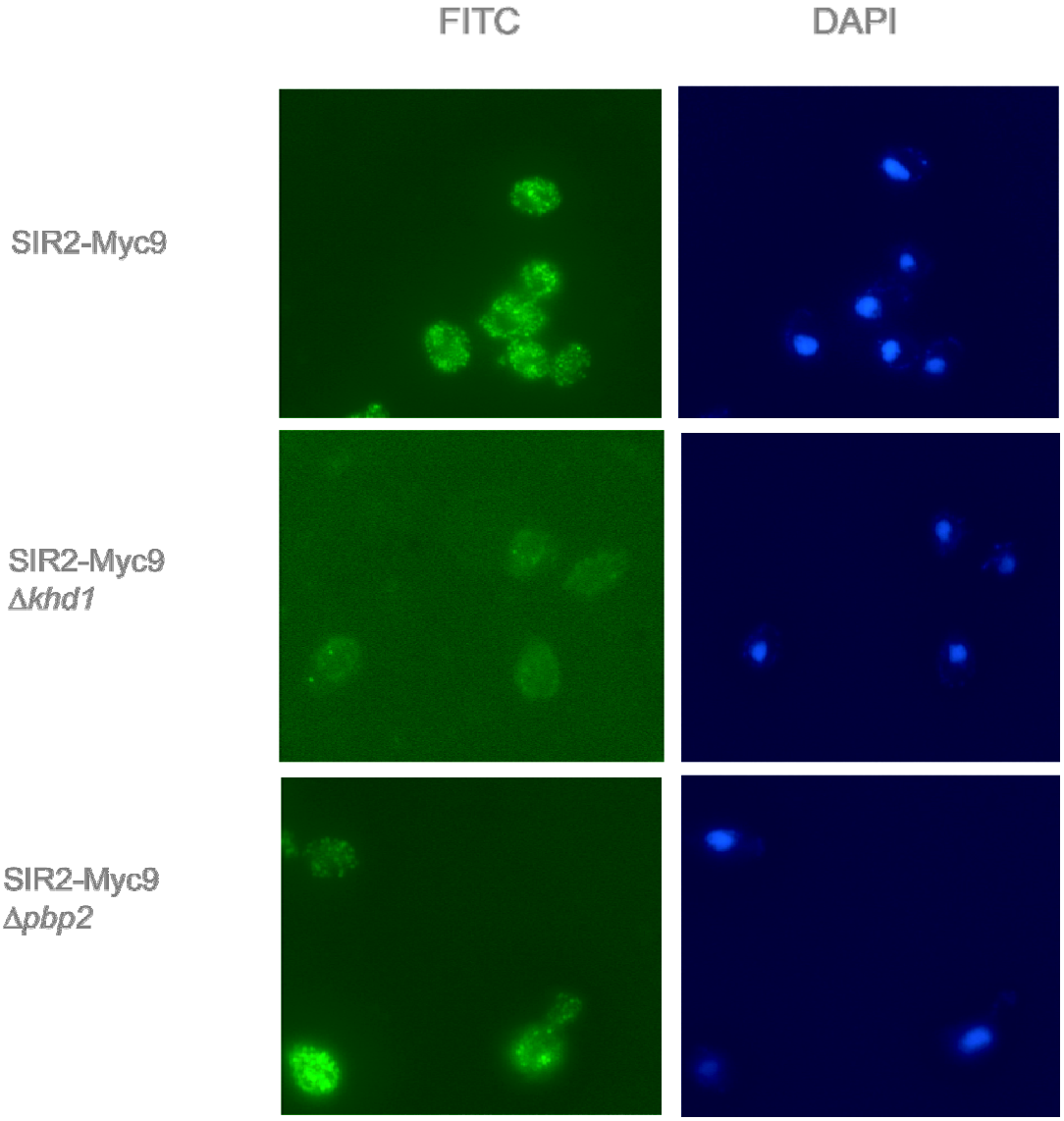


Fig. 27 - *SIR2* mRNA levels, measured by Northern Blot (n=2)

7.6 Does this decrease of Sir2p amount also lead to a displacement of Sir2p from subtelomeric regions, thus further explaining why a desilencing is observed?

Fig. 28 - Indirect Immunofluorescence.
Imaging of Sir2-myc9 on wt, *Δkhd1* and *Δpbp2*. Primary – 9E10, anti myc 1:200, Secondary - rabbit anti mouse Alexa 488.



As can be seen in figure 28, almost no signal was observed for Sir2p-myc in a *Δkhd1*. This result, consistent with the very low abundance on the western blot level (Fig. 26), does not allow any conclusion on the localization of Sir2p in a *Δkhd1*.

7.7 Can the desilencing effect observed for Δrpd3 Δkhd1 be rescued by providing an excess of SIR2?

It has been shown that overexpression of *SIR2* leads to cell death (Holmes *et al.*, 1997) and that this effect can be rescued by an upregulation of Histone H4 (Matecic *et al.*, 2002).

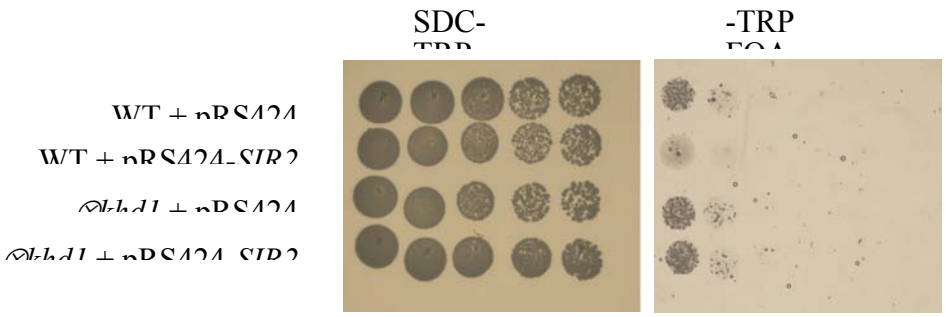


Fig. 29 - Serial dilutions and growth analysis.

We observed that *SIR2* driven from a multicopy plasmid causes indeed death on FOA for the wildtype but does not do so for *Δkhd1*. This result indicates that our Sir2p downregulation model for *Δkhd1* is true (Fig. 29).

7.8 Can a Δrpd3 Δkhd1 desilencing effect be rescued with a lower copy number SIR2?

As can be seen in figure 30, a CEN-plasmid bourne-*SIR2* increases cell viability of *Δrpd3 Δkhd1* on 5'FOA containing media, but does not restore complete silencing, indicating that in this double mutant combination, other factors are also affected, apart from Sir2p.

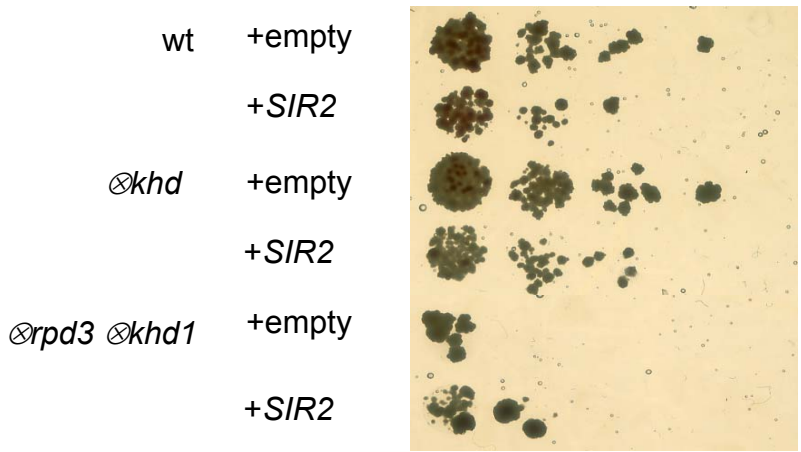


Fig. 30 - Serial dilutions of CEN-plasmid bourne copies of *sir2* (or empty) containing cells.

7.9 Does a Sir2p downregulation, a deacetylase lead to a change in acetylation levels of Histone H4K16?

Sir2p has been shown to actively deacetylate Histone H4K16, and determine the establishment of the euchromatin/heterochromatin boundary (Suka *et al.*, 2002). Could *ΔKHD1* influence changes in heterochromatin/euchromatin formation, namely an increase in Histone H4K16, consistent with the downregulation of Sir2p?

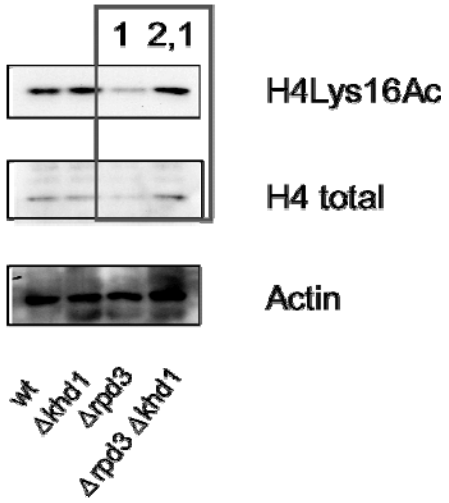


Fig. 31 - Changes in histone modifications?
Western blot of whole cell extracts. Quantification performed using Imagequant 5.0 (N=3).

Table 2 - Histone H4 acetylation levels.

	HH4K8/H4	HH4K12/H4	HH4k16/H4
Wt	**0,24**	**0,75**	**2,205**
Δkhd1	0,215	0,55	2,41
Δrpd3	0,395	1,30	**1,65**
Δkhd1 Δrpd3	**0,09**	**0,93**	**3,4**
Δsir2	0,28	**1,49**	2,085
*Δ*yku70	0,14	0,265	**2,515**
*Δ*yku70 *Δ*khd1	0,13	0,3	**1,015**
Δrad27	**1,415**	**2,27**	**3,08**
Δrad27 Δkhd1	0,36	1,27	1,97

Table 3 - Histone H4 acetylation levels

	HH4k8/Actin	HH4k12/Actin	HH4k16/Actin
Wt	**1,02**	**1**	**1,72**
Δkhd1	1,03	1	**0,64**
Δrpd3	1,03	0,97	**0,06**
Δkhd1 Δrpd3	1,18	0,92	**3,97**
Δrif1	**22,89**	**1,04**	**4,1**
Δrif1 Δkhd1	**2,18**	**0,96**	**1,03**
Δpbp2	1,02	1	0,97
Δpbp2 Δrpd3	0,73	0,9	0,26
Δsir2	1,02	0,99	**2,8**
Δrad27	1,05	0,96	1
Δrif2	**9,67**	0,44	0,93
Δrif2 Δkhd1	**37,78**	0,93	1,11
Δrif1 Δpbp2	1,04	0,98	**1,12**
Δrif1 Δpbp2 Δkhd1	0,97	0,99	**0,43**

RPD3 disruption shows consistently less Histone H4 and the disruption of *KHD1* reverts this effect. The acetylation levels of Histone H4 on Lys 8 and 12 are not significantly different as to explain the changes observed in silencing, when actin is used as a loading control. We looked at the acetylation levels on Lys16, as it is known to be the boundary of telomeric heterochromatin (Suka *et al.*, 2002). This residue shows a significant increase of acetylation in *Δrpd3 Δkhd1* as compared to *Δrpd3*, or even to *Δkhd1* indicating that transcription is therefore more active in this region.

When Histone H4 total levels are taken as a loading control the picture looks somewhat different. Still, there is 2-fold increase for HH4K16Ac between *Δrpd3 Δkhd1* and *Δrpd3*. In addition, the levels of Histone H4 K8Ac are also decreased in *Δrpd3 Δkhd1*.

We observed that *Δsir2* shows almost no changes in HistoneH4 acetylation when HistoneH4 is taken as a loading control, but on the other hand when actin is taken as a reference one can see a 1,7x increase on HH4K16Ac. When Δ*khd1* is analysed in combination with other mutants we observed that it changes the acetylation levels of HH4K8 and of HH4K16, when in combination with Δ*rif1* and of HH4K8 when in combination with Δ*rif2*.

Table 4 - Histone H3 Trimethylation levels.

	H3K4Me3/H3	**H3K36Me3/H3**	**H3K79Me3/H3**
Wt	0,93	1,2	1,63
Δkhd1	0,97	1,38	1,78
Δrpd3	1,01	1,5	**5,51**
Δkhd1 Δrpd3	**1,67**	1,59	**2,5**
Δsir2	0,91	0,78	1,5
*Δ*yku70	**1,56**	0,85	**2,41**
*Δ*yku70 *Δ*khd1	**11,95**	**12,62**	**15,24**
Δrad27	1,05	1,12	1,47
Δrad27 Δkhd1	0,92	1,7	**3,21**

Another histone modification that regulates transcription is Histone H3 trimethylation (Schneider *et al.*, 2004). We observed that an additional *Δkhd1* to *Δrpd3* leads to an increase of H3K4Me3 and a decrease in H3K79Me3 as compared to *Δrpd3*. In addition, we observed that *Δkhd1 Δyku70* leads to dramatic increases in the trimethylation levels for all three lysines analysed, when compared to the wildtype or even the single mutants. Furthermore, the *Δrad27 Δkhd1* also shows an increased H3K79Me3 level as compared to both single mutations.

7.10 Is there a relationship to the telomerase RNA subunit?

As *KHD1* encodes an mRNA binding protein with a translation repressor function, and the telomerase is an enzyme with an RNA subunit, we wanted to see if there was any connection between the two. For this we analysed gene transcriptional silencing when additional copies of the telomerase RNA subunit, *TLC1*, were supplied.

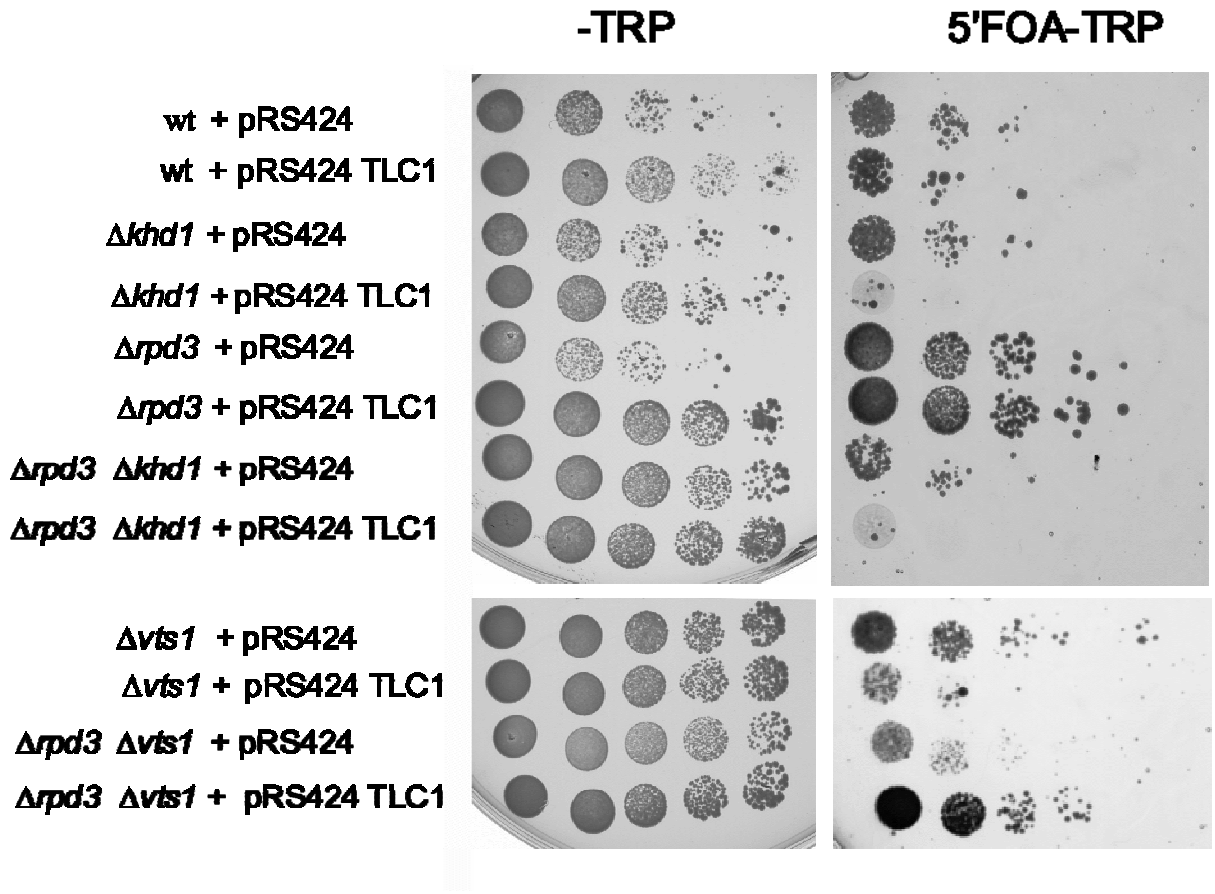


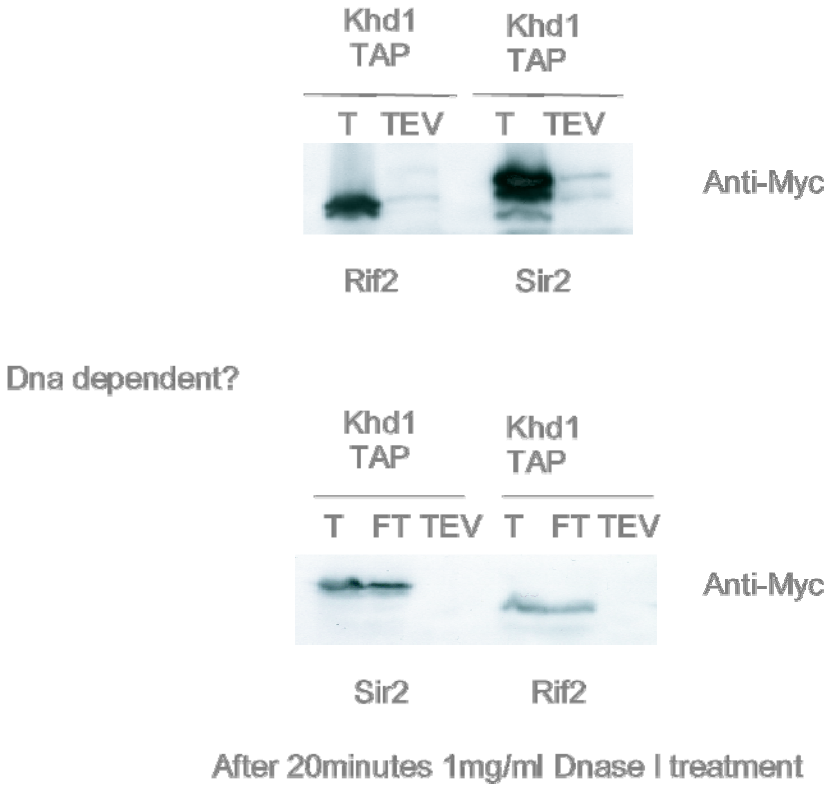
Fig. 32 - Telomerase RNA subunit affects telomere gene transcriptional silencing of mRNA binding proteins mutants?
Serial dilutions and growth for 72hours at 30 degrees.

We observed that an overexpression of *TLC1* leads to death of *Δkhd1* in 5'FOA containing media. We were also able to determine that this effect is not a general translation repressor or even a general mRNA binding protein defect, since *Δvts1* fails to show a similar phenotype, although exhibiting less fitness.

7.11 Is Khd1p a part of a heterochromatin remodelling complex?

As we have shown that *Δkhd1* causes a loss of telomeric silencing, downregulates Sir2p and plays a role in stabilizing the telomere when an excess of *TLC1* is provided, we were wondering if Khd1p is a part of any telomeric protein complex. To address this question, a tandem affinity purification was performed and the co-purification of specific proteins, Sir2p and Rif2p, was investigated. Both can indeed be purified when using Khd1p as bait, but this interaction seems to be DNA dependent (Fig. 33).

Fig. 33 – Is Khd1p a part od a chromatin remodelling complex?
Khd1-TAP TEV-cleaved eluates were probed on western blot for Rif2-myc9p and for Sir2-myc9p. The same experiment was performed after a 20minutes DNAse I treatment.



7.12 Is KHD1 involved in DNA repair?

Denisenko and co-workers proposed that Khd1p is involved in the Telomeric Rapid Deletion Pathway (Denisenko *et al.*, 2002). A major complex involved in this process is the Mre11p/Rad50p/Xrs2p complex. This complex is responsible for homologous recombination events in the telomeric regions leading to Telomeric Rapid Deletion events, i.e. telomere length reduction and is also responsible for non-homologous End-joining and DNA recombinational repair.

We decided to analyse the relationship between this complex and Khd1p and to see if *KHD1* played also a role in DNA repair.

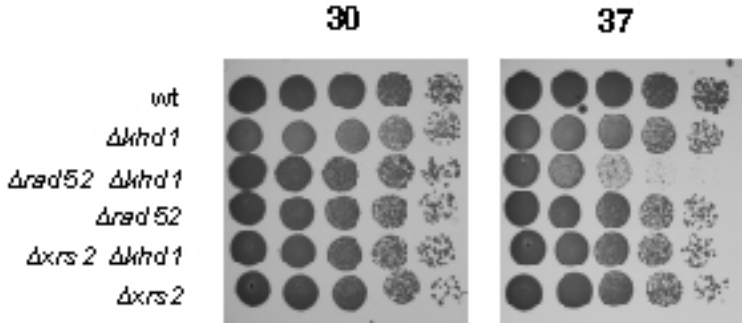


Fig. 34 - Temperature sensitivity of Δ*khd1* Δ*rad52.*
10-fold serial dilutions of overnight cultures were plated onto YEPD plates and incubated at different temperatures.

As shown previously *khd1* plays a role in telomere maintenance. Telomere maintenance and DSB repair are intimately linked. Telomeres are double stranded breaks (DSB's) *per se*, that surprisingly escape the recognition mechanism. Recognition of the double stranded break is mediated by *RAD52*, that distinguishes between DSB's and telomeres (Fig. 19). Another gene that is involved in both telomere maintenance and DSB repair is *YKU70*. This gene binds the telomerase RNA and acts in double stranded break repair by non-homologous end joining. We tested *KHD1* for synthetic lethality with *RAD52* and *YKU70* and both turned out to be negative. We were able to determine that Δ*rad52* Δ*khd1* cells are Synthethic Growth defective at 37ºC while at 30ºC no impaired growth was observed. Furthermore, this observed growth defect was specific for Δ*rad52* Δ*khd1* as Δ*xrs2* Δ*khd1* cells do not exhibit it. This result indicates that *KHD1* can contribute to a pathway parallel to *RAD52*.

7.13 Involved in which pathway of DNA repair?

Proteins involved in telomeric silencing are also related to DNA damage and repair pathways. Defects in DNA repair pathways are usually detected with the help of DNA damaging agents such as MethylMethanoSulphate (MMS), Hydroyurea, ionizing radiation, UV radiation and others.

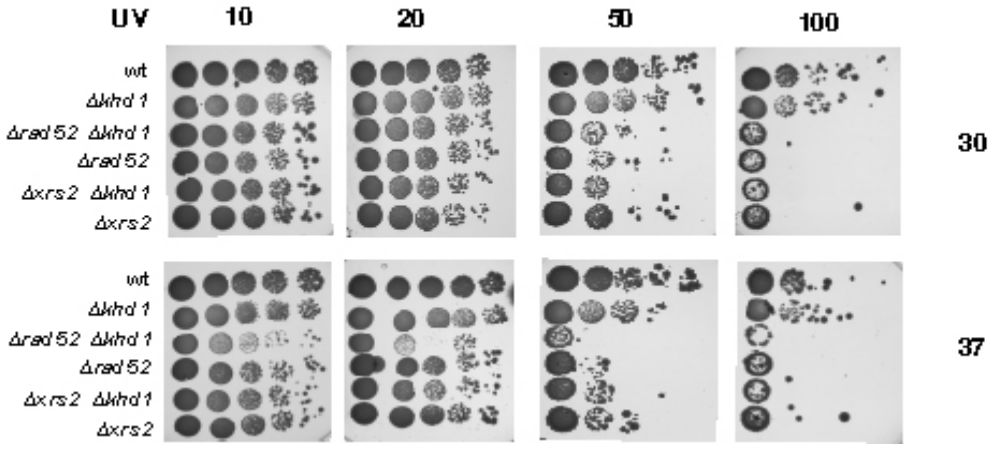
MethylMethanoSulphonate, although initially thought to cause alkylation of the DNA and thus induce DSBs, causes stalling of the replication fork and mutants in the homologous recombination pathway cannot overcome this fork stalling and thus exhibit extreme sensitivity to this agent (Lundin *et al.*, 2005).

Ultraviolet radiation causes thimidine-dimer formation, that cannot pair with the opposing strand, thus leading to a bulge of the DNA molecules. As the DNA replication machinery progresses it can cause stalling, mispairing and mutations (Matsumura and Ananthaswamy, 2004; Davies *et al.,* 2002; Schreier *et al.*, 2007).

Hydroxyurea is a DNA replication inhibitor that causes ribonucleotide depletion by inhibiting the ribonucleotide reductase and can ultimately result in DNA double strand breaks due to the extended replication forks (Platt *et al.*, 2008).

Cells were subjected to increasing amounts of UV radiation exposure and their viability was measured. *khd1* disrupted cells showed no increased sensitivity to UV radiation as compared to the wildtype, in normal growth conditions (Fig. 35). However a *Δrad52 Δkhd1* survived poorly when subjected to elevated temperature and to UV radiation (Fig. 35), indicating an intolerance to this combination of effects, not observable in the single mutants. This intolerance is however not significantly higher than the growth defect observed for growth at 37 degrees.

In addition, a *Δkhd1 Δxrs2* showed an increased sensitivity to 50μJ and to 100μJ as compared to the



single mutants, indicating that indeed *khd1* plays a role in UV damage induced repair.

Fig. 35 - UV radiation sensitivity essay.

10-fold serial dilutions of an overnight culture were plated on YEPD and then subjected to increasing doses of UV radiation. Plates were then incubated at 30 and at 37 degrees.

In order to test if *ΔKHD1* is also sensitive to damage-inducing chemical agents, cells were subjected to increasing concentrations of hydroxyurea. We observed that Δ*khd1* caused no change in sensitivity to hydroxyurea (Fig. 36), indicating that Δ*khd1* does not play a role in replication.

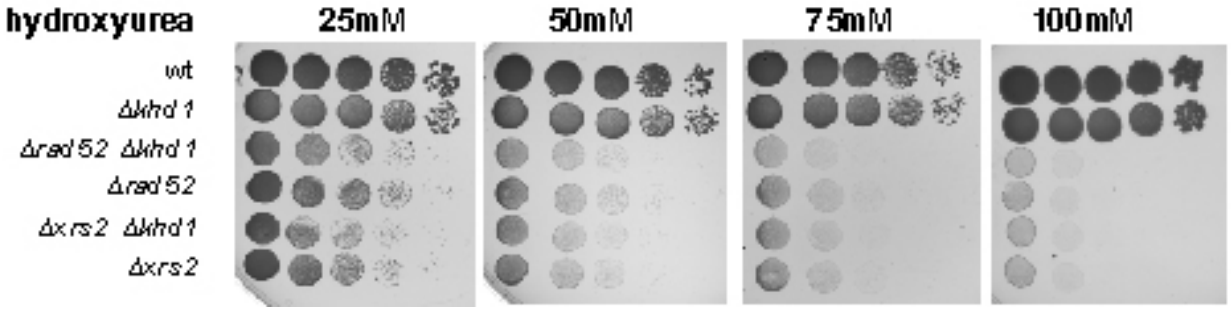


Fig. 36 - Hydroxyurea sensitivity essay.
10-fold serial dilutions of an overnight culture were plated on media containing increasing concentration of Hydroxyurea.

The same experiments were performed with increasing concentrations of MMS. As Δ*khd1* showed an increased sensitivity phenotype to 0,05% MMS than wildtype cells (data not shown), we decided to repeat the same experiments with different combinations of mutants from the contributing double strand break repair pathways, to try to elucidate with which Khd1p, could be involved.

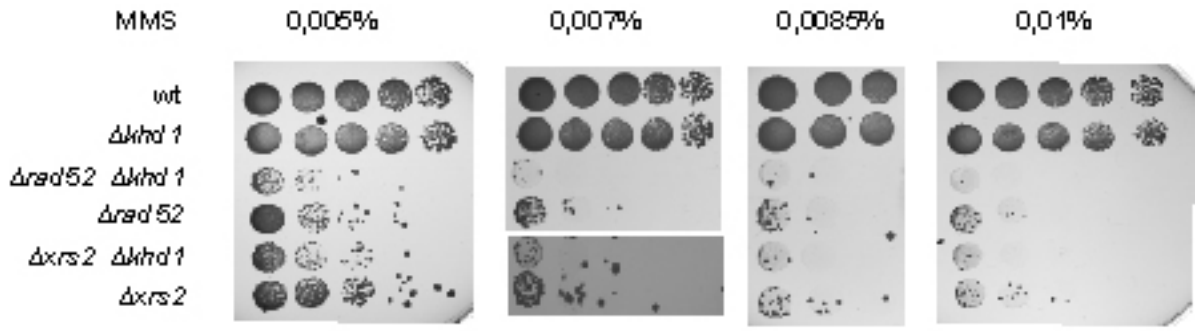


Fig. 37 - MethylMethanoSulphonate sensitivity essay.
10-fold serial dilutions of an overnight culture were plated on media containing increasing concentration of Hydroxyurea.

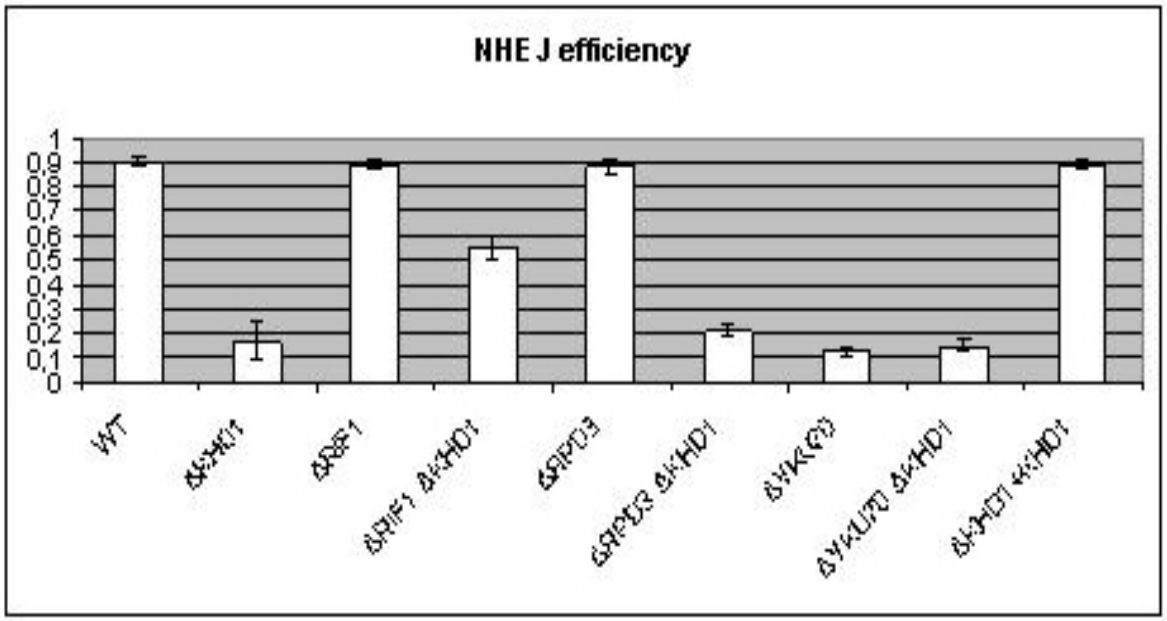
We observed that a Δ*rad52* Δ*khd1* shows an increased sensitivity to MMS than the single Δ*rad52*

mutant, for all MMS concentrations tested (Fig. 37). In addition, the same is true for Δ*xrs2* Δ*khd1*.

7.14 Involved in Non Homologous End Joining?

Non homologous end joining is a process where loose ends of DNA are ligated to other DNA ends, and therefore, repaired. This process normally does not require sequence homology between fragments to take place. We decided to investigate if *KHD1* played a role in this process.

Fig. 38 - Non Homologous End Joining Efficiency.
An overnight culture was diluted to 0.2 and let grow exponentially. When OD600=1 was reached cells were transformed with 0,5µg DNA of a linear plasmid and of a circular plasmid in parallel and plated out in selective media. Plates were incubated 3 days and cfus were counted. Bars represent the ratio between number of cfus with linear plasmid/ number of cfus with circular plasmid.



We observed that *KHD1* alone leads to a dramatic decrease in NHEJ, which can be rescued when supplying an additional *KHD1* copy on a plasmid. As *khd1* has been shown to be involved in telomeric maintenance we also wanted to see if it was a general telomeric maintenance defect. Therefore we analysed *Δrpd3* and *Δrif1* deletions, both alone and in combination with *Δkhd1*. We observed that these genes when disrupted alone do not affect NHEJ but when in combination with a *KHD1* disruption, a NHEJ defect is also observed (Fig. 38). This finding was extremely surprising, as Khd1p has been proposed to be a translation repressor and an mRNA-binding protein (Irie *et al.*, 2002; Paquin *et al.*, 2007). In addition, and even more surprising, was the observation that *KHD1* is epistatic to *YKU70* (Fig. 38). This finding would point to a direct role of Khd1p on NHEJ or a

regulation of a factor that is essential for NHEJ.

7.15 NHEJ coupled to a specific KH-domain?

Khd1p contains 3-KH domains, that are known to bind nucleic acids. We wanted to investigate if we could couple this NHEJ defect to any of the KH domains. We observed that a point mutant in KHdomain3-L284R, still rescues the NHEJ defect but a point mutant in KHdomain2-I183R no longer does so (Fig. 39).

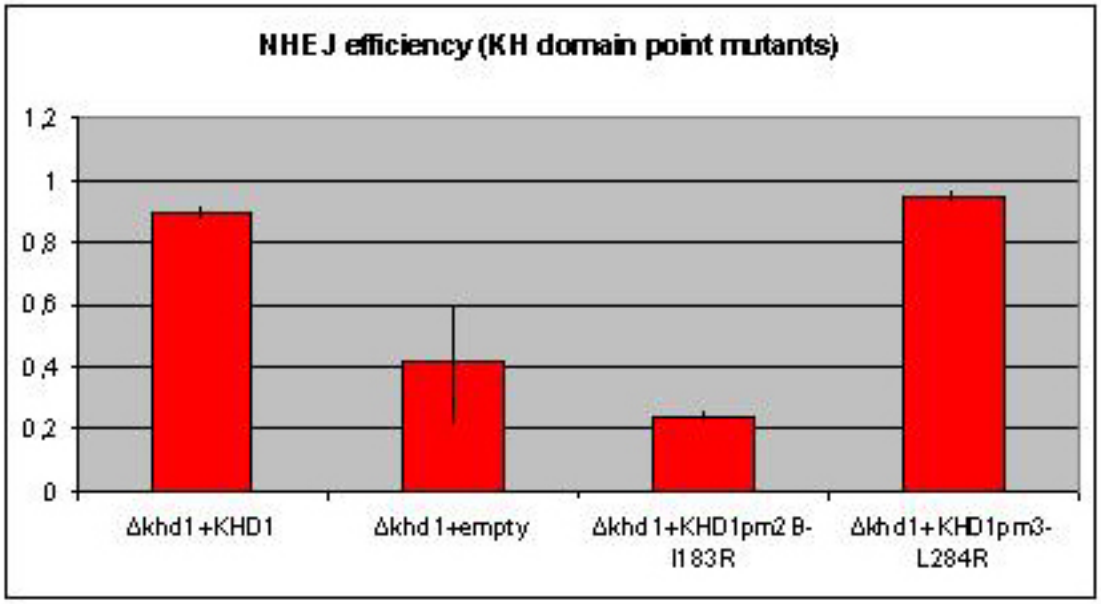


Fig. 39 - Non-Homologous End Joining rescue with plasmid bourne-*KHD1* and *KHD1*-point mutants.

7.16 VTS1, another translation repressor involved in Telomeric Silencing?

VTS1 is a gene that has been shown to be functional homologue to *Drosophila* Smaug protein (SMG1) (Aviv *et al.*, 2003; Aviv *et al.*, 2006). Smaug binds poly-C regions and acts as a translation repressor of *oskar* mRNA, during development of the *Drosophila* embryo (Dahanukar *et al.*, 1999; Zaessinger *et al.*, 2006). Smaug has been proposed also to be involved in mRNA deadenylation via the *CCR4/NOT* complex and thus control stability of the target mRNA (Zaessinger *et al.*, 2006).

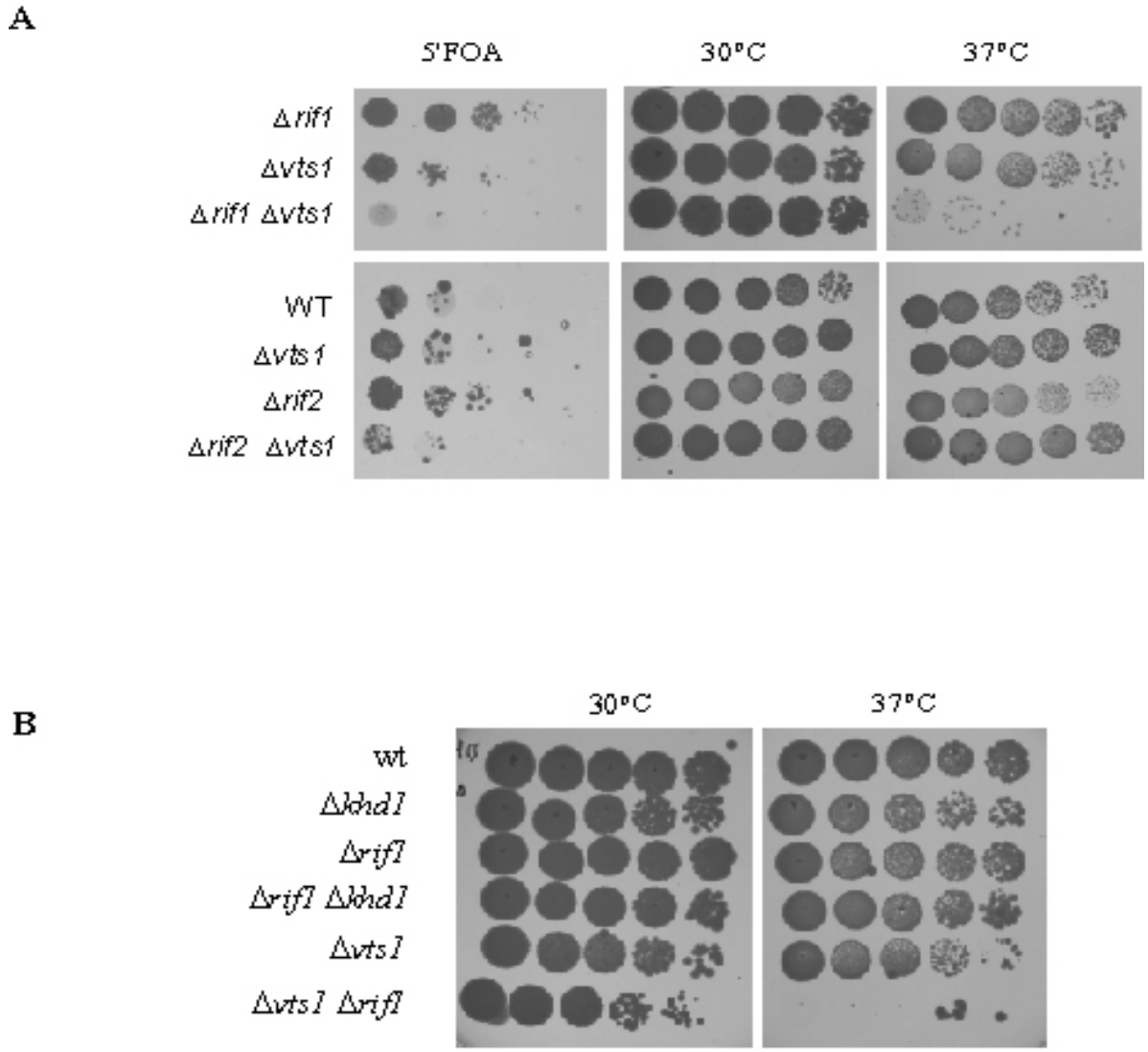
7.17 Could another protein that has been shown to be involved in mRNA localization and translation repression be also involved in telomeric silencing and stability? Is it a process specific to Khd1p?

As can be seen in figure 40 *Δvts1* alone shows no effect on telomeric silencing, the same as observed for *Δkhd1* alone. But in combination with other mutants, *Δvts1* behaves differently than *Δkhd1* (Figs. 21, 23 and 40). Whereas *khd1* disruption showed a severe silencing deffect in addition to a *Δrpd3*, a *Δrpd3 Δvts1* shows no effect (Fig. 21), indicating that it is not translation *per se* that is affecting the genomic stability but rather a *khd1* specific effect. On the other hand, *Δkhd1 Δrif1* showed a slighter increased silencing effect whereas *Δvts1 Δrif1* shows a dramatic loss of telomeric silencing (Figs. 23 and 40). In addition, this *Δvts1 Δrif1* double mutant is temperature sensitive whereas the single mutants are not (Fig. 40).

7.18 Are these effects specific to *Δrif1* alone or are they also true for Δ*rif2*?

Rif2p is a protein partner of Rif1p, and they both act in binding to Rap1p at the telomere and regulate telomere length together (Wotton and Shore, 1997).

Fig. 40 - Role for *VTS1* in telomeric silencing?
A – Serial dilutions and growth in differential conditions, to address silencing.
B – Serial dilutions and temperature sensitivity determination.



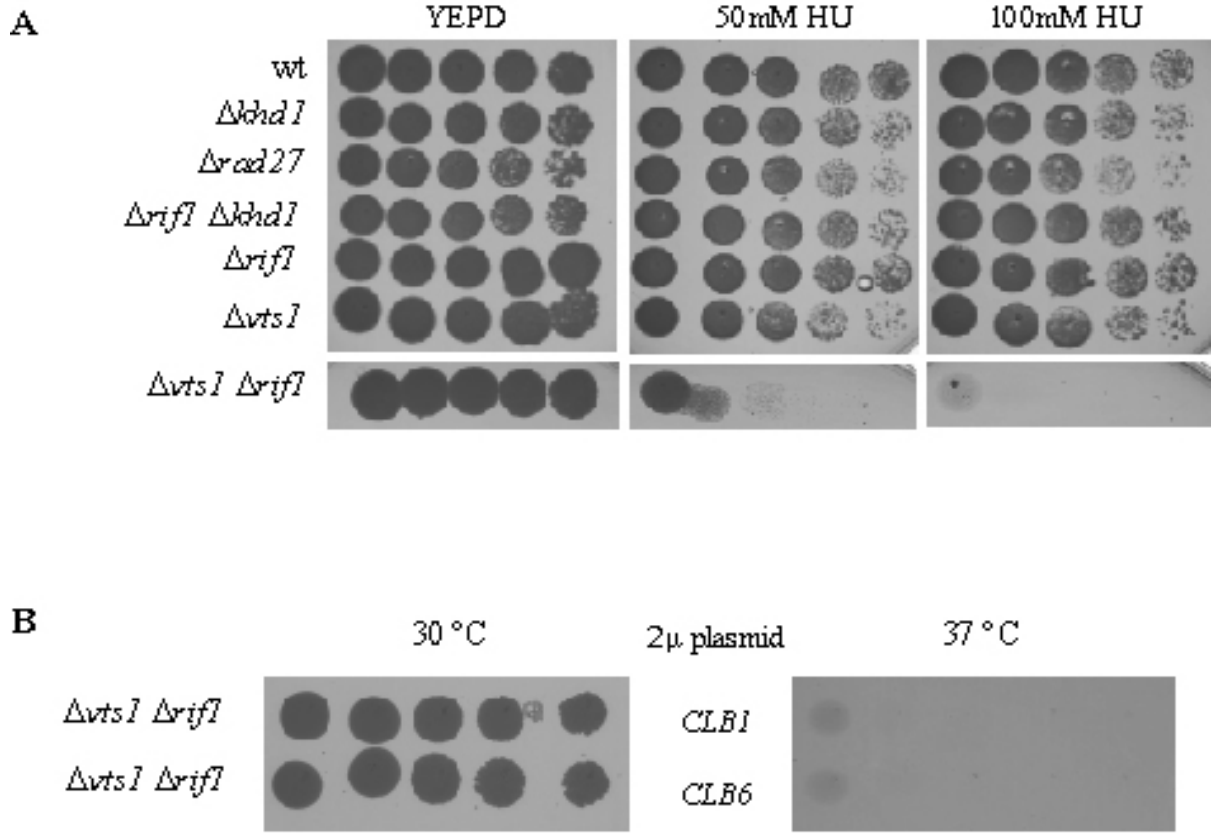
We have observed that *Δrif2 Δvts1* does not behave as *Δrif1 Δvts1* (Fig. 40), as it is not dead on 5'FOA and also not sensitive to 37ºC, suggesting that the effects observed are specific to *Δrif1 Δvts1*, being the first described events where Rif1p and Rif2p act independently.

7.19 Does *Δvts1 Δrif1* show a replication defect?

A temperature sensitivity phenotype in combination to *rif1* disruption can be found associated to loss of silencing, to loss of telomere capping and to very short telomeres (e.g. *Yku70*) and we analysed whether this was also the case for Δ*rif1* Δ*vts1*.

To address this question we analysed these mutants in media containing incrasing concentrations of hydroxyurea.

Fig. 41 - DNA replication defect?
A – Hydroxyurea sensitivity assay.
B – Temperature sensitivity rescue with high-copy cyclins.



We observed that *Δvts1 Δrif1* is extremely sensitive to hydroxyurea (Fig. 41A), a drug that inhibits DNA replication and S-phase progression, indicating a DNA replication problem. This hydroxyurea sensitivity was not overcome with a high-copy plasmid contaning *CLB1* or *CLB6* (Fig. 41B). As *CLB1* is a G2-M cyclin (Surana *et al.*, 1991) and *CLB6* is a DNA-synthesis activator cyclin (Schwob and Nasmyth, 1993; both reviewed in Lew *et al.*, 1997), we ruled out that the arrest would be at these two stages. These results led us to try to map the point of the replication problem. We wanted to check if the cells were arrested at G1-S or at G2-M or not at all, by using FACS analysis.

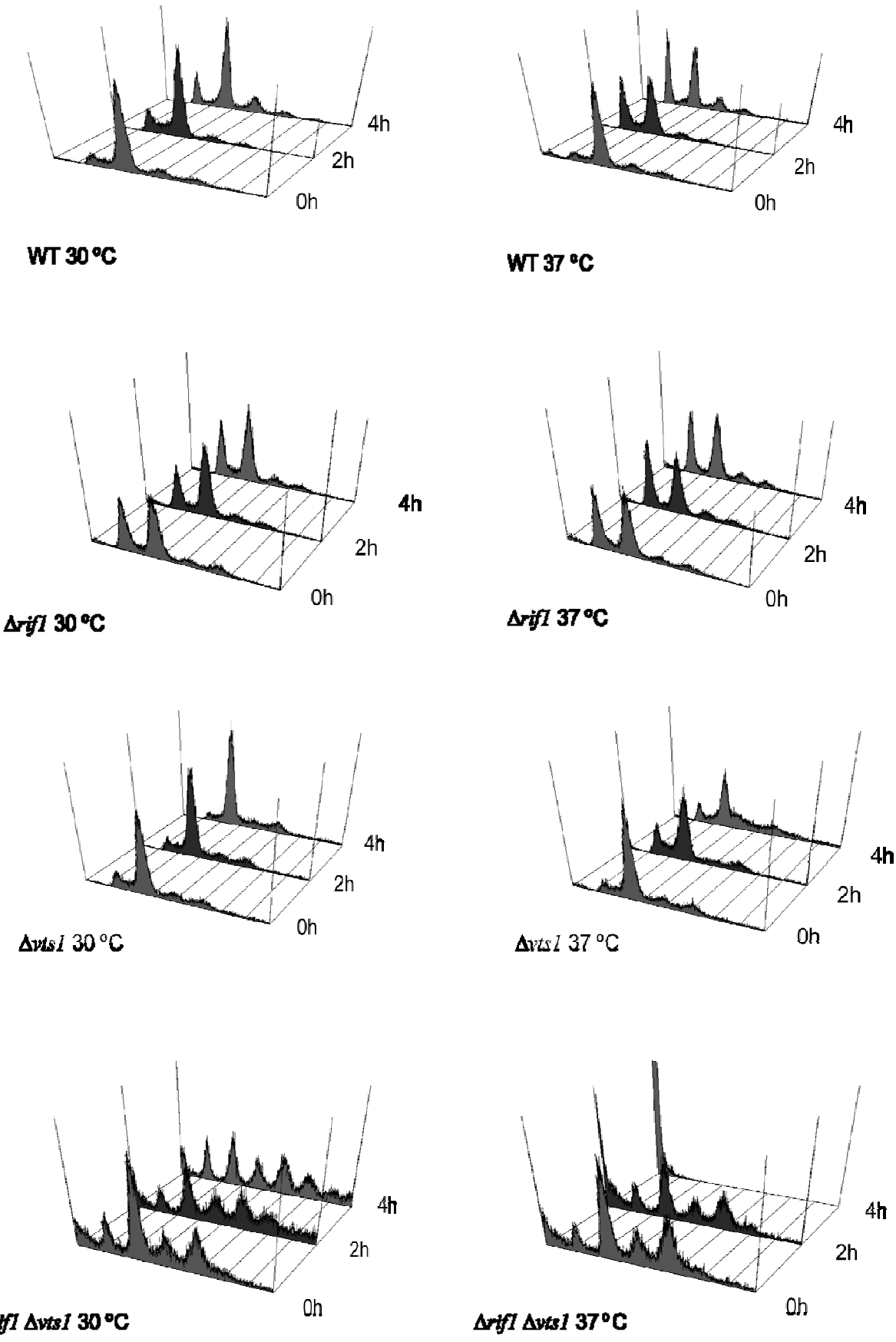
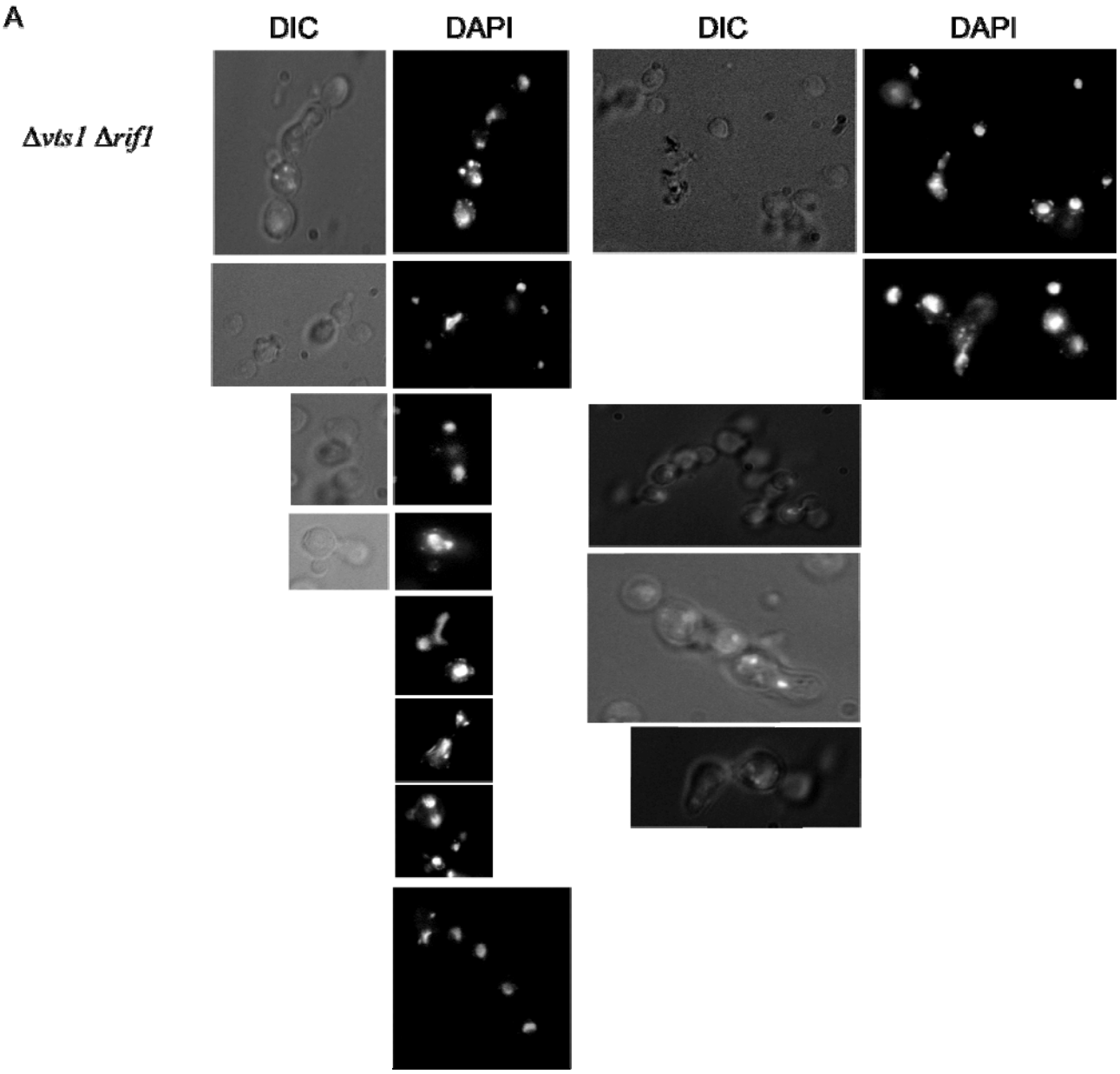


Fig. 42 - Cell Cycle Arrest?
FACS analysis.

The FACS analysis revealed that the *Δvts1 Δrif1* mutant (Fig. 42), already at 30ºC shows a severe difference to the wildtype or even to the single mutants. We observed (Fig. 42) that this mutant increases DNA content, indicating that DNA replication occurs but that citokinesis does not occur properly, as has been observed for Guanydine depleted cells (Sagot *et al.*, 2005). In order to confirm these results we have also looked at the cells in the microscope. (Fig. 43). In fact, *Δrif1 Δvts1* cells accumulate chains of multiple buds, showing indeed a defect in cytokinesis. In addition these mutants also show a number of tripartide spindles (Fig. 43), lagging and bridging chromosomes (Fig. 43), indicating a defect in chromosome segregation aswell, most likely linked to anaphase checkpoint recognition defect.

Fig. 43 - Defects in cell division?
DAPI staining of Δ*rif1* Δ*vts1* cells.



How can a cytoplasmic protein that is involved in translation regulation be involved in telomeric silencing and cause such dramatic effects in combination with *Δrif1*? Is there a direct nuclear function associated to *VTS1*?

7.20 Is Vts1p a shuttling protein?

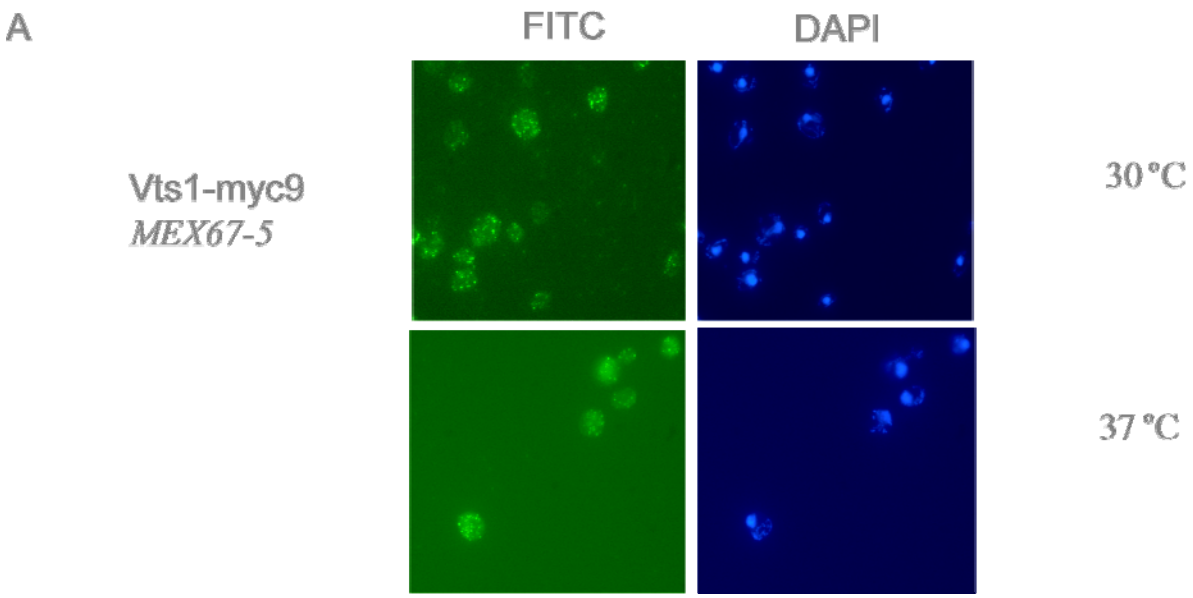


Fig. 44 - Vts1p in nucleus?
A – Immunofluorescence using a *mex67* temperature sensitive allele, that blocks nuclear mRNA export.

As can be seen in the figure 44, Vts1-myc9 is steady state cytoplasmic and cannot be trapped in the nucleus in an mRNA dependent manner. Khd1-myc9 and She2p, two mRNA binding proteins have been shown to accumulate in the nucleus, using the same assay (Du *et al.*, 2008; Kruse *et al.*, 2002).

7.21 Could it be due to telomeric length regulation control? Could Δ*vts1* reduce so dramatically the telomere that renders the cell uncapable of dividing?

Could Vts1p be a part of the Rap1-Rif1/2 counting model?

We observed that *vts1* disruption does not by itself give a shorter telomere than wildtype. The combination of *Δrif1* with *Δvts1* gives an extremely surprising result as the whole subtelomeric region is lost in these mutants, as seen in figure 45.

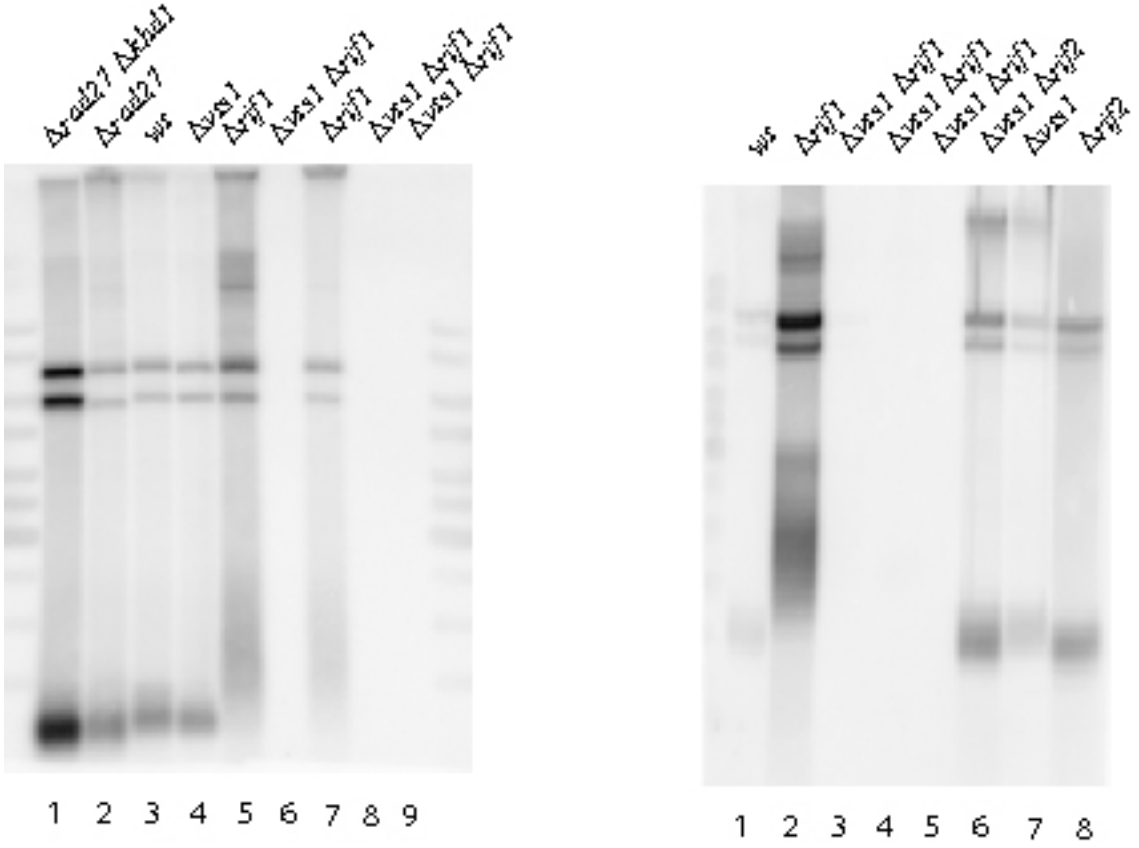


Fig. 45 - Telomeric Length analysis
Southern Blot probed with TG1-3 repeat probe.

We also analysed telomeric length in the *Δrif2 Δvts1* mutants to see if they also showed a total loss of the subtelomeric region. We observed that indeed these mutant cells do not, showing that this phenotype is specific for *Δvts1 Δrif1* (Fig. 45).

8. DISCUSSION

Telomeres are the ends of chromosomes. These DNA regions are places of intense chromatin remodelling and one of the three loci, together with the mating type and rDNA loci, where gene transcriptional silencing occurs.

KHD1 has already been shown to play a role in telomere positioning effect (TPE) and to bind to subtelomeric DNA (Denisenko *et al.*, 2002). *PBP2,* or polyA-binding protein-2, encodes for another 3-KH domain containing protein, with a 30% similarity to *KHD1*, has been shown to also play a role in telomeric gene transcriptional silencing and in combination with *Δkhd1* contribute to an elongated telomeric region (Denisenko *et al.*, 2002). This elongation was suggested to be related to a defect in the telomeric rapid deletion pathway (Denisenko *et al.*, 2002).

In order to study TPE a plate based assay, that measures activation of a normally silenced *URA3* marker positioned at the telomere, was used (Fig. 19).

8.1 KHD1 involved in telomeric silencing?

Results presented in this work show that *khd1* disrupted cells have no drammatic phenotype in telomeric gene transcriptional silencing, when compared to the wildtype (Fig. 20). Surprisingly, a double *rpd3 khd1* deletion loses telomeric gene transcriptional silencing characteristic to *Δrpd3*. *RPD3* encodes for a Histone H3 deacetylase and influences general acetylation levels and at the telomeric region induces gene transcriptional silencing (Rundlett *et al.*, 1996; Sun and Hampsey, 1999). The fact this additional *khd1* disruption causes such a drammatic loss of silencing shows that *khd1* really plays a role in the telomeric gene transcriptional silencing process. In order to prove that this Δ*rpd3* Δ*khd1* loss of silencing phenotype is a *khd1* specific event we also chose to test whether *pbp2* also caused the same phenotype. *Δpbp2 Δrpd3* mutant cells do not behave significantly different from *Δpbp2* mutant cells, although showing a partially weaker silencing than *rpd3* disrupted cells. This could mean that *pbp2* is epistatic to *rpd3*. The fact that *Δpbp2 Δrpd3* show a less pronounced desilencing phenotype than *Δkhd1 Δrpd3* argues for a *khd1* specific effect. As changes in strength of telomeric positioning effect can be due to several different pathways and events, it is conceivable that in the same read-out, growth or no growth on 5'FOA, several effects are contained. Telomeric gene transcriptional silencing can change as a result of changes in telomeric length (Wotton and Shore, 1997), of changes in heterochromatin formation (Rundlett *et al.*, 1996) or even as a result of changes in the formation of the silencing complex *SIR2-3-4*

(Aparicio *et al.*, 1991; Moazed *et al.*, 1997). The Sir2, Sir3, and Sir4 proteins are limiting within the cell, leading to an internal competition between the telomeres and the HM loci (Buck and Shore, 1995) as well as between the telomeres and the rDNA (Smith *et al.,* 1998) for these proteins. The observation that *Δkhd1 Δrpd3* does not differ significantly in phenotype from both *Δsir2 Δrpd3*, mutant that does not form a functional *SIR* complex (Aparicio *et al.*, 1991; Moazed *et al.*, 1997), and *Δsas2 Δrpd3*, mutant that reduces *SIR* complex deposition at the telomeres (Suka *et al.*, 2002), does not allow us to conclude whether it could be a *SIR* complex formation event or a *SIR* complex localization and targeting. Considering the fact that Khd1p has been proposed to be a translation repressor we also tested whether another translation repressor, like Vts1p (Aviv *et al.*, 2002), also plays a role in TPE. Analysis of *vts1* deletion mutants in the telomeric gene transcriptional silencing assay elicited some very interesting results (discussed later). In fact, as observed for *khd1* disruption, *Δvts1* shows no effect on TPE alone. On the other hand, *Δvts1* does not influence transcriptional silencing in *Δrpd3* mutant cells. This is in contrast to what was observed for *Δkhd1 Δrpd3* mutant cells. Khd1p could play a direct role in the TPE process or it could be an indirect event, linked to the mRNA translation repressor function and associated to the specific mRNAs it binds to. Unfortunately, until now only *ASH1* mRNA has been shown to be bound by Khd1p (Irie *et al.*, 2002).

An answer to these questions and a clearer picture on these *Δkhd1* and *Δpbp2* effects in TPE is still needed.

8.2 Other roles in TPE for KHD1 in combination with other mutants?

In order to get a clearer picture about the role of *KHD1* in TPE, we combined a *khd1* disruption with deletions from other genes already established to be involved in TPE. We observed that *Δkhd1* increases silencing of *Δrif1* and of *Δrif2* mutant. In contrast a *pbp2* deletion in a Δ*rif1* causes the exact opposite, reversing the silencing effect observed for *Δrif1*. This indicates that i) *PBP2* might counteract *RIF* complex function in telomeric length regulation, ii) can cause a change in heterochromatin establishment, leading to a lower transcriptional activation at the telomeric site, or iii) might contribute to *SIR* complex deposition at the telomere.

8.3 Is KHD1 involved in telomeric length regulation?

Although it has been described for both *Δkhd1* and *Δpbp2* to cause telomere elongation (Denisenko *et al*., 2002), results presented in this work have not shown the same. In fact, there was no

difference observed for *khd1* or *pbp2* disruptions as compared to the wildtype. Furthermore, both show no change in telomeric length in combination with ⊗*rpd3*, indicating that it is not telomeric length regulation to cause the phenotype changes observed on the TPE assay.

Nevertheless, in support of the idea that *khd1* plays a role in telomeric length regulation, we observed that in *Δrif1* cells, which already have an extended telomeric region, an additional *khd1* deletion causes a further elongation. As *Δrif1* is already transcriptionally silenced (Marsellach *et al.*, 2006), this additional *khd1* disruption shows no detectable increase in the silencing phenotype observed for the TPE assay. *Δpbp2,* however, showed no such effect in combination with Δ*rif1*. In fact, a double *pbp2 rif1* disruption showed an elongated telomeric region but showed a loss of silencing as compared to a single *Δrif1*.These results point to a rescue of the silencing defect caused by *RIF1* deletion due to the deletion of *PBP2*, which is independent from telomeric length. In addition these findings also confirmed that in combination with Δ*rif1* both Δ*khd1* and Δ*pbp2* contribute to telomeric elongation.

Another interesting result is that a combination of *Δrad27 Δrif1 Δkhd1* showed an elongated telomeric region, indicating that all three mutations regulate telomeric length in different ways. It is conceivable that this is so, taking into account that *rif1* is responsible for shortening the telomere with each cell division (Wotton and Shore, 1997), *rad27* is responsible for okazaki fragment maturation and *rad27* disruption causes an elongated lagging strand (reviewed in Liu *et al.*, 2004), *khd1,* which has been implicated in the telomeric rapid deletion pathway (Denisenko *et al.*, 2002), thus cummulatively contributing to the elongation of the telomeric region.

The longest telomeric region was observed for *Δrif1 Δrif2 Δkhd1*, which adds evidence to the fact that indeed *Δkhd1* plays a role in telomeric elongation, since this triple mutant shows an even more elongated telomere than *Δrif1 Δrif2*.

8.4 Can KHD1 be involved in Sir2p downregulation?

The observation that a double *khd1 rpd3* deletion mimicks a *Δsir2 Δrpd3* or even a *Δsas2 Δrpd3* led us to address the question whether Δ*khd1* was responsible for Sir2p downregulation or for delocalization of the *SIR* complex from the telomere, thus causing the loss of silencing observed (discussed above).

When Sir2p-myc9 levels were analysed in *Δkhd1* and in *Δpbp2*, we observed that they remained unchanged in *Δpbp2* but were significantly reduced in *Δkhd1*, independent of their respective *SIR2* mRNA levels, arguing for a post transcriptional regulation event in regard to *Δkhd1*. These results

clearly show that *pbp2* and *khd1* are acting in different pathways to regulate silencing and TPE. This downregulation of Sir2p-Myc9 could be an explanation for the weaker silencing observed for the *rpd3 khd1* disrupted cells.

To address the question whether Sir2p-myc9 is correctly localized, indirect immunofluorescence was performed. There was no observable difference in localization of Sir2p-Myc9 between wildtype and *Δpbp2*. The very low abundance of Sir2p-myc9 in *Δkhd1* cells did not allow any conclusion regarding subcellular localization using the same technique (Fig. 28).

Gene transcriptional silencing occurs at three loci, rDNA, telomeres and mating type loci. Sir2, Sir3, and Sir4 proteins are limiting within the cell, leading to an internal competition between the telomeres and the HM loci (Buck and Shore, 1995) as well as between the telomeres and the rDNA (Smith *et al.,* 1998) for these proteins. The decrease of Sir2p in *Δkhd1* cells could explain why non-conventional mating occurs more frequently in *khd1* disrupted cells than it does for the wildtype cells (data not shown), since the mating type loci might be less effectively silenced and the available Sir2p would be somehow tethered to the rDNA loci and to the telomeres. Sir2p deposition at rDNA loci prevents homologous recombination events that create rDNA circles that cause cellular ageing (Park *et al.*, 1999; Kaeberlin *et al.*, 1999; Gottlieb and Esposito, 1989; Guarante, 2001). The fact that there is less Sir2p in a *khd1* disrupted cell could mean that it ages faster than a wildtype. It would be interesting to investigate if this is true and if so, determine how faster does it age. Taking into account that *khd1* has sequence homology to hnRNP K and hnRNP K has been shown to be involved in telomere regulation (Marsich *et al.*, 2001; Bandiera *et al.*, 2003) it would be interesting to test whether hnRNP K also affects levels of Sirtuins and thus aging regulation. It would also be interesting to determine whether there is a correlation between Khd1p function in yeast and hnRNP K functions in a more complex and regulated system, such as the human cell.

8.5 Can the observed Δrpd3 Δkhd1 desilencing phenotype be rescued?

The results presented and discussed above show that disruption of *khd1* causes a decrease of Sir2p. Previous observations from other groups have shown that a *sir2* disruption causes loss of gene transcriptional silencing (Shore *et al.*, 1984) and *sir2* overexpression causes chromosome instability, leading even to chromosomal loss (Holmes *et al.*, 1997). Although the loss of silencing is an expected outcome for a *sir2* disruption, as the *SIR* complex cannot be properly formed, assembled and deposited at the silenced loci, it is not so intuitive that a *SIR2* overexpression is detrimental for cell viability. A putative explanation could be that too much deacetylation of histone tails occurs (Braunstein *et al.*, 1993) and so the overall level of transcription is severely reduced,

thus causing cell death in 5'FOA, an additional stressing factor. In fact, *SIR2* expressed from a multicopy plasmid is indeed lethal for a wildtype cell. Interestingly this is not the case for *Δkhd1* disrupted cells (Fig. 30).

When a *SIR2* expressed from a low-copy plasmid, a partial rescue of gene transcriptional silencing could be observed for *rpd3 khd1* double deletion, indicating that *SIR2* is indeed one of the factors affected. The fact that the rescue observed is not complete might be due to the fact that *SIR2* levels are very tightly balanced, as both an excess and a loss of *SIR2* lead to desilencing. Another possible reason would be that another factor involved in telomeric gene transcriptional silencing is also affected by *Δkhd1* and its loss is not completely rescued by additional copies of *SIR2*.

8.6 Does Δkhd1 lead to a change in histone modifications?

Sir2p is an essential enzyme in the formation of the euchromatin/heterochromatin boundary. This is an important step in regulating transcriptional silencing or desilencing. In this regard, the levels of acetylation of the histone tails are an important determinant for the establishment of this boundary. Acetylation of Lysine 16 of Histone H4 (HH4K16Ac) is the hallmark for heterochromatin boundary formation at the telomere and it is regulated by the competition between Sir2p, a histone deacetylase and Sas2p, an histone acetyl-transferase (Suka *et al.*, 2002; Kimura *et al.*, 2002).

In order to explain the differences in silencing observed for *Δkhd1 Δrpd3* and for *Δpbp2 Δrpd3*, the acetylation levels of Histone H4 Lysines 8 (K8), 12 (K12) and 16 (K16) were analysed. Although the levels for K8 and K12 were not significantly different in order to explain the changes in silencing observed, the changes in K16 acetylation were very striking. The fact that in *Δkhd1* cells no changes in Histone H4 acetylation levels were observed, could mean that both Sir2p and Sas2p are downregulated or that another deacetylase, at least partially takes over Sir2p function. To better answer this question the amount of Sas2p should also be determined in order to rule out Sas2p downregulation.

Whereas *Δpbp2 Δrpd3* showed no significant difference, in *Δkhd1Δrpd3* cells Histone H4K16 was 3-fold more acetylated than on *rpd3* disrupted cells, consistent with the previously observed downregulation of Sir2p function and with a more transcriptionally active region.

The effects caused by *pbp2* disruption and *khd1* disruption, in spite of the domain similarity between these two genes, are not functional related. This is shown by the observation that *pbp2* disruption causes no significant change on Histone H4 acetylation and that *khd1* deletion has, probably via Sir2p. It is conceivable that the phenotype observed for *Δpbp2* in desilencing of *rpd3* disruption is related to histone H3 modifications, since *rpd3* is a deacetylase of histone H3 tails and

so therefore its influence on telomeric silencing would be indirect. Furthermore, one can also hypothesize that *pbp2* could contribute to trymethylation of histone H3 tails, an important regulator of transcription (Tamaru *et al.*, 2003), and thus activate transcription at the telomeric region, resulting in the loss of silencing observed for *Δpbp2 Δrpd3*.

In summary,

- *KHD1* plays a role in telomeric gene silencing. *PBP2*, a gene quite similar to *KHD1*, in that it also encodes for a 3-KH domain containing protein, also plays an almost similar role. These roles were proven to be independent of telomeric length and again, in this read-out, both *Δpbp2* and *Δkhd1* show similar results, leading to elongated telomeres (as proposed by Denisenko *et al.*, 2002) only in combination with *Δrif1*.
- However, when levels of Sir2p-Myc9 are analysed in both *khd1* and *pbp2* deletion mutants, *Δkhd1* shows a clear difference whereas *Δpbp2* shows no difference. These results clearly indicate that the mechanism uncovered involving an increase of Histone H4K16Ac and a decrease of Sir2p-Myc9 in regard to a *khd1* disruption, is specific for *khd1*.
- The fact that *Δpbp2* causes a similar silencing rescue of *Δrpd3*, but does so independently of telomeric length control, Sir2p amount and of Histone H4K16Ac regulation suggests that *pbp2* is involved in an entirely different pathway that does not involve Sir2p or changes in Histone H4K16Ac.

8.7 Is Khd1p a part of an heterochromatin regulator complex?

The observation that *Δkhd1* causes a Sir2p downregulation led us to analyse whether Khd1p is a part of the *SIR* complex or a Sir2p interacting partner.

The observation that *Δkhd1* causes a further telomeric elongation for *Δrif1* and for *Δrif2* led us to investigate whether Khd1p was a part of the *RIF* protein complex.

Results in figure 33 show that Khd1p can co-purify both Sir2p and Rif2p, but does so only in the presence of DNA. These results suggest that these interactions are likely to be indirect and require DNA. The previous observation that Khd1p binds to subtelomeric DNA (Denisenko *et al.*, 2002) seems to support this DNA mediated indirect Khd1p-Sir2p and Khd1p-Rif2p interaction hypothesis.

8.8 Is KHD1 gene transcriptional silencing influenced by telomerase RNA subunit overexpression?

Telomerase is an holoenzime, composed of three proteins, Est1-3p and an RNA subunit, the *TLC1* RNA. Proteins involved in telomeric silencing regulation, such as Est1-4p and Yku70p, have been shown to bind *TLC1* (Peterson *et al.*, 2001; Stellwagen *et al.*, 2003; Lustig AJ, 2004) and be able to target it to the telomere, to tether it to its proper location.

Results presented in figure 32 indicate that a multicopy plasmid containing *TLC1* proves detrimental for growth of any *khd1* disrupted cell tested. This means that an overexpressed *TLC1* causes a loss of telomeric gene transcriptional silencing in a *khd1* disrupted cell. This observation is specific for *Δkhd1*, as a deletion of another mRNA binding protein, *Δvts1*, which is also a translation repressor, does not show a similar phenotype. In fact, these observations suggest that Khd1p also binds to *TLC1* and contributes to the efficient tethering of *TLC1* to the telomere. This hypothesis is supported by the model that an overexpressed *TLC1* would titer Yku70p/Yku80p away from the telomere and would therefore lead to desilencing, as no *SIR* complex could be targeted and anchored at the telomeric region, via Sir4p-Yku70p binding.

Furthermore, there is another complex of proteins apart from the telomerase and Yku70p that bind *TLC1*, which is the Sm complex – small nuclear ribonucleoprotein particle (Seto *et al.*, 1999). This complex is proposed to be involved in the intracellular trafficking and maturation of the *TLC1* RNA and it would be interesting to see if *KHD1*, being a shuttling protein, is somehow functionally related to this Sm complex. As *TLC1* is a poly-adenylated RNA that is not translated and as Khd1p is a translation repressor, it is conceivable that the Khd1p-*TLC1* interaction is an important part of telomerase biogenesis.

Recently, increasing evidence has implicated mRNA surveillance mechanisms in telomerase function and biogenesis (Azzalin *et al.*, 2006; Azzalin *et al.*, 2007; Gallardo *et al.*, 2008). As *KHD1* was shown to play a role in mRNA surveillance and NMD (Section 4.1) it is possible that *KHD1* plays a role also in regulating telomeric basal transcription events, by aiding the *UPF* pathway in dealing with excess transcripts originated in this manner (Azzalin *et al.*, 2006). In addition, *VTS1* is involved in *CCR4-POP2-NOT* function in deadenylation as a part of mRNA surveillance mechanisms and mRNA turnover. The observation that *Δvts1* does not show a similar phenotype to *khd1* disruption, seems to contradict this mRNA surveillance function hypothesis for *KHD1*, but it is possible that as *TLC1* is not really a mature mRNA it eludes the *CCR4-POP2-NOT* machinery.

In order to understand whether a link between Khd1p-*TLC1* exists, a direct binding of Khd1p to *TLC1* RNA should be investigated further. If there is a direct connection between Khd1p and *TLC1,* experiments should be designed to determine whether Khd1p and *TLC1* RNA shuttle together (Fig.

46) and whether this binding is relevant for the translationally repressed trafficking.

In addition, if the high-copy-*TLC1* induced loss of silencing observed for *khd1* disrupted cells (Fig. 32) is related to Yku70p/80p delocalization from the telomere, a rescue of this silencing defect should be observed, when a high copy-Yku70/80 heterodimer is supplied to the cells.

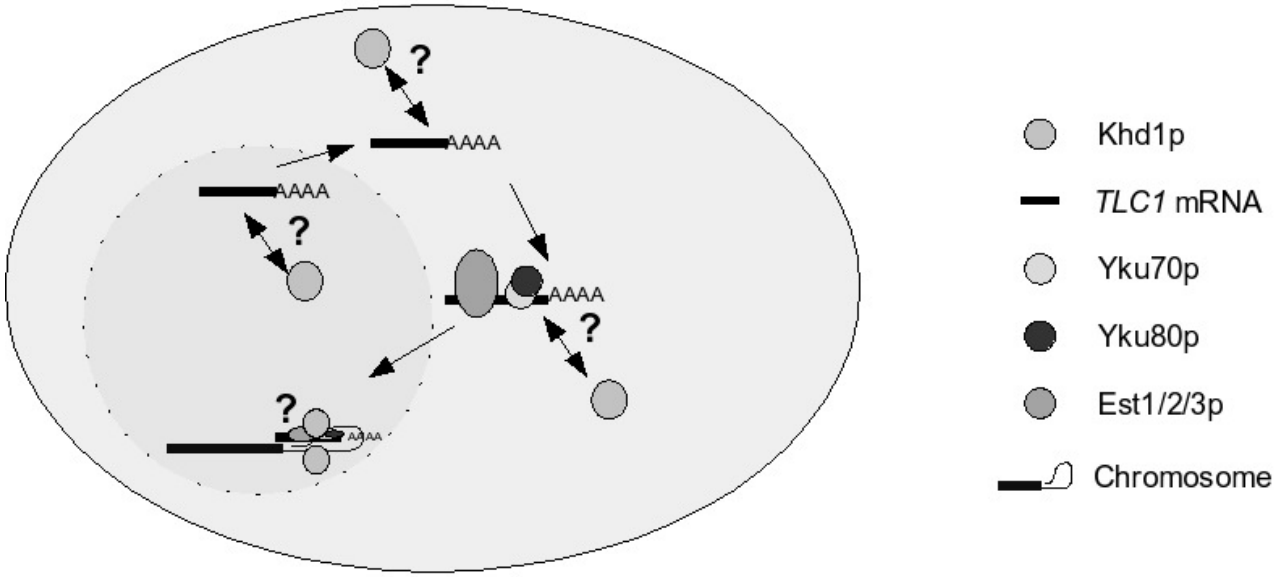


Fig. 46 - Putative Model for the connection between Khd1p and *TLC1*. As the binding between Khd1p and *TLC1* RNA is still unconfirmed, it is unclear whether this model will hold. After confirmation of the Khd1p – *TLC1* interaction it would be important to know where in the cell do they interact, whether they shuttle together from the nucleus to the cytoplasm, whether Khd1p is a translation repressor to *TLC1* providing insights into what is the relevance of this interaction.

8.9 Is KHD1 involved in DNA repair?

Genes that are involved in telomeric gene transcriptional silencing are generally also involved in the DNA damage repair pathways. DNA repair is an extremely conserved process throughout evolution (Cromie *et al.*, 2001). The major players that are involved in this process are conserved, with a few exceptions (Reviewed in Li and Heyer, 2008; Weterings and Chen, 2008; Shrivastav *et al*., 2008; Phillips and McKinnon, 2007). A telomere, is, in its essence a double stranded DNA break, that is not recognized as such and is not repaired and ligated to other lose DNA ends. If this occured it would be detrimental for the cell, in terms of chromosome segregation and cell cycle progression (reviewed in Viscardi *et al.*, 2005). A telomere could therefore be envisioned as a DNA damage that escapes repair. This escape is mediated by *RAD52*, that distinguishes double strand breaks (DSB's) from telomeres. Another gene that is involved in both pathways is the yeast Ku homolog, *YKU70*, which binds to *TLC1* RNA and acts in DSB repair and non-homologous end joining (NHEJ)

(Boulton and Jackson, 1996). A protein complex that is involved in both telomere maintenance and DSB repair is the *MRE11/RAD50/XRS2* complex (Wilson *et al.*, 1999). It is responsible for homologous recombination events, both in the telomeric rapid deletion pathway, that relies on homologous recombination events to reduce long telomeric DNA regions to wildtype in a single cell division (Li and Lustig, 1996), and in the DSB repair pathway. Denisenko and Bomsztky proposed in 2002 that *KHD1* is involved in the Telomeric Rapid Deletion Pathway. To assess the validity of this hypothesis, and determine whether *KHD1* is, as the MRX complex (Ivanov *et al*., 1992), also a *RAD52*-epistasis group member, an analysis of the genetic relationship between *KHD1* and this complex was performed, as well with other members of the DNA DSB repair pathway. Not surprisingly, no genetic relationship was found between *khd1* and *yku70*, *khd1* and *rad52* or *khd1* and *xrs2*, under normal growing conditions. At an elevated growth temperature *rad52 khd1* double disruption showed a slower growth phenotype. This observation cannot be attributed to any specific function of *RAD52* or *KHD1* but suggests that there is a relationship between DSB repair and *KHD1*. Supporting this hypothesis is the observation that mutations in genes involved in DNA related events, such as mitosis progression are temperature sensitive with *RAD52*, e.g. *BUB2* and *BUB3* (Myung *et al.*, 2004). It is conceivable that one such relationship, if existing, would be more prominent under DNA damage stress, which will be discussed further in the following sections.

8.10 Is KHD1 involved in Double Strand Break Repair?

DNA damage repair can be normally assessed with studies on sensitivity to damage inducing drugs or radiation. Methyl-methanosulphonate, although initially thought to cause alkylation of the DNA and thus induce DSBs, causes stalling of the replication fork and mutants in the homologous recombination pathway cannot overcome this fork stalling and thus exhibit extreme sensitivity to this agent (Lundin *et al.*, 2005). UV radiation, one example of radiation commonly used, induces thymidine dimer formation and can also cause stalling of the replication fork, and in addition nucleotide misincorporation and thus lead to deleterious mutations (Matsumu and Ananthaswamy, 2004). *Δkhd1* cells showed no increased sensitivity to UV radiation. Nor did they show any increased sensitivity in combination with *Δrad52* when grown at 30 degrees. *Δkhd1* did however show a decreased viability with *Δxrs2* at high UV dosage (50μJ). The DNA repair defect caused by *Δkhd1* is even more pronounced when *Δrad52 Δkhd1* irradiated cells were grown at 37 degrees (Fig. 35). In this combination of conditions, *Δrad52 Δkhd1* mutant cells exhibit an extremely reduced fitness, indicating indeed a defect in DNA repair, although the fact that this double mutant

combination leads to synthetic growth defect at elevated temperatures makes the results harder to interpret.

The fact that both *Δxrs2 Δkhd1* and *Δrad52 Δkhd1* show no elevated sensitivity to increasing concentrations of hydroxyurea (Fig. 36), a DNA replication inhibitor, points to a direct role of *KHD1* in DNA repair, independent of DNA replication.

This defect in DNA repair is even more evident when the results for sensitivity to MMS are analysed. Again, although *Δkhd1* alone shows no defect in regard to MMS sensitivity alone it does so with *Δrad52* and also with *Δxrs2*, as seen for all MMS concentrations tested. These findings support the idea that *KHD1* plays indeed a role in DNA DSB repair. The above results would indicate that *RAD52* and *KHD1* contribute to different pathways in DSB repair. As *XRS2* is involved in both already characterized DSB repair pathways (Fig. 19), it is not surprising that *khd1* deletion also shows a DNA repair defect with *xrs2* deletion. The results mentioned above suggest that *KHD1* can play a role not in recombinational repair (*rad52* dependent) but in non homologous end joining, a DSB repair pathway that does not require *RAD52* but does *YKU70*/*YKU80*. The possibility that *KHD1*, a translation repressor involved in mRNA localization could also be involved in NHEJ and DNA related events is an extremely interesting possibility. One could fit a model to *KHD1* that includes first, a cytoplasmic function, in translation repression and mRNA localization and second, a nuclear function, related to telomeres and NHEJ, that could also involve *TLC1* RNA. This model could also be fitted with the observation that Khd1p can be trapped in the nucleus in an mRNA dependent manner, clearly showing that it is a shuttling protein and that it could have a nuclear function (Du *et al.*, 2008).

8.11 Is KHD1 involved in Non-Homologous End Joining?

Non homologous end joining is a process where loose ends of DNA are ligated, and hence, repaired (Hefferin *et al.*, 2005; Daley *et al.*, 2005). This process, that does not need sequence homology between ends, requires a functional Yku70/80 heterodimer, to recognize the double stranded break and initiate the repair process (Boulton and Jackson, 1996; Boulton and Jackson, 1996; Boulton and Jackson, 1998) (Figs. 18 and 19). The presented results in figure 38 show a decrease in NHEJ efficiency assigned to *KHD1* disruption in all mutant combinations analysed, except for *YKU70 KHD1* double disruption. This latter result, indicates epistasis between *KHD1* and *YKU70* in regard to NHEJ, supporting the proposed hypothesis that *KHD1* plays a role in NHEJ. Although the result in itself is clear, it is not clear whether it really is due to *Δkhd1* directly or to any of the mRNAs whose translation it regulates. This hypothesis cannot be disproven nor can the model for the two

independent and separate functions, a cytoplasmic function and a nuclear function for *KHD1*. Adding to this unclear situation is the fact that there are genes that are involved in NHEJ that show almost no change in strength of telomeric gene transcritptional silencing. Some of these genes show an increased non-conventional mating frequency and show different MMS sensitivities - e.g. *POL4* (Wilson and Lieber, 1999; Sterling *et al.*, 2006) or show an effect for only a part of the observed *Δkhd1* phenotypes, e.g. *SIR2* (loss of *SIR2* leads to telomeric gene transcriptional desilencing, NHEJ defect and MMS sensitivity - Cohen *et al.*, 2004). One cannot rule out a pleiotropic effect related to mRNAs that are misregulated by *KHD1* disruption that cause the observed phenotypes. Further experiments on this issue are required in order to assert which of the models is true.

8.12 KHD1 disruption NHEJ deffect can be assigned to KH domain-2

KH-domains, or hnRNP K homology domains, are capable of binding nucleic acids. In FMR1 a point mutation in KH domain-3, Ile304Asn, was described to cause loss of FMR1 association to mRNA in polysomes (Laggerbauer *et al.*, 2001). After alignment and structure modelling of each of the KH domains of Khd1p, we generated point mutants I59R, I68R, I183R in Khd1p that should disrupt the KH domain folds for KH domains 1 and 2 and a L284R on the third KH domain, corresponding to Ile304Asn of FMR-1, that should disrupt polysome association (Siomi *et al.*, 1994; Laggerbauer *et al.*, 2001). The observation that point mutant L284R is still able to rescue the NHEJ defect of *Δkhd1* (Fig. 39) indicates that this point mutant does not disrupt Khd1p function in the NHEJ context. On the other hand, the point mutant I183R no longer rescues this NHEJ defect and therefore one can assume that the KH domain 2 is the one that is responsible for Khd1p role in NHEJ. This result suggests that KH domain 2 either binds directly to DNA or binds an mRNA required for proper NHEJ. In order to elucidate this point, gel shift assays with subtelomeric DNA and *ASH1*-mRNA should be performed to determine whether this point mutant still binds nucleic acids and if not, which one does it no longer bind. This information could direct further research into the relevance of *KHD1* in this process.

In addition, it would be interesting to see if a point mutant in KH domain-1 also still rescues the NHEJ in order to confirm that KH-domain 2 alone is responsible for this NHEJ effect.

8.13 Possible functional significance of KHD1/hnRNP K involvement in NHEJ:

Inherited mutations that affect DNA repair genes are strongly associated with high cancer risks in

humans. Hereditary nonpolyposis colorectal cancer (HNPCC) is strongly associated with specific mutations in the DNA mismatch repair pathway. *BRCA1* and *BRCA2*, two genes that when mutated confer a hugely increased risk of breast cancer on carriers, are both associated with a large number of DNA repair pathways, especially NHEJ and homologous recombination. If hnRNP K can be tracked down to show the same defect as *Δkhd1* in NHEJ, then a direct connection between other types of cancer, not due to *BRCA1* or *BRCA2* mutations, can be looked for and studied.

In summary

- *KHD1* is involved in DNA repair, showing increased sensitivity to UV and to MMS but not to hydroxyurea.
- *KHD1* contributes to NHEJ function.
- A KH-domain2 point mutant, Khd1p-I183R mutant cannot rescue the NHEJ defect observed for *KHD1* disruption.

8.14 VTS1, another translation factor involved in telomeric silencing?

The fact that *KHD1* is a translation repressor involved in mRNA localization and also plays a role in telomere gene transcriptional silencing, DSB repair and NHEJ originated interesting new ideas on the role of translation repressors in the tight control of telomere maintenance and DNA related events. *VTS1* is a translation repressor, homologous to Smaug (Aviv *et al.* 2003; Aviv *et al.*, 2006) and was initially found to be involved in vesicle trafficking (Dilcher *et al.*, 2001). *VTS1* disruption causes a slighty stronger telomeric gene transcriptional silencing. Interestingly, *VTS1 RIF1* double disrupted cells exhibit a temperature sensitivity and a complete gene transcriptional desilencing, indicating a telomere and DNA associated phenotype. This is a quite striking event as a *RIF1* disruption normally causes a transcriptional silence at the telomere site due to an overextended telomeric region (Marsellach *et al.*, 2006; Levy *et al.*, 2004; Mishra *et al.*, 1999; Wotton *et al.*, 1997). In addition, *KHD1 RIF1* double disruption showed a slightly better than *ΔRIF1* gene transcriptional silencing, pointing that it is not general translation repressor related but a *Δvts1* specific event. Surprisingly, the fact that *RIF2 VTS1* double disruption shows no temperature sensitive phenotype nor does it show such extreme gene transcriptional desilencing, suggests that it is a *Δrif1 Δvts1* combination specific event. This is even more surprising, when taking into account that *RIF1* and *RIF2* have always been considered to act together, as a part of the same protein complex (Wotton and Shore, 1997) and this is the first indication of an event where they act separately.

8.15 Does ΔVTS1 ΔRIF1 cause a cell cycle progression defect?

Is it sensitive to hydroxyurea?

Hydroxyurea is a drug that inhibits S-phase progression and is commonly used to assess defects in cell cycle progression. As a DNA replication inhibitor, causes ribonucleotide depletion and results in DNA double strand breaks near replication forks. In these growth conditions, *Δrif1 Δvts1* definitely shows sensitivity to increasing concentrations of hydroxyurea whereas neither the single mutants nor *Δrif2 Δvts1* do. These findings seem to indicate that *Δrif1 Δvts1* have a cell cycle progression defect, although it cannot be ruled out that a differential cell wall permeability could explain the differences in hydroxyurea sensitivity. Furthermore, one other hypothesis that cannot also be ruled out is that both temperature stress, 5'FOA stress and hydroxyurea stress can easily tip the scale in a weak survival balance. In other words, it can be that *Δrif1 Δvts1* is already so sick that an additional stress causes its death. This hypothesis was then disproven by FACS analysis of *vts1 rif1* double deletion, that show an increase in DNA content, possibly due to a citokinesis defect, as shown by the increased 2n, 3n, 4n peaks in the FACS analysis. This has also been shown to occur with guanydine depleted cells (Sagot *et al.*, 2005) that also fail to complete proper cytokinesis. This cytokinesis defect hypothesis was then confirmed by a microscope analysis of these mutant cells (Fig. 43). *Rif1 vts1* double mutant cells accumulate multiple buds, thus explaining the single increment in DNA content. Furthermore, they also show a number of tripartite spindles, lagging and bridging chromosomes, which is an indicator of a defect in chromosome segregation, most likely related to anaphase checkpoint defects and spindle assembly, such as those related to *MAD* and *BUB* genes, to *NDC80* complex and to *CBF3* complex (Martinez-Exposito *et al.*, 1999; Gillet *et al.*, 2004; Gardner *et al.*, 2001; McCleland *et al.*, 2003).

Most importantly, *Δrif2 Δvts1* showed no similar phenotype, supporting the initial observation that it is a *Δrif1 Δvts1* combination specific effect.

As *VTS1* encodes a translation repressor, it cannot be ruled out that the observed phenotype is a result of a misregulation of specific mRNAs. In a recent publication, a set of 79 mRNAs were shown to be bound and regulated by Vts1p (Aviv *et al.*, 2006). Of these, several were cell cycle related, such as *CLN1*, a cyclin, *PHO85*, a cyclin dependent kinase and 6 other cell cycle, spindle orientation and polarization related genes. The most relevant could be *NNF1* (Shan *et al.*, 1997), which has been shown to be involved in chromosomal segregation and kinetochore attachment, nuclear envelope integrity and other cell cycle regulated processes. It is therefore conceivable that this defect observed for *Δrif1 Δvts1* is an underlying effect due solely to *VTS1* disruption that the

additional *RIF1* disruption brings into the spotlight.

8.16 Can VTS1 encode also a shuttling protein, with a translation repressor function in the cytoplasm and another function in the nucleus?

Some proteins can move in and out of the nucleus and are thus called shuttling proteins. She2p and Khd1p are two examples (Kruse *et al.*, 2002; Du *et al.*, 2008) that have already been discussed in previous sections. They can be trapped in the nucleus in an mRNA dependent manner, by use of a temperature sensitive allele of an mRNA nuclear export factor, *MEX67* (Segref *et al.*, 1999). Using the same tool and the same set up, Vts1p was shown not to accumulate in the nucleus in an mRNA dependent manner. The fact that it does not accumulate in the nucleus in an mRNA dependent manner does not mean altogether that it does not shuttle. In fact, other protein factors such as karyopherins (reviewed in Koehler and Hurt, 2007) are involved in the import and export of proteins and further experiments with these mutants would eventually show whether Vts1p is a shuttling protein.

8.17 Is the phenotype observed related to telomeric length regulation?

Rif1p is a part of the *RIF* complex that interacts with Rap1p (Wotton *et al.*, 1997). *Δrif1* causes an elongated telomere, due to loss of negative length regulation. The fact that *Δrif1 Δvts1* shows a total loss of the subtelomeric region whereas a *Δrif2 Δvts1* does not, further strengthens the model that it is *Δrif1 Δvts1* specific observation. The recent observation that SMG1 deletion causes telomeric instability and can lead to telomere loss (Azzalin *et al.*, 2006) strengthens the hypothesis that the same is true for the *Δrif1 Δvts1* double mutant analysed. The observations presented above, hydroxyurea sensitivity, mitotic spindle misorientation and tribudded cells, point also to a ribonucleotide reductase defect and it would be interesting to determine whether the telomeric instability observed for *Δrif1 Δvts1* can be linked to this enzymatic defect.

In summary

- *Δrif1 Δvts1* cells show telomeric gene transcriptional desilencing and temperature sensitivity.
- *Δrif1 Δvts1* cells are sensitive to hydorxyurea and exhibit abnormal cell cycle progression, both evidenced by the FACS analysis results – increase of DNA content – and by the microscopic data – abnormal spindles and citokinesis defect.

- The *Δrif1 Δvts1* cells analysed suffered telomeric catastrophy, as seen by the southern blot results.
- All these observations were made only for *Δrif1 Δvts1* and not for *Δrif2 Δvts1,* indicating a specific deffect associated to *Δrif1 Δvts1* cells, independently of *RIF2* function.
- This is the first indication of a process where Rif1p and Rif2p do not act together.

Although on the one hand the connection between *Δrif1 Δvts1* and cell cycle progression is extremely interesting, on the other hand the possibility that it all might be due to a misregulation of mRNAs due to *VTS1* disruption, makes it difficult to analyse. It would be interesting to see if any of the identified mRNAs bound by Vts1p (Aviv *et al*., 2006) rescues, either by over-expression or by deletion, the cytokinesis defect and/or the spindle orientation defect. Telomere checkpoint defects can also be associated to lagging and bridging chromosomes, as seen for spindle orientation mutants, which adds in complexity (Musaro *et al.*, 2008; Lazzaro *et al.*, 2008; Review in Viscardi *et al.*, 2005).

As Smaug is an extremely conserved translational repressor, the fact that a whole new field of study on Smaug function can now be available can be of a crucial importance and might bring several important hints in cell cycle control related to translation repression.

9. REFERENCES:

- Aboussekhra A, Biggerstaff M, Shivji MK, Vilpo JA, Moncollin V, Podust VN, Protić M, Hübscher U, Egly JM, Wood RD. 1995. Mammalian DNA nucleotide excision repair reconstituted with purified protein components. Cell. 1995 Mar 24;80(6):859-68.
- Alarcón VB, Elinson RP. 2001. RNA anchoring in the vegetal cortex of the Xenopus oocyte. J Cell Sci. 2001 May;114(Pt 9):1731-41.
- Allsopp RC, Vaziri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, Greider CW, Harley CB. 1992. Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci U S A. 1992 Nov 1;89(21):10114-8.
- Aparicio OM, Billington BL, Gottschling DE. 1991. Modifiers of position effect are shared between telomeric and silent mating-type loci in S. cerevisiae. Cell 66(6):1279-87
- Arn EA, Cha BJ, Theurkauf WE, Macdonald PM. 2003. Recognition of a bicoid mRNA Localization Signal by a Protein Complex Containing Swallow, Nod, and RNA Binding Proteins. Developmental Cell, Volume 4, Number 1, January 2003 , pp. 41-51(11).
- Aronov S, Gelin-Licht R, Zipor G, Haim L, Safran E, Gerst JE. 2007. mRNAs Encoding Polarity and Exocytosis Factors Are Cotransported with the Cortical Endoplasmic Reticulum to the Incipient Bud in Saccharomyces cerevisiae. Mol Cell Biol 27(9):3441-55.
- Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. Eds. Short protocols in molecular biology 4th edition. Current Portocols in Molecular Biology 1999, John Wiley and Sons, NY.
- Aviv T, Lin Z, Ben-Ari G, Smibert CA, Sicheri F. 2006. Sequence-specific recognition of RNA hairpins by the SAM domain of Vts1p. Nat Struct Mol Biol 13(2):168-76
- Aviv T, Lin Z, Lau S, Rendl LM, Sicheri F, Smibert CA. 2003. The RNA-binding SAM domain of Smaug defines a new family of post-transcriptional regulators. Nat Struct Biol 10(8):614-21
- Azzalin CM, Lingner J. 2006. The double life of UPF1 in RNA and DNA stability pathways. Cell Cycle. 2006 Jul;5(14):1496-8. Epub 2006 Jul 17
- Azzalin CM, Reichenbach P, Khoriauli L, Giulotto E, Lingner J. 2007. Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends. Science. 2007 Nov 2;318(5851):798-801. Epub 2007 Oct 4
- Bandiera A, Tell G, Marsich E, Scaloni A, Pocsfalvi G, Akintunde Akindahunsi A, Cesaratto L, Manzini G. 2003. Cytosine-block telomeric type DNA-binding activity of hnRNP proteins

from human cell lines. Arch Biochem Biophys. 2003 Jan 15;409(2):305-14.

•Bao K, Cohen SN. 2001. Terminal proteins essential for the replication of linear plasmids and chromosomes in Streptomyces. Genes Dev. 2001 Jun 15;15(12):1518-27.

•Baroudy BM, Venkatesan S, Moss B. 1983. Structure and replication of vaccinia virus telomeres. Cold Spring Harb Symp Quant Biol. 1983;47 Pt 2:723-9.

•Bashirullah A, Cooperstock RL, Lipshitz HD. 1998. RNA localization in development. Annu Rev Biochem. 1998;67:335-94. Review.

•Baumann P and Cech TR. 2000. Protection of telomeres by the Ku protein in fission yeast. Mol Biol Cell 11(10):3265-75

•Baumann P, Cech TR. 2001. Pot1, the putative telomere end-binding protein in fission yeast and humans. Science. 2001 May 11;292(5519):1171-5. Erratum in: Science 2001 Jul 13;293(5528):214.

•Beattie TL, Zhou W, Robinson MO, Harrington L. 2001. Functional multimerization of the human telomerase reverse transcriptase. Mol Cell Biol 21(18):6151-60.

•Berman J, Tachibana CY, Tye BK. 1986. Identification of a telomere-binding activity from yeast. Proc Natl Acad Sci U S A. 1986 Jun;83(11):3713-7.

•Bertrand E, Chartrand P, Schaefer M, Shenoy SM, Singer RH, Long RM. 1998. Localization of ASH1 mRNA particles in living yeast. Mol Cell 1998 Oct;2(4):437-45.

•Bezzubova OY, Schmidt H, Ostermann K, Heyer WD, Buerstedde JM. 1993. Identification of a chicken RAD52 homologue suggests conservation of the RAD52 recombination pathway throughout the evolution of higher eukaryotes. Nucleic Acids Res 21(25):5945-9.

•Bibikova M, Wu B, Chi E, Kim KH, Trautman JK, Carroll D. 1998. Characterization of FEN-1 from Xenopus laevis. cDNA cloning and role in DNA metabolism. J Biol Chem 273(51):34222-9

•Blackburn EH. 2000. Telomere states and cell fates. Nature. 2000 Nov 2;408(6808):53-6

•Blasco MA, Funk W, Villeponteau B, Greider CW. 1995. Functional characterization and developmental regulation of mouse telomerase RNA. Science. 1995 Sep 1;269(5228):1267-70.

•Bobola N, Jansen RP, Shin TH, Nasmyth K. 1996. Asymmetric accumulation of Ash1p in postanaphase nuclei depends on a myosin and restricts yeast mating-type switching to mother cells. Cell 1996 Mar 8;84(5):699-709.

•Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE. 1998. Extension of life-span by introduction of telomerase into normal human cells. Science. 1998 Jan 16;279(5349):349-52

•Boehl F, Kruse C, Frank A, Ferring D, Jansen RP. 2000. She2p, a novel RNA-binding protein

tethers ASH1 mRNA to the Myo4p myosin motor via She3p. EMBO J. 2000 Oct 16;19(20):5514-24.

•Boulton SJ and Jackson SP. 1996. Identification of a Saccharomyces cerevisiae Ku80 homologue: roles in DNA double strand break rejoining and in telomeric maintenance. Nucleic Acids Res 24(23):4639-48

•Boulton SJ and Jackson SP. 1996. Saccharomyces cerevisiae Ku70 potentiates illegitimate DNA double-strand break repair and serves as a barrier to error-prone DNA repair pathways. EMBO J 15(18):5093-103

•Boulton SJ and Jackson SP. 1998. Components of the Ku-dependent non-homologous end-joining pathway are involved in telomeric length maintenance and telomeric silencing. EMBO J 17(6):1819-28

•Braunstein M, Rose AB, Holmes SG, Allis CD, Broach JR. 1993. Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. Genes Dev 7(4):592-604

•Brendza RP, Serbus LR, Duffy JB, Saxton WM. 2000. A function for kinesin I in the posterior transport of oskar mRNA and Staufen protein. Science 2000 Sep 22;289(5487):2120-2

•Bretscher A. 2003. Polarized growth and organelle segregation in yeast: the tracks, motors, and receptors. J Cell Biol. 2003 Mar 17;160(6):811-6. Review.

•Broccoli D, Smogorzewska A, Chong L, de Lange T. 1997. Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. Nat Genet. 1997 Oct;17(2):231-5.

•Broccoli D, Young JW, de Lange T. 1995. Telomerase activity in normal and malignant hematopoietic cells. Proc Natl Acad Sci U S A. 1995 Sep 26;92(20):9082-6.

•Brocke KS, Neu-Yilik G, Gehring NH, Hentze MW, Kulozik AE. 2002. The human intronless melanocortin 4-receptor gene is NMD insensitive. Hum Mol Genet 2002 Feb 1:11(3):331-5

•Brown, V., Jin, P., Ceman, S., Darnell, J.C., O'Donnell, W.T., Tenenbaum, S.A., Jin, X., Feng, Y., Wilkinson, K.D., Keene, J.D. et al., 2001. Cell 107, pp. 477–487.

•Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. 1995. Telomere elongation in immortal human cells without detectable telomerase activity. EMBO J. 1995 Sep 1;14(17):4240-8.

•Buchman AR, Kimmerly WJ, Rine J, Kornberg RD. 1988. Two DNA-binding factors recognize specific sequences at silencers, upstream activating sequences, autonomously replicating sequences, and telomeres in Saccharomyces cerevisiae. Mol Cell Biol. 1988 Jan;8(1):210-25.

•Buck SW and Shore D. 1995. Action of a RAP1 carboxy-terminal silencing domain reveals an underlying competition between HMR and telomeres in yeast. Genes Dev 9(3):370-84

•Burkhart BA, Alcorta DA, Chiao C, Isaacs JS, Barrett JC. 1999. Two posttranscriptional pathways that regulate p21(Cip1/Waf1/Sdi1) are identified by HPV16-E6 interaction and correlate with life span and cellular senescence. Exp Cell Res. 1999 Feb 25;247(1):168-7.
•Cabanas MJ, Vazquez D, Modolell J. 1978. Dual interference of hygromycin B with ribosomal translocation and with aminoacyl-tRNA recognition. Eur J Biochem. 87(1):21-27
•Cao Q, Richter JD. 2002. Dissolution of the maskin-eIF4E complex by cytoplasmic polyadenylation and poly(A)-binding protein controls cyclin B1 mRNA translation and oocyte maturation. EMBO J. 2002 Jul 15;21(14):3852-62.
•Carr AM, Hoekstra, MF. 1993. The cellular responses to DNA damage. Trends in Cell Biol 5, 32-40.
•Carson JH, Worboys K, Ainger K, Barbarese E. 1997. Translocation of myelin basic protein mRNA in olygodendrocytes requires microtubules and kinesin. Cell Motil Cytoskeleton 1997; 38(4):318-28.
•Catlett NL, Duex JE, Tang F, Weisman L. 2000. Two distinct regions in a yeast myosin-V tail domain are required for the movement of different cargoes. J Cell Biol 2000 Aug 7;150(3):513-26.
•Challberg MD, Desiderio SV, Kelly TJ Jr.. 1980. Adenovirus DNA replication in vitro: characterization of a protein covalently linked to nascent DNA strands. Proc Natl Acad Sci U S A. 1980 Sep;77(9):5105-9.
•Chartrand P, Meng XH, Huttelmaier S, Donato D, Singer RH. 2002. Asymmetric sorting of Ash1p in yeast results from inhibition of translation by localization elements in the mRNA. Mol Cell 2002 Dec;10(6):1319-30.
•Chartrand P, Meng XH, Singer RH, Long RM. 1999. Structural elements required for the localization of ASH1 mRNA and of a green fluorescent protein reporter particle in vivo. Curr Biol 1999 Mar 25;9(6):333-6.
•Chen DC, Yang BC, Kuo TT. 1992. One step transformation of yeast in stationary phase. Curr Genet 1992 Jan;21(1):83-4.
•Chen JL, Blasco MA, Greider CW. 2000. Secondary structure of vertebrate telomerase RNA. Cell. 2000 Mar 3;100(5):503-14.
•Chin L, Artandi SE, Shen Q, Tam A, Lee SL, Gottlieb GJ, Greider CW, DePinho RA. 1999. p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. Cell. 1999 May 14;97(4):527-38.
•Chong L, van Steensel B, Broccoli D, Erdjument-Bromage H, Hanish J, Tempst P, de Lange T. 1995. A human telomeric protein. Science. 1995 Dec 8;270(5242):1663-7.

•Clark A, Meignin C, Davis I. 2007. A Dynein-dependent shortcut rapidly delivers axis determination transcripts into the Drosophila oocyte. Development 134, 1955-65 (2007).
•Cohen *et al.*, 2004
•Coller J and Parker R. 2004. Eukaryotic mRNA decapping. Annu Rev Biochem. 2004;73:861-90.
•Conrad MN, Wright JH, Wolf AJ, Zakian VA. 1990. RAP1 protein interacts with yeast telomeres in vivo: overproduction alters telomere structure and decreases chromosome stability. Cell. 1990 Nov 16;63(4):739-50.
•Corral-Debrinski M, Blugeon C, Jacq C. 2000. In yeast, the 3' untranslated region or the presequence of ATM1 is required for the exclusive localization of its mRNA to the vicinity of mitochondria. Mol Cell Biol. 2000 Nov;20(21):7881-92.
•Cosma MP, Tanaka T, Nasmyth K. 1999. Ordered recruitment of transcription and chromatin remodelling factors to a cell cycle and developmentally regulated promoter. Cell 1999 Apr 30;97(3):299-311.
•Counter CM, Botelho FM, Wang P, Harley CB, Bacchetti S. 1994. Stabilization of short telomeres and telomerase activity accompany immortalization of Epstein-Barr virus-transformed human B lymphocytes. J Virol. 1994 May;68(5):3410-4.
•Counter CM, Meyerson M, Eaton EN, Weinberg RA. 1997. The catalytic subunit of yeast telomerase. Proc Natl Acad Sci U S A. 1997 Aug 19;94(17):9202-7
•Cromie GA, Connelly JC, Leach DR. 2001. Recombination at double-strand breaks and DNA ends: conserved mechanisms from phage to humans. Mol Cell. 2001 Dec;8(6):1163-74. Review.
•Cunningham KS, Dodson RE, Nagel MA, Shapiro DJ, Schoenberg DR. 2000. Vigilin binding selectively inhibits cleavage of the vitellogenin mRNA 3'-untranslated region by the mRNA endonuclease polysomal ribonuclease 1. PNAS November 7, 2000; vol. 97; no. 23; 12498-12502
•Dahanukar A, Walker JA, Wharton RP. 1999. Smaug, a novel RNA-binding protein that operates a translational switch in Drosophila. Mol Cell. 1999 Aug;4(2):209-18.
•Dahlen M, Olsson T, Kanter-Smoler G, Ramne A, Sunnerhagen P. 1998. Regulation of telomere length by checkpoint genes in Schizosaccharomyces pombe. Mol Biol Cell. 1998 Mar;9(3):611-21.
•Daley JM, Palmbos PL, Wu D, Wilson TE. 2005. Nonhomologous end joining in yeast. Annu Rev Genet 39:431-51
•Darzacq X, Powrie E, Gu W, Singer RH, Zenklusen D. 2003. RNA asymmetric distribution and daughter/mother differentiation in yeast. Curr Opin Microbiol 2003 Dec;6(6)614-20.

•Davies H.; Bignell G. R.; Cox C.; 2002. "Mutations of the BRAF gene in human cancer". Nature 417: 949-954.
•Davies J, Davis BD. 1968. Misreading of ribonucleic acid code words induced by aminoglycoside antibiotics. The effect of drug concentration.J Biol Chem. 243(12):3312-6
•de Bruin D, Zaman Z, Liberatore RA, Ptashne M. 2001. Telomere looping permits gene activation by a downstream UAS in yeast. Nature. 2001 Jan 4;409(6816):109-13.
•de Lange, T. 2005. Telomere-related genome instability in cancer. Cold Spring Harb Symp Quant Biol. 2005;70:197-204. Review.
•Debrauwere *et al.*, 2001
•Delanuoe R, Davis I. 2005. Dynein anchors its mRNA cargo after apical transport in the Drosophila blastoderm embryos. Cell 2005 Jul 15,122(1)97-106
•Deng Y, et al. . 2008. Translation of ASH1 mRNA is repressed by Puf6p-Fun12p/eIF5B interaction and released by CK2 phosphorylation. Genes Dev 22(8):1037-50
•Denisenko O and Bomsztyk K. 2002. Yeast hnRNP K-like genes are involved in regulation of the telomeric position effect and telomere length. Mol Cell Biol 22(1):286-97
•Dice JF. 1993. Cellular and molecular mechanisms of aging. Physiol Rev. 1993 Jan;73(1):149-59. Review
•Dilcher M, Kohler B, von Mollard GF. 2001. Genetic interactions with the yeast Q-SNARE VTI1 reveal novel functions for the R-SNARE YKT6. J Biol Chem 276(37):34537-44
•Doma MK and Parker R. 2006. Endonucleolytic cleavage of eukaryotic mRNAs with stalls in translation elongation. Nature 440(7083):561-4
•Du TG, Jellbauer S, Müller M, Schmid M, Niessing D, Jansen RP. 2008. Nuclear transit of the RNA-binding protein She2 is required for translational control of localized ASH1 mRNA. EMBO Rep. 2008 Jun 20.
•Du TG, Schmid M, Jansen RP. 2007. Why cells move messages: the biological functions of mRNA localization. Semin Cell Dev Biol. 2007 Apr;18(2):171-7. Epub 2007 Feb 6. Review.
•Dunham MA, Neumann AA, Fasching CL, Reddel RR. 2000. Telomere maintenance by recombination in human cells. Nat Genet. 2000 Dec;26(4):447-50.
•Ephrussi A, Dickinson LK, Lehmann R. 1991. Oskar organizes the germ plasm and directs localization of the posterior determinant nanos. Cell. 1991 Jul 12;66(1):37-50.
•Erkmann JA, Kutay U. 2004. Nuclear export of mRNA: from the site of transcription to the cytoplasm. Exp Cell Res. 2004 May 15;296(1):12-20.
•Estrada P, Kim J, Coleman J, Walker L, Dunn B, Takizawa P, Novick P, Ferro-Novick S. 2003. Myo4p and She3p are required for cortical ER inheritance in Saccharomyces cerevisiae. J

Cell Biol 163(6):1255-66

•Evans SK and Lundblad V, 1999. Est1 and Cdc13 as comediators of telomerase access. Science. 1999 Oct 1;286(5437):117-20.

•Farina KL, Singer RH. 2002. The nuclear connection in RNA transport and localization. Trends Cell Biol 2002 Oct;12(10):466-72.

•Feldmann H, Driller L, Meier B, Mages G, Kellermann J, Winnacker EL. 1996. HDF2, the second subunit of the Ku homologue from Saccharomyces cerevisiae. J Biol Chem. 1996 Nov 1;271(44):27765-9.

•Feldmann H, Winnacker EL. 1993. A putative homologue of the human autoantigen Ku from Saccharomyces cerevisiae. J Biol Chem. 1993 Jun 15;268(17):12895-900

•Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J, et al. 1995. The RNA component of human telomerase. Science. 1995 Sep 1;269(5228):1236-41

•Ferrezuelo F, Steiner B, Aldea M, Futcher B. 2002. Biogenesis of yeast telomerase depends on the importin mtr10. Mol Cell Biol. 2002 Sep;22(17):6046-55

•Fisher TS, Taggart AK, Zakian VA. 2004. Cell cycle-dependent regulation of yeast telomerase by Ku. Nat Struct Mol Biol. 2004 Dec;11(12):1198-205. Epub 2004 Nov 7.

•Frey S, Pool M, Seedorf M. 2001. Scp160p, an RNA-binding, polysome-associated protein, localizes to the endoplasmic reticulum of Saccharomyces cerevisiae in a microtubule-dependent manner. J Biol Chem 2001 May 11;276(19):15905-12.

•Friedberg EC, Walker GC and Siede W. 1995. DNA Repair and Mutagenesis, ASM Press, Washington DC.

•Friedman DS, Vale RD. 1999. Single molecule analysis of kinesin motility reveals regulation by the cargo-binding tail domain. Nat Cell Biol 1999 Sep;1(5):293-7.

•Gallardo F, Olivier C, Dandjinou AT, Wellinger RJ, Chartrand P. 2008. TLC1 RNA nucleo-cytoplasmic trafficking links telomerase biogenesis to its recruitment to telomeres. EMBO J. 2008 Mar 5;27(5):748-57. Epub 2008 Feb 14

•Galy V, Gadal O, Fromont-Racine M, Romano A, Jaciquier A, Nehrbass U. 2004. Nuclear retention of unspliced mRNAs in yeast is mediated by perinuclear Mlp1. Cell 2004 Jan 9; 116(1):63-73.

•Gardner RD, Poddar A, Yellman C, Tavormina PA, Monteagudo MC, Burke DJ. 2001. The spindle checkpoint of the yeast Saccharomyces cerevisiae requires kinetochore function and maps to the CBF3 domain. Genetics. 157:1493–1502.

•Garvik B, Carson M, Hartwell L. 1995. Single stranded DNA arising at telomeres in cdc13

mutants may constitute a specific signal for the RAD9 checkpoint. Mol Cell Biol, 15, 6128-38.
•Gavin AC, Aloy P, Grandi P, Krause R, Boesche M, Marzioch M, Rau C, Jensen LJ, Bastuck S, Dumpelfeld B, Edelmann A, Heurtier MA, Hoffman V, Hoefert C, Klein K, Hudak M, Michon AM, Schelder M, Schirle M, Remor M, Rudi T, Hooper S, Bauer A, Bouwmeester T, Casari G, Drewes G, Neubauer G, Rick JM, Kuster B, Bork P, Russell RB, Superti-Furga G. 2006. Proteome survey reveals modularity of the yeast cell machinery. Nature 440(7084):631-6
•Gavin AC, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, Schultz J, Rick JM, Michon AM, Cruciat CM, Remor M, Hofert C, Schelder M, Brajenovic M, Ruffner H, Merino A, Klein K, Hudak M, Dickson D, Rudi T, Gnau V, Bauch A, Bastuck S, Huhse B, Leutwein C, Heurtier MA, Copley RR, Edelmann A, Querfurth E, Rybin V, Drewes G, Raida M, Bouwmeester T, Bork P, Seraphin B, Kuster B, Neubauer G, Superti-Furga G. 2002. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature 415(6868):141-7
•Gavis ER, Lehmann R. 1994. Translational regulation of nanos by RNA localization. Nature. 1994 May 26;369(6478):315-8.
•Gietz RD, Schiestl RH. Applications of High efficiency lithium acetate transformation of intact yeast cells using single stranded nucleic acids as carrier. Yeast 1991 Apr;7(3)253-63.
•Gillett ES, Espelin CW, Sorger PK. 2004. Spindle checkpoint proteins and chromosome–microtubule attachment in budding yeast. J. Cell Biol., Feb 2004; 164: 535 - 546.
•Gilson E, Laroche T, Gasser SM. 1993. Telomeres and the functional architecture of the nucleus. Trends Cell Biol. 1993 Apr;3(4):128-34.
•Gonzalez A, Jimenez A, Vazquez D, Davies JE, Schindler D. 1978. Studies on the mode of action of hygromycin B, an inhibitor of translocation in eukaryotes.Biochim Biophys Acta. 521(2):459-69
•Gonzalez I, Buonomo SB, Nasmyth K, von Ahsen U. 1999. ASH1 mRNA localization in yeast involves multiple seccondary structural elements and Ash1 protein translation. Curr Biol 1999 Mar 25;9(6):337-40.
•Gotta M, Laroche T, Formenton A, Maillet L, Scherthan H, Gasser SM. 1996. The clustering of telomeres and colocalization with Rap1, Sir3, and Sir4 proteins in wild-type Saccharomyces cerevisiae. J Cell Biol. 1996 Sep;134(6):1349-63.
•Gottlieb S and Esposito RE. 1989. A new role for a yeast transcriptional silencer gene, SIR2, in regulation of recombination in ribosomal DNA. Cell 56(5):771-6
•Gottlieb TM, Jackson SP. 1993. The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. Cell. 1993 Jan 15;72(1):131-42.
•Gottschling DE, Aparicio OM, Billington BL, Zakian VA. 1990. Position effect at S. cerevisiae

telomeres: reversible repression of Pol II transcription. Cell. 1990 Nov 16;63(4):751-62.
•Govindan B, Bowser R, Novick P. 1995. The role of Myo2, a yeast class V myosin, in vesicular transport. J Cell Biol. 1995 Mar;128(6):1055-68.
•Govindan B, Novick P. 1995. Development of cell polarity in budding yeast. J Exp Zool. 1995 Dec 1;273(5):401-24. Review.
•Grandin N, Damon C, Charbonneau M. 2000. Cdc13 cooperates with the yeast Ku proteins and Stn1 to regulate telomerase recruitment. Mol Cell Biol. 2000 Nov;20(22):8397-408.
•Grandin N, Damon C, Charbonneau M. 2001. Cdc13 prevents telomere uncapping and Rad50-dependent homologous recombination. EMBO J. 2001 Nov 1;20(21):6127-39.
•Grandin N, Reed SI, Charbonneau M. 1997. Stn1, a new Saccharomyces cerevisiae protein, is implicated in telomere size regulation in association with Cdc13. Genes Dev. 1997 Feb 15;11(4):512-27.
•Gravel S, Larrivée M, Labrecque P, Wellinger RJ. 1998. Yeast Ku as a regulator of chromosomal DNA end structure. Science. 1998 May 1;280(5364):741-4.
•Greider CW, Blackburn EH. 1985. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell. 1985 Dec;43(2 Pt 1):405-13.
•Greider CW, Blackburn EH. 1987. The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. Cell. 1987 Dec 24;51(6):887-98.
•Greider CW, Blackburn EH. 1989. A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature. 1989 Jan 26;337(6205):331-7.
•Griffith JD, Comeau L, Rosenfield S, Stansel RM, Bianchi A, Moss H, de Lange T. 1999. Mammalian telomeres end in a large duplex loop. Cell. 1999 May 14;97(4):503-14.
•Grollman AP. 1967. Inhibitors of protein biosynthesis. II. Mode of action of anisomycin. J. Biol. Chem. 242 (13): 3226-33.
•Gu W, Deng Y, Zenklusen D, Singer RH. 2004. A new yeast PUF family protein, Puf6p, represses ASH1 mRNA translation and is required for its localization. Genes Dev 18(12):1452-65
•Guarante L. 2001. SIR2 and aging--the exception that proves the rule. Trends Genet 17(7):391-2
•Haarer BK, Petzold A, Lillie SH, Brown SS. 1994. Identification of MYO4, a second class V myosin gene in yeast. J Cell Sci 1994 Apr; 107(Pt4):1055-64.
•Hansen *et al.*, 2000
•Hardy CF, Sussel L, Shore D. 1992. A RAP1-interacting protein involved in transcriptional

silencing and telomere length regulation. Genes Dev. 1992 May;6(5):801-14.
•Harnpicharnchai P, Jakovljevic J, Horsey E, Miles T, Roman J, Rout M, Meagher D, Imai B, Guo Y, Brame CJ, Shabanowitz J, Hunt DF, Woolford JL Jr. 2001. Composition and functional characterization of yeast 66S ribosome assembly intermediates. Mol Cell 2001 Sep;8(3):505-15.
•Hausner TP, Geigenmuller U, Nierhaus KH. 1988. The allosteric three-site model for the ribosomal elongation cycle. New insights into the inhibition mechanisms of aminoglycosides, thiostrepton, and viomycin.J Biol Chem. 263(26):13103-11.
•Hayflick L and Moorehead PS. 1961. Exp Cell Res 25, 585-621.
•Hayflick L. 1965. The limited in vitro lifetime of human diploid cell strains. Exp Cell Res. 37, 614-636.
•Hefferin ML and Tomkinson AE. 2005. Mechanism of DNA double-strand break repair by non-homologous end joining. DNA Repair (Amst) 4(6):639-48
•Hemann MT, Greider CW. 1999. G-strand overhangs on telomeres in telomerase-deficient mouse cells. Nucleic Acids Res. 1999 Oct 15;27(20):3964-9.
•Hieronymus H and Silver PA. 2004. A systems view of mRNP biology. GENES & DEVELOPMENT 18:2845-2860.
•Hoek KS, Kidd GJ, Carson JH, Smith R. 1998.hnRNP A2 selectively binds the cytoplasmic transport sequence of myelin basic protein mRNA. Biochemistry. 1998 May 12;37(19):7021-9.
•Holmes SG, Rose AB, Steuerle K, Saez E, Sayegh S, Lee YM, Broach JR. 1997. Hyperactivation of the silencing proteins, Sir2p and Sir3p, causes chromosome loss. Genetics 145(3):605-14
•Hooijberg E, Ruizendaal JJ, Snijders PJ, Kueter EW, Walboomers JM, Spits H. 2000. Immortalization of human CD8+ T cell clones by ectopic expression of telomerase reverse transcriptase. J Immunol. 2000 Oct 15;165(8):4239-45.
•Hsu HL, Gilley D, Blackburn EH, Chen DJ. 1999. Ku is associated with the telomere in mammals. Proc Natl Acad Sci U S A. 1999 Oct 26;96(22):12454-8.
•Hsu HL, Gilley D, Galande SA, Hande MP, Allen B, Kim SH, Li GC, Campisi J, Kohwi-Shigematsu T, Chen DJ. 2000. Ku acts in a unique way at the mammalian telomere to prevent end joining. Genes Dev. 2000 Nov 15;14(22):2807-12.
•Hwang PI, Chu G, Asahara H and Linn S. 1995. FASEB J 9, A1398.
•Iborra FJ, Pombo A, Jackson DA, Cook PR.(1996) Active RNA polymerases are localized within discrete transcription "factories' in human nuclei. J. Cell Sci. 109 (6):1427-1436
•Irie K, Tadauchi T, Takizawa PA, Vale RD, Matsumoto K, Herskowitz I. 2002. The Khd1 protein, which has three KH RNA-binding motifs, is required for proper localization of ASH1

mRNA in yeast. EMBO J 21(5):1158-67

•Irion U and St. Johnston D. 2007. bicoid RNA localization requires specific binding of an endosomal sorting complex. Nature 445, 554-558 (1 February 2007). Received 20 October 2006; Accepted 1 December 2006

•Irion U, Adams J, Chang CW, St Johnston D. 2006. Miranda couples oskar mRNA/Staufen complexes to the bicoid mRNA localization pathway. Dev Biol. 2006 Sep 15;297(2):522-33. Epub 2006 May 26.

•Irion U, Adams J, Chang CW, St Johnston D. 2006. Miranda couples oskar mRNA/Staufen complexes to the bicoid mRNA localization pathway. Dev Biol. 2006 Sep 15;297(2):522-33. Epub 2006 May 26.

•Ishikawa K, Catlett NL, Nocak JL, Tang F, Nau JJ, Weismann LS. 2003. Identification of an organelle specific myosin V receptor. J Cell Biol 2003 Mar 17;160(6):887-97.

•Itoh T, Watabe A, Toh-E A, Matsui Y. 2002. Complex formation with Ypt11p, a rab-type small GTPase, is essential to facilitate the function of Myo2p, a class V myosin, in mitochondrial distribution in Saccharomyces cerevisiae. Mol Cell Biol 2002 Nov;22(22):7744-57.

•Ivy JM, Klar AJ, Hicks JB. 1986. Cloning and characterization of four SIR genes of Saccharomyces cerevisiae. Mol Cell Biol. 1986 Feb;6(2):688-702.

•Jambhekar A and Derisi J. 2007. Cis-acting determinants of asymmetric, cytoplasmic RNA transport. RNA 2007 13: 625-642

•Jambhekar, A., McDermott, K., Sorber, K., Shepard, K.A., Vale, R.D., Takizawa, P.A., and DeRisi, J.L. 2005. Unbiased selection of localization elements reveals cis-acting determinants of mRNA bud localization in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. 102: 18005–18010.

•Jansen RP, Dowzer C, Michaelis C, Galova M, Nasmyth K. 1996. Mother cell specific HO expression in budding yeast depends on the unconventional myosin Myo4p and other cytoplasmic proteins. Cell 1996 Mar 8; 84(5):687-97.

•Januschke J, Gervais L, Dass S, Kaltschmidt J, Lopez-Schier H, Johnston D, Brand A, Roth S, Guichet A. 2002A. Polar transport in the Drosophila oocyte requires Dynein and Kinesin I cooperation. Curr Biol. 12, 1971-81.

•Januschke J, Gervais L, Dass S, Kaltschmidt J, Lopez-Schier H, Johnston D, Brand A, Roth S, Guichet A. 2002B. Polar transport in the Drosophila oocyte requires Dynein and Kinesin I cooperation. Curr Biol. 12, 1971-81.

•Jensen TH, Dower K, Libri D, Rosbash M. 2003. Early formation of mRNP: license for export or quality control? Mol Cell 2003 May; 11(5):1129-38

•Jeske M, Meyer S, Temme C, Freudenreich D, Wahle E. 2006. Rapid ATP-dependent deadenylation of nanos mRNA in a cell-free system from Drosophila embryos. J Biol Chem. 2006 Sep 1;281(35):25124-33. Epub 2006 Jun 22.

•Johnston GC, Prendergast JA, Singer RA. 1991. The Saccharomyces cerevisiae Myo2 gene encodes an essential myosin for vectorial transport of vesciles. J Cell Biol 1991 May;113(3):539-51.

•Johnstone O, Lasko P. 2001. Translational regulation and RNA localization in Drosophila oocytes and embryos. Annu Rev Genet. 2001;35:365-406. Review.

•Jonhson *et al.*, 1995

•K. L. Farina, S. Huttelmaier, K. Musunuru, R. Darnell, and R. H. Singer. 2003. Two ZBP1 KH domains facilitate {beta}-actin mRNA localization, granule formation, and cytoskeletal attachment. J. Cell Biol., January 2, 2003; 160(1): 77 – 87.

•Kaeberlin *et al.*, 1999;

•Kao HI, Henricksen LA, Liu Y, Bambara RA. 2002. Cleavage specificity of Saccharomyces cerevisiae flap endonuclease 1 suggests a double-flap structure as the cellular substrate. J Biol Chem. 2002 Apr 26;277(17):14379-89. Epub 2002 Feb 1

•Karlseder J, Broccoli D, Dai Y, Hardy S, de Lange T. 1999. p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. Science. 1999 Feb 26;283(5406):1321-5

•Kaytor, M.D. and Orr, H.T., 2001. Cell 107, pp. 555–557.

•Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. Specific association of human telomerase activity with immortal cells and cancer. Science. 1994 Dec 23;266(5193):2011-5.

•Kim SH, Kaminker P, Campisi J. 1999. TIN2, a new regulator of telomere length in human cells. Nat Genet. 1999 Dec;23(4):405-12.

•Kim-Ha J, Kerr K, MacDonald PM. 1995. Translation regulation of oskar mRNA by bruno, an ovarian RNA-binding protein, is essential. Cell 1995 May 5;81(3):403-12.

•Kimura A, Umehara T, Horikoshi M. 2002. Chromosomal gradient of histone acetylation established by Sas2p and Sir2p functions as a shield against gene silencing. Nat Genet 32(3):370-7

•Kislauskis EH, Singer RH. 1992. Determinants of mRNA localization. Curr Opin Cell Biol. 1992 Dec;4(6):975-8. Review.

•Kloc M, Etkin LD. 1994. Delocalization of Vg1 mRNA from the vegetal cortex in Xenopus oocytes after destruction of Xlsirt RNA. Science. 1994 Aug 19;265(5175):1101-3.

•Knop M, Siegers K, Pereira G, Zachariae W, Winsor B, Nasmyth K, Schiebel E. 1999. Epitope

tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. Yeast 1999 Jul; 15(10B):963-72.

•Koehler A and Hurt E. 2007. Exporting RNA from the nucleus to the cytoplasm. Nat Rev Mol Cel Biol 2007 Oct;8:761-773.

•Komili S, Farny NG, Roth FP, Silver PA. 2007. Functional specificity among ribosomal proteins regulates gene expression. Cell. 2007 Nov 2;131(3):557-71.

•Kozubowski, L., Larson, J. R., and Tatchell, K. 2005. Role of the septin ring in the asymmetric localization of proteins at the mother-bud neck in Saccharomyces cerevisiae. Mol. Biol. Cell 16, 3455–3466.

•Kruk PA, Rampino NJ, Bohr VA. 1995. DNA damage and repair in telomeres: relation to aging. Proc Natl Acad Sci USA, 92, 258-62.

•Kruse C, Jaedicke A, Beaudouin J, Bohl F, Ferring D, Guttler T, Ellenberg J, Jansen RP. 2002. Ribonucleoprotein-dependent localization of the yeast class V myosin Myo4p. J Cell Biol. 2002 Dec 23;159(6):971-82. Epub 2002 Dec 23.

•Kurtz S and Shore D. 1991. RAP1 protein activates and silences transcription of mating type genes in yeast. Genes Dev, 5, 616-28.

•Kwon S, Schnapp BJ. 2001. RNA localization: SHEdding loght on the RNA-motor linkage. Curr Biol 2001 Mar 6;11(5):166-8

•Kyungjae Myung, Stephanie Smith, and Richard D. Kolodner. 2004. Mitotic checkpoint function in the formation of gross chromosomal rearrangements in Saccharomyces cerevisiae. PNAS, November 9, 2004, vol. 101(45)15980-15985

•Lacal JC, Carrasco L. 1983. Antiviral effects of hygromycin B, a translation inhibitor nonpermeant to uninfected cells. Antimicrob Agents Chemother 24(2):273-5.

•Laggerbauer B, Ostareck D, Keidel EM, Ostareck-Lederer A, Fischer U. 2001. Evidence that fragile X mental retardation protein is a negative regulator of translation. Hum Mol Genet. 2001 Feb 15;10(4):329-38

•Lang BD, Fridovich-Keil JL. 2000. Scp160p, a multiple KH-domain protein, is a component of mRNP complexes in yeast. Nucleic Acids Res 2000 Apr 1;28(7):1576-84.

•Lazzaro F, Sapountzi V, Granata M, Pellicioli A, Vaze M, Haber JE, Plevani P, Lydall D, Muzi-Falconi M. 2008. Histone methyltransferase Dot1 and Rad9 inhibit single-stranded DNA accumulation at DSBs and uncapped telomeres. EMBO J. 2008 Apr 17; [Epub ahead of print]

•Lécuyer E, Yoshida H, Parthasarathy N, Alm C, Babak T, Cerovina T, Hughes TR, Tomancak P, Krause HM. 2007. Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function. Cell. 2007 Oct 5;131(1):174-87.

•Lee HW, Blasco MA, Gottlieb GJ, Horner JW 2nd, Greider CW, DePinho RA. 1998. Essential role of mouse telomerase in highly proliferative organs. Nature. 1998 Apr 9;392(6676):569-74.
•Lee TI, Rinaldi NJ, Robert F, Odom DT, Bar-Joseph Z, Gerber GK, Hannett NM, Harbison CT, Thompson CM, Simon I, Zeitlinger J, Jennings EG, Murray HL, Gordon DB, Ren B, Wyrick JJ, Tagne JB, Volkert TL, Fraenkel E, Gifford DK, Young RA. 2002. Transcriptional regulatory networks in Saccharomyces cerevisiae. 2002 Oct 25;298(5594):799-804.
•Lei EP, Krebber H, Silver PA. 2001. Messenger RNAs are recruited for nuclear export during transcription. Genes Dev 2001 Jul 15;15(14):1771-82.
•Lendvay TS, Morris DK, Sah J, Balasubramanian B, Lundblad V. 1996. Senescence mutants of Saccharomyces cerevisiae with a defect in telomere replication identify three additional EST genes. Genetics. 1996 Dec;144(4):1399-412
•Levy DL and Blackburn EH. 2004. Counting of Rif1p and Rif2p on Saccharomyces cerevisiae telomeres regulates telomere length. Mol Cell Biol 24(24):10857-67
•Lew DJ, et al. 1997. Cell cycle control in Saccharomyces cerevisiae. Pp. 607-695 in The Molecular and Cellular Biology of the Yeast Saccharomyces: Cell Cycle and Cell Biology, edited by Pringle JR, Broach JR and Jones EW. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press
•Li AM, Watson A, Fridovich-Keil JL. 2003. Scp160p associates with specific mRNAs in yeast. Nucleic Acids Res 2003 Apr 1;31(7):1830-7.
•Li B, Oestreich S, de Lange T. 2000. Identification of human Rap1: implications for telomere evolution. Cell. 2000 May 26;101(5):471-83.
•Li X, Heyer WD. 2008. Homologous recombination in DNA repair and DNA damage tolerance. Cell Res. 2008 Jan;18(1):99-113. Review.
•Lichy JH, Horwitz MS, Hurwitz J. 1981. Formation of a covalent complex between the 80,000-dalton adenovirus terminal protein and 5'-dCMP in vitro. Proc Natl Acad Sci U S A. 1981 May;78(5):2678-82.
•Lieber MR. 1997. The FEN-1 family of structure-specific nucleases in eukaryotic DNA replication, recombination and repair. Bioessays. 1997 Mar;19(3):233-40. Review.
•Lillie SH, Brown SS. 1994. Immunofluorescence localization of the unconventional myosin, Myo2p, and the putative kinesin-related protein, Smy1p, to the same regions of polarized growth in Saccharomyces cerevisiae. J Cell Biol 1994 May;125(4):825-42.
•Lin JJ, Zakian VA. 1996. The Saccharomyces CDC13 protein is a single-strand TG1-3 telomeric DNA-binding protein in vitro that affects telomere behavior in vivo. Proc Natl Acad Sci U S A. 1996 Nov 26;93(24):13760-5.

•Lingner J, Cech TR, Hughes TR, Lundblad V. 1997b. Three Ever Shorter Telomere (EST) genes are dispensable for in vitro yeast telomerase activity. Proc Natl Acad Sci U S A. 1997 Oct 14;94(21):11190-5.
•Lingner J, Cech TR. 1996. Purification of telomerase from Euplotes aediculatus: requirement of a primer 3' overhang. Proc Natl Acad Sci U S A. 1996 Oct 1;93(20):10712-7
•Lingner J, Hendrick LL, Cech TR. 1994. Telomerase RNAs of different ciliates have a common secondary structure and a permuted template. Genes Dev. 1994 Aug 15;8(16):1984-98
•Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V, Cech TR. 1997a. Reverse transcriptase motifs in the catalytic subunit of telomerase. Science. 1997 Apr 25;276(5312):561-7.
•Liu Y, Kao HI, Bambara RA. 2004. Flap endonuclease 1: a central component of DNA metabolism. Annu Rev Biochem 73:589-615
•Lombard DB and Guarente L. 2000. Nijmegen breakage syndrome disease protein and MRE11 at PML nuclear bodies and meiotic telomeres. Cancer Res. 2000 May 1;60(9):2331-4.
•Long RM, Gu W, Lorimer E, Singer RH, Chartrand P. 2000. She2p is a novel RNA binding protein that recruits the Myo4p-She3p complex to ASH1 mRNA. EMBO J 2000 Dec 1;19(23):6592-601.
•Long RM, Gu W, Meng X, Gonsalvez G, Singer RH, Chartrand P. 2001. An exclusively nuclear RNA-binding protein affects asymmetric localization of ASH1 mRNA and Ash1p in yeast. J Cell Biol 2001 Apr 16;153(2):307-18.
•Long RM, Singer RH, Meng X, Gonzalez I, Nasmyth K, Jansen RP. 1997. Mating type switching in yeast is controlled by asymmetric localization of ASH1 mRNA. Science 1997 Jul 18;277(5324):383-7,
•Longhese MP, Paciotti V, Neecke H, Lucchini G. 2000. Checkpoint proteins influence telomeric silencing and length maintenance in budding yeast. Genetics 155(4):1577-91.
•Lowell JE and Pillus L. 1998. Telomere tales: chromatin, telomerase and telomere function in Saccharomyces cerevisiae. Cell Mol Life Sci 54(1):32-49
•Ludwig A, Saretzki G, Holm PS, Tiemann F, Lorenz M, Emrich T, Harley CB, von Zglinicki T. 2001. Ribozyme cleavage of telomerase mRNA sensitizes breast epithelial cells to inhibitors of topoisomerase. Cancer Res. 2001 Apr 1;61(7):3053-61
•Lundin C, North M, Erixon K, Walters K, Jenssen D, Goldman ASH and Helleday T. 2005. Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable in vivo DNA double-strand breaks. Nucleic Acids Research 33 (12): 3799–3811.
•Lustig AJ and Petes TD. 1986. Identification of yeast mutants with altered telomere structure.

Proc Natl Acad Sci U S A 83(5):1398-402
•Lustig AJ. 2004. Telomerase RNA: a flexible RNA scaffold for telomerase biosynthesis. Curr Biol 14(14):R565-7
•Lydall D, Weinert T. 1995. Yeast checkpoint genes in DNA damage processing: implications for repair and arrest. Science. 1995 Dec 1;270(5241):1488-91.
•MacDougall N, Clark A, MacDougall E, Davis I. 2003. Drosophila gurken (TGFalpha) mRNA localizes as particles that move within the oocyte in two dynein dependent steps. Dev Cell 2003 Mar, 4(3), 307-19.
•Macquat LE. 2004. Nonsense mediated mRNA decay: Splicing, Translation and mRNP dynamics. Nat Rev Mol Cell Biol 2004 Feb; 5:89-99
•Mages GJ, Feldmann HM, Winnacker EL. 1996. Involvement of the Saccharomyces cerevisiae HDF1 gene in DNA double-strand break repair and recombination. J Biol Chem 271(14):7910-5
•Makarov VL, Hirose Y, Langmore JP. 1997. Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. Cell. 1997 Mar 7;88(5):657-66.
•Maldonado E, Shiekhattar R, Sheldon M, Cho H, Drapkin R, Rickert P, Lees E, Anderson CW, Linn S, Reinberg D. 1995. A human RNA polymerase II complex associated with SRB and DNA-repair proteins. Nature. 1996 May 2;381(6577):86-9. Erratum in: Nature 1996 Nov 28;384(6607):384
•Marc P, Margeot A, Devaux F, Bluegeon C, Corral-Cebrinsky M, Jacq C. 2002. Genome-wide analysis of mRNAs targeted to yeast mitochondria. EMBO Rep 2002 FEB;3(2)159-64
•Marsellach FX, Huertas D, Azorin F. 2006. The multi-KH domain protein of Saccharomyces cerevisiae Scp160p contributes to the regulation of telomeric silencing. J Biol Chem 281(26):18227-35
•Marsich E, Bandiera A, Tell G, Scaloni A, Manzini G. 2001. A chicken hnRNP of the A/B family recognizes the single-stranded d(CCCTAA)(n) telomeric repeated motif. Eur J Biochem. 2001 Jan;268(1):139-48.
•Martin SG, Laroche T, Suka N, Grunstein M, Gasser SM. 1999. Relocalization of telomeric Ku and SIR proteins in response to DNA strand breaks in yeast. Cell. 1999 May 28;97(5):621-33.
•Martinez-Exposito MJ, Kaplan KB, Copeland J, Sorger PK. 1999. Retention of the BUB3 checkpoint protein on lagging chromosomes. Proc Natl Acad Sci U S A. 1999 Jul 20;96(15):8493-8.
•Matecic M, Stuart S, Holmes S. 2002. Sir2 induced inviability is suppressed by Histone H4 overexpression. Genetics 162:973-76.

•Matsumu, Y. & Ananthaswamy, H. N. 2004. “Toxic effects of ultraviolet radiation on the skin”, Toxicology and Applied Pharmacology: 298-308
•Maxon ME, Herskowitz I. 2001. Ash1p is a site-specific DNA binding protein that actively represses transcription. Proc Natl Acad Sci USA 2001 Feb 13;98(4):1495-500,
•McBride HJ, Sil A, Measday V, Yu Y, Moffat J, Maxon ME, Herskowitz I, Andrews B, Stillman DJ. 2001. The protein kinase Pho85 is required for asymmetric accumulation of the Ash1 protein in Saccharomyces cerevisiae. Mol Microbiol 2001 Oct; 42(2):345-53.
•McCleland ML, Gardner RD, Kallio MJ, Daum JR, Gorbsky GJ, Burke DJ, and Stukenberg PT. 2003. The highly conserved Ndc80 complex is required for kinetochore assembly, chromosome congression, and spindle checkpoint activity. Genes Dev. 17:101–114.
•Meier B, Driller L, Jaklin S, Feldmann HM. 2001. New function of CDC13 in positive telomere length regulation. Mol Cell Biol 21(13):4233-45.
•Michelson R and Weinert T. 1999. Sensor-less checkpoint activation? Nature Cell Biology 1, E177 - E179.
•Mishra K and Shore D. 1999. Yeast Ku protein plays a direct role in telomeric silencing and counteracts inhibition by rif proteins. Curr Biol 9(19):1123-6
•Moazed D, Kistler A, Axelrod A, Rine J, Johnson AD. 1997. Silent information regulator protein complexes in Saccharomyces cerevisiae: a SIR2/SIR4 complex and evidence for a regulatory domain in SIR4 that inhibits its interaction with SIR3. Proc Natl Acad Sci U S A 94(6):2186-91
•Moazed, D. and H.F. Noller. 1987. Interaction of antibiotics with functional sites in 16S ribosomal RNA. Nature 327(6121): 389-94.
•Muenchow S, Sauter C, Jansen RP. 1999. Association of the class V myosin Myo4p with a localized messenger RNA in budding yeast depends on the She proteins. J Cell Sci 1999 May;112 (Pt10):1511-8.
•Musarò M, Ciapponi L, Fasulo B, Gatti M, Cenci G. 2008. Unprotected Drosophila melanogaster telomeres activate the spindle assembly checkpoint. Nat Genet. 2008 Mar;40(3):362-6. Epub 2008 Feb 3.
•Nasmyth K, Jansen RP. 1997. The cytoskeleton in mRNA localization and cell differentiation. Curr Opin Cell Biol. 1997 Jun;9(3):396-400.
•Nasmyth K. 1993. Regulating the HO endonuclease in yeast. Curr Opin Cell Biol 1997 Jun;9(3):396-400.
•Niessing D, Huttelmaier S, Zenklusen D, Singer RH, Burley SK. 2004. She2p is a novel RNA binding protein with a basic helical hairpin motif. Cell 119(4):491-502

•Nissan, T.A., Bassler, J., Petfalski, E., Tollervey, D. and Hurt, E.2002. 60S pre-ribosome formation viewed from assembly in the nucleolus until export to the cytoplasm. Embo J, 21, 5539-5547.

•NJ Pokrywka and EC Stephenson. 1991. Microtubules mediate the localization of bicoid RNA during Drosophila oogenesis. Development, Vol 113, Issue 1 55-66.

•Nugent CI, Bosco G, Ross LO, Evans SK, Salinger AP, Moore JK, Haber JE, Lundblad V. 1998. Telomere maintenance is dependent on activities required for end repair of double-strand breaks. Curr Biol 8(11):657-60

•Oleynikov, Y. and Singer, R.H.. 2003. Real-time visualization of ZBP1 association with beta-actin mRNA during transcription and localization. Curr Biol, 13, 199-207.

•Olivier, C., Poirier, G., Gendron, P., Boisgontier, A., Major, F., and Chartrand, P. 2005. Identification of a conserved RNA motif essential for She2p recognition and mRNA localization to the yeast bud. Mol. Cell. Biol. 25: 4752–4766.

•Osada S, Sutton A, Muster N, Brown CE, Yates III JR, Sternglanz R and Workman JL. 2001. The yeast SAS (something about silencing) protein complex contains a MYST-type putative acetyltransferase and functions with chromatin assembly factor ASF1. Genes Dev 15(23):3155-68

•Otto C, Almqvist E, Hayden M, Andrew S. 2001. The "flap" endonuclease gene FEN1 is excluded as a candidate gene implicated in the CAG repeat expansion underlying Huntington disease. Clin Genet 59(2):122-7

•Palladino F, Laroche T, Gilson E, Axelrod A, Pillus L, Gasser SM. 1993. SIR3 and SIR4 proteins are required for the positioning and integrity of yeast telomeres. Cell. 1993 Nov 5;75(3):543-55.

•Pan X, Ye P, Yuan DS, Wang X, Bader JS, Boeke JD. 2006. A DNA integrity network in the yeast Saccharomyces cerevisiae. Cell 124(5):1069-81

•Paquin N, Menade M, Poirier G, Donato D, Drouet E, Chartrand P. 2007. Local Activation of Yeast ASH1 mRNA Translation through Phosphorylation of Khd1p by the Casein Kinase Yck1p. Mol Cell 26(6):795-809

•Park PU, Defossez PA, Guarente L. 1999. Effects of mutations in DNA repair genes on formation of ribosomal DNA circles and life span in Saccharomyces cerevisiae. Mol Cell Biol 19(5):3848-56

•Peterson SE, Stellwagen AE, Diede SJ, Singer MS, Haimberger ZW, Johnson CO, Tzoneva M, Gottschling DE. 2001. The function of a stem-loop in telomerase RNA is linked to the DNA repair protein Ku. Nat Genet 27(1):64-7

•Phillips ER, McKinnon PJ. 2007. DNA double-strand break repair and development. Oncogene. 2007 Dec 10;26(56):7799-808. Review.
•Platt OS. 2008. "Hydroxyurea for the treatment of sickle cell anemia". N. Engl. J. Med. 358 (13): 1362–9.
•Polotnianka RM, Li J, Lustig AJ. 1998. The yeast Ku heterodimer is essential for protection of the telomere against nucleolytic and recombinational activities. Curr Biol. 1998 Jul 2;8(14):831-4
•Porter SE, Greenwell PW, Ritchie KB, Petes TD. 1996. The DNA-binding protein Hdf1p (a putative Ku homologue) is required for maintaining normal telomere length in Saccharomyces cerevisiae. Nucleic Acids Res. 1996 Feb 15;24(4):582-5.
•Prescott J and Blackburn EH. 1997. Functionally interacting telomerase RNAs in the yeast telomerase complex. Genes Dev. 1997 Nov 1;11(21):2790-800.
•Pruyne DW, Schott DH, Bretscher A. 1998. Tropomyosin-containing actin cables direct Myo2p-dependent polarized delivery of secretory vesicles in budding yeast. J Cell Biol 1998 Dec 28;143(7):1931-45.
•Pryde FE, Louis EJ. 1999. Limitations of silencing at native yeast telomeres. EMBO J. 1999 May 4;18(9):2538-50
•Puig O, Caspary F, Rigaut G, Rutz B, Bouveret E, Bragado-Nilsson E, Wilm M, Seraphin B. 2001. The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods 24(3):218-29
•Qi H and Zakian VA. 2000. The Saccharomyces telomere-binding protein Cdc13p interacts with both the catalytic subunit of DNA polymerase alpha and the telomerase-associated est1 protein. Genes Dev. 2000 Jul 15;14(14):1777-88.
•Reagan MS, Pittenger C, Siede W, Friedberg EC. 1995. Characterization of a mutant strain of Saccharomyces cerevisiae with a deletion of the RAD27 gene, a structural homolog of the RAD2 nucleotide excision repair gene. J Bacteriol. 1995 Jan;177(2):364-71
•Resnick MA and Martin P. 1976. The repair of double-strand breaks in the nuclear DNA of Saccharomyces cerevisiae and its genetic control. Mol Gen Genet. 1976 Jan 16;143(2):119-29.
•Riechmann V, Ephrussi A. 2001. Axis formation during Drosophila oogenesis. Curr Opin Genet Dev. 2001 Aug;11(4):374-83. Review.
•Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Seraphin B. 1999. A generic protein purification method for protein complex characterization and proteome exploration. Nat Biotechnol 17(10):1030-2
•Ritchie KB and Petes TD. 2000. The Mre11p/Rad50p/Xrs2p complex and the Tel1p function in

a single pathway for telomere maintenance in yeast. Genetics. 2000 May;155(1):475-9.

•Ritchie KB, Mallory JC, Petes TD. 1999. Interactions of TLC1 (which encodes the RNA subunit of telomerase), TEL1, and MEC1 in regulating telomere length in the yeast Saccharomyces cerevisiae. Mol Cell Biol. 1999 Sep;19(9):6065-75.

•Romero DP, Blackburn EH. 1991. A conserved secondary structure for telomerase RNA. Cell. 1991 Oct 18;67(2):343-53.

•Rongo C, Gavis ER, Lehmann R. 1995. Localization of oskar RNA regulates oskar translation and requires Oskar protein. Development. 1995 Sep;121(9):2737-46

•Rossi *et al.*, 2006

•Rother S and Strasser K. 2007. The RNA polymerase II CTD kinase Ctk1 functions in translation elongation. Genes Dev 21(11):1409-21

•Roy N and Runge KW. 1999. The ZDS1 and ZDS2 proteins require the Sir3p component of yeast silent chromatin to enhance the stability of short linear centromeric plasmids. Chromosoma. 1999 Jul;108(3):146-61. Erratum in: Chromosoma 2000 Mar;108(8):533.

•Rudolph KL, Chang S, Lee HW, Blasco M, Gottlieb GJ, Greider C, DePinho RA. 1999. Longevity, stress response, and cancer in aging telomerase-deficient mice. Cell. 1999 Mar 5;96(5):701-12

•Rundlett SE, Carmen AA, Kobayashi R, Bavykin S, Turner BM, Grunstein M. 1996. HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. Proc Natl Acad Sci U S A 93(25):14503-8

•Saguez C, Olesen JR and Jensen TH. 2005. Formation of export-competent mRNP: escaping nuclear destruction. Current Opinion in Cell Biology. Volume 17, Issue 3, June 2005, Pages 287-293

•Saint-Georges Y, Garcia M, Delaveau T, Jourdren L, Le Crom S, Lemoine S, Tanty V, Devaux F, Jacq C. 2008. Yeast mitochondrial biogenesis: a role for the PUF RNA-binding protein Puf3p in mRNA localization. PLoS ONE 3(6):e2293.

•Samper E, Goytisolo FA, Slijepcevic P, van Buul PP, Blasco MA. 2000. Mammalian Ku86 protein prevents telomeric fusions independently of the length of TTAGGG repeats and the G-strand overhang. EMBO Rep. 2000 Sep;1(3):244-52.

•Saxton W. 2001. Microtubules, Motors, and mRNA Localization MechanismsWatching Fluorescent Messages Move. Cell , Volume 107 , Issue 6 , Pages 707 - 710.

•Schmid M, Jaedicke A, Du TG, Jansen RP. 2006. Coordination of endoplasmic reticulum and mRNA localization to the yeast bud. Curr Biol 16(15):1538-43

•Schneider R, Bannister AJ, Myers FA, Thorne AW, Crane-Robinson C, Kouzarides T. 2004.

Histone H3 lysine 4 methylation patterns in higher eukaryotic genes. Nat Cell Biol. 2004 Jan;6(1):73-7. Epub 2003 Dec 7

•Schnorrer F, Bohmann K, Nüsslein-Volhard C. 2000. The molecular motor dynein is involved in targeting swallow and bicoid RNA to the anterior pole of Drosophila oocytes. Nat Cell Biol. 2000 Apr;2(4):185-90.

•Schott DH, Ho J, Pruyne D, Bretscher A. 1999. The COOH-terminal domain of Myo2p, a yeast myosin V, has a direct role in secretory vesicle targeting. J Cell Biol 1999 Nov 15:147(4):791-808.

•Schott DH, Huffaker T, Bretscher A. 2002. Microfilaments and microtubules: the news from yeast. Curr Opin Microbiol 2002 Dec;5(6):564-74.

•Schreier WJ, Schrader TE, Koller FO, Gilch G, Crespo-Hernández CG, Swaminathan VN, Carell T, Zinth W, Kohler B. 2007. Thymine Dimerization in DNA Is an Ultrafast Photoreaction. Science 2 February 2007: Vol. 315. no. 5812, pp. 625 – 629

•Schwob E and Nasmyth K. 1993. CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in Saccharomyces cerevisiae. Genes Dev 7(7A):1160-75

•Segref A, Sharma K, Doye V, Hellwig A, Huber J, Lührmann R, Hurt E. 1997. Mex67p, a novel factor for nuclear mRNA export, binds to both poly(A)+ RNA and nuclear pores. EMBO J. 1997 Jun 2;16(11):3256-71.

•Seto AG, Zaug AJ, Sobel SG, Wolin SL, Cech TR. 1999. Saccharomyces cerevisiae telomerase is an Sm small nuclear ribonucleoprotein particle. Nature 401(6749):177-80

•Shan X, Xue Z, Euskirchen G, Mélèse T. 1997. NNF1 is an essential yeast gene required for proper spindle orientation, nucleolar and nuclear envelope structure and mRNA export. J Cell Sci. 1997 Jul;110 (Pt 14):1615-24.

•Sharanov YS, Zvereva MI, Dontsova OA. 2006. Saccharomyces cerevisiae telomerase subunit Est3p binds DNA and RNA and stimulates unwinding of RNA/DNA heteroduplexes. FEBS Lett. 2006 Aug 21;580(19):4683-90. Epub 2006 Jul 24.

•Shay JW and Wright WE. 1996. Telomerase activity in human cancer. Curr Opin Oncol. 1996 Jan;8(1):66-71. Review.

•Shay JW, Wright WE, Werbin H. 1993. Toward a molecular understanding of human breast cancer: a hypothesis. Breast Cancer Res Treat. 1993;25(1):83-94. Review.

•Shepard KA, Gerber AP, Jambhekar A, Takizawa PA, Brown PO, Herschlag D, DeRisi JL, Vale RD. 2003. Widespread cytoplasmic mRNA transport in yeast: identification of 22 bud localized transcripts using DNA microarray analysis. Proc Natl Acad Sci USA 2003 Sep 30;100(20):11429-34.

•Shore D and Nasmyth K. 1987. Purification and cloning of a DNA binding protein from yeast that binds to both silencer and activator elements. Cell. 1987 Dec 4;51(5):721-32.
•Shore D, Squire M, Nasmyth KA. 1984. Characterization of two genes required for the position-effect control of yeast mating-type genes. EMBO J 3(12):2817-23
•Shrivastav M, De Haro LP, Nickoloff JA. 2008. Regulation of DNA double-strand break repair pathway choice. Cell Res. 2008 Jan;18(1):134-47. Review.
•Sikorski RS, Hieter P. 1989. A system of shuttling vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics. 1989 May;122(1)19-22
•Sil A, Herskowitz I. 1996. Identification of asymmetrically localized determinant, Ash1p, required for lineage-specific transcription of the yeast HO gene. Cell 1996 Mar 8;84(5):711-22.
•Singer MS and Gottschling DE. 1994. TLC1: template RNA component of Saccharomyces cerevisiae telomerase. Science 266(5184):404-9
•Siomi H, Choi M, Siomi MC, Nussbaum RL, Dreyfuss G. 1994. Essential role for KH domains in RNA binding: impaired RNA binding by a mutation in the KH domain of FMR1 that causes fragile X syndrome. Cell. 1994 Apr 8;77(1):33-9.
•Slack C, Overton PM, Tuxworth RI and Chia W. 2007. Asymmetric localisation of Miranda and its cargo proteins during neuroblast division requires the anaphase-promoting complex/cyclosome. Development 134, 3781-3787 (2007).
•Smilenov LB, Morgan SE, Mellado W, Sawant SG, Kastan MB, Pandita TK. 1997. Influence of ATM function on telomere metabolism. Oncogene. 1997 Nov 27;15(22):2659-65.
•Smith JS, Brachmann CB, Pillus L, Boeke JD. 1998. Distribution of a limited Sir2 protein pool regulates the strength of yeast rDNA silencing and is modulated by Sir4p. Genetics 149(3):1205-19
•Smith S, Giriat I, Schmitt A, de Lange T. 1998. Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. Science. 1998 Nov 20;282(5393):1484-7
•Smogorzewska A, de Lange T. 2004. Regulation of telomerase by telomeric proteins. Annu Rev Biochem. 2004;73:177-208. Review.
•Smogorzewska A, van Steensel B, Bianchi A, Oelmann S, Schaefer MR, Schnapp G, de Lange T. 2000. Control of human telomere length by TRF1 and TRF2. Mol Cell Biol. 2000 Mar;20(5):1659-68
•Sommers CH, Miller EJ, Dujon B, Prakash S, Prakash L. 1995. Conditional lethality of null mutations in RTH1 that encodes the yeast counterpart of a mammalian 5'- to 3'-exonuclease required for lagging strand DNA synthesis in reconstituted systems. J Biol Chem. 1995 Mar 3;270(9):4193-6.

•Spahn CM and Prescott CD. 1996. Throwing a spanner in the works: antibiotics and the translation apparatus. J Mol Med 74(8): 423-39.
•Spellman PT, Sherlock G, Zhang MQ, Iyer VR, Anders K, Eisen MB, Brown PO, Botstein D, Futcher B. 1998. Comprehensive identification of cell cycle regulated genes of the yeast Saccharomyces cerevisiae by microarray hybridization. Mol Biol Cell 1998 Dec;9(12):3273-97.
•Sperger JM and Cech TR, 2001. A stem-loop of Tetrahymena telomerase RNA distant from the template potentiates RNA folding and telomerase activity. Biochemistry. 2001 Jun 19;40(24):7005-16.
•St Johnston D, Brown NH, Gall JG, Jantsch M. 1992. A conserved double-stranded RNA-binding domain. Proc Natl Acad Sci U S A. 1992 Nov 15;89(22):10979-83.
•St Johnston D. 2005. Moving messages: the intracellular localization of mRNAs. Nat Rev Mol Cell Biol. 2005 May;6(5):363-75. Review.
•Stansel MR, de Lange T, Griffith JD. 2001. T-loop assembly in vitro involves binding of TRF2 near the 3' telomeric overhang. EMBO J. 2001 Oct 1;20(19):5532-40.
•Stellwagen AE, Haimberger ZW, Veatch JR, Gottschling DE. 2003. Ku interacts with telomerase RNA to promote telomere addition at native and broken chromosome ends. Genes Dev 17(19):2384-95
•Sterling CH and Sweasy JB. 2006. DNA polymerase 4 of Saccharomyces cerevisiae is important for accurate repair of methyl-methanesulfonate-induced DNA damage. Genetics 172(1):89-98
•Stewart M. 2007. Ratcheting mRNA out of the nucleus. Mol Cell. 2007 Feb 9;25(3):327-30
•Strahl-Bolsinger S, Hecht A, Luo K, Grunstein M. 1997. SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. Genes Dev. 1997 Jan 1;11(1):83-93.
•Stutz F, Izaurralde E. 2003. The interplay of nuclear mRNP assembly, mRNA surveillance and export. Trends Cell Biol. 2003 Jun;13(6):319-27.
•Suka N, Luo K, Grunstein M. 2002. Sir2p and Sas2p opposingly regulate acetylation of yeast histone H4 lysine16 and spreading of heterochromatin. Nat Genet 32(3):378-83
•Sun ZW and Hampsey M (1999) A general requirement for the Sin3-Rpd3 histone deacetylase complex in regulating silencing in Saccharomyces cerevisiae. Genetics 152(3):921-32
•Sundell CL, Singer RH. 1991. Requirement of microfilaments in sorting of actin messenger RNA. Science 1991 Sep 13;253(5025):1275-7.
•Surana U, et al. 1991. The role of CDC28 and cyclins during mitosis in the budding yeast S. cerevisiae. Cell 65(1):145-61
•Sylvestre J, Margeot A, Jacq C, Dujardin G, Corral-Debrinski M. 2003. The role of the 3'

untranslated region in mRNA sorting to the vicinity of mitochondria is conserved from yeast to human cells. Mol Biol Cell. 2003 Sep;14(9):3848-56. Epub 2003 Jun 13

•Symington, LS. 1998. Homologous recombination is required for the viability of rad27 mutants. Nucleic Acids Res. 1998 Dec 15;26(24):5589-95.

•Szentirmay MN and Sawadogo M. 2000. Spatial organization of RNA polymerase II transcription in the nucleus. Nucleic Acids Research, 2000, Vol. 28, No. 10 2019-2025

•Takizawa PA, Sil A, Swedlow JR, Heskowitz I, Vale RD. 1997. Actin-dependent localization of an RNA encoding a cell fate determinant in yeast. Nature 1997 Sep 4;389(6646):90-3

•Takizawa PA, Vale RD. 2000. The myosin motor, Myo4p, binds ASH1 mRNA via the adapter protein, She3p. Proc Natl Acad Sci USA. 2000 May 9;97(10):5273-8.

•Tamaru H, Zhang X, McMillen D, Singh PB, Nakayama J, Grewal SI, Allis CD, Cheng X, Selker EU. 2003. Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in Neurospora crassa. Nat Genet. 2003 May;34(1):75-9

•Tang F, Kauffman EJ, Novak JL, Nau JJ, Catlett NL, Weisman LS. 2003. Regulated degradation of a class V myosin receptor directs movement of the yeast vacuole. Nature 2003 Mar 6;422(6927):87-92.

•Teixeira MT, Forstemann K, Gasser SM, Lingner J. 2002. Intracellular trafficking of yeast telomerase components. EMBO Rep. 2002 Jul;3(7):652-9

•Teo SH, Jackson SP. 2001. Telomerase subunit overexpression suppresses telomere-specific checkpoint activation in the yeast yku80 mutant. EMBO Rep. 2001 Mar;2(3):197-202

•Tishkoff DX, Filosi N, Gaida GM, Kolodner RD. 1997. A novel mutation avoidance mechanism dependent on S. cerevisiae RAD27 is distinct from DNA mismatch repair. Cell. 1997 Jan 24;88(2):253-63.

•Ule J, Jensen K, Mele A, Darnell RB. 2005. CLIP: a method for identifying protein-RNA interaction sites in living cells. Methods. 2005 Dec;37(4):376-86.

•Ule J, Jensen KB, Ruggiu M, Mele A, Ule A, Darnell RB. 2003. CLIP identifies Nova-regulated RNA networks in the brain. Science. 2003 Nov 14;302(5648):1212-5.

•van Gent DC, van der Burg M. 2007. Non-homologous end-joining, a sticky affair. Oncogene. 2007 Dec 10;26(56):7731-40. Review.

•van Steensel B and de Lange T. 1997. Control of telomere length by the human telomeric protein TRF1. Nature. 1997 Feb 20;385(6618):740-3.

•van Steensel B, Smogorzewska A, de Lange T. 1998. TRF2 protects human telomeres from end-to-end fusions. Cell. 1998 Feb 6;92(3):401-13.

•Vaziri H, Benchimol S. 1998. Reconstitution of telomerase activity in normal human cells

leads to elongation of telomeres and extended replicative life span. Curr Biol. 1998 Feb 26;8(5):279-82.

•Vaziri H, Schächter F, Uchida I, Wei L, Zhu X, Effros R, Cohen D, Harley CB. 1993. Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. Am J Hum Genet. 1993 Apr;52(4):661-7.

•Vaziri H, Squire JA, Pandita TK, Bradley G, Kuba RM, Zhang H, Gulyas S, Hill RP, Nolan GP, Benchimol S. 1999. Analysis of genomic integrity and p53-dependent G1 checkpoint in telomerase-induced extended-life-span human fibroblasts. Mol Cell Biol. 1999 Mar;19(3):2373-9.

•Vicens Q and Westhof E. 2001. Crystal Structure of Paromomycin Docked into the Eubacterial Ribosomal Decoding A Site. Structure 9 (8): 647–658

•Virta-Pearlman V, Morris DK, Lundblad V. 1996. Est1 has the properties of a single-stranded telomere end-binding protein. Genes Dev. 1996 Dec 15;10(24):3094-104

•Viscardi V, Clerici M, Cartagena-Lirola H, Longhese MP. 2005. Telomeres and DNA damage checkpoints. Biochimie. 2005 Jul;87(7):613-24. Epub 2004 Dec 9. Review.

•Wang MJ, Lin YC, Pang TL, Lee JM, Chou CC, Lin JJ. 2000. Telomere-binding and Stn1p-interacting activities are required for the essential function of Saccharomyces cerevisiae Cdc13p. Nucleic Acids Res. 2000 Dec 1;28(23):4733-41.

•Wang SS and Zakian VA. 1990. Sequencing of Saccharomyces telomeres cloned using T4 DNA polymerase reveals two domains. Mol Cell Biol. 1990 Aug;10(8):4415-9.

•Watson J. 1972. Origin of concatemeric T7 DNA. Nat New Biol. 1972 Oct 18;239(94):197-201

•Wenz C, Enenkel B, Amacker M, Kelleher C, Damm K, Lingner J. 2001. Human telomerase contains two cooperating telomerase RNA molecules. EMBO J. 2001 Jul 2;20(13):3526-34

•Weterings E, Chen DJ. 2008. The endless tale of non-homologous end-joining. Cell Res. 2008 Jan;18(1):114-24. Review.

•White LK, Wright WE, Shay JW. 2001. Telomerase inhibitors. Trends Biotechnol. 2001 Mar;19(3):114-20. Review

•Wilhelm JE, Hilton M, Amos Q, Henzel WJ. 2003. Cup is an eIF4E binding protein required for both the translational repression of oskar and the recruitment of Barentsz. J Cell Biol 2003 Dec 22;163(6):1192-204

•Wilhelm JE, Mansfield J, Hom-Booher N, Wang S, Turck CW, Hazelrigg T, Vale RD. 2000. Isolation of a ribonucleoprotein complex involved in mRNA localization in Drosophila oocytes. J Cell Biol 2000 Feb 7; 148(3):427-40

•Wilson S, Warr N, Taylor DL, Watts FZ. 1999. The role of Schizosaccharomyces pombe

Rad32, the Mre11 homologue, and other DNA damage response proteins in non-homologous end joining and telomere length maintenance. Nucleic Acids Res 27(13):2655-61

•Wilson TE and Lieber MR. 1999. Efficient processing of DNA ends during yeast nonhomologous end joining. Evidence for a DNA polymerase beta (Pol4)-dependent pathway. J Biol Chem 274(33):23599-609

•Winters E, Baroudy BM, Moss B. 1985. Molecular cloning of the terminal hairpin of vaccinia virus DNA as an imperfect palindrome in an Escherichia coli plasmid. Gene. 1985;37(1-3):221-8.

•Wintersberger U, Kuhne C, Karwan A. 1995. Scp160p, a new yeast protein associated with the nuclear membrane and the endoplasmic reticulum, is necessary for maintenance of exact ploidy. Yeast 1995 Aug;11(10):929-44.

•Wotton JM and Shore D. 1997. A novel Rap1p-interacting factor, Rif2p, cooperates with Rif1p to regulate telomere length in Saccharomyces cerevisiae. Genes Dev. 1997 Mar 15;11(6):748-60.

•Wright JH, Gottschling DE, Zakian VA. 1992. Saccharomyces telomeres assume a non-nucleosomal chromatin structure. Genes Dev. 1992 Feb;6(2):197-210.

•Wright WE, Shay JW. 2001. Cellular senescence as a tumor-protection mechanism: the essential role of counting. Curr Opin Genet Dev. 2001 Feb;11(1):98-103. Review.

•Wright WE, Tesmer VM, Liao ML, Shay JW. 1999. Normal human telomeres are not late replicating. Exp Cell Res. 1999 Sep 15;251(2):492-9.

•Wu KJ, Grandori C, Amacker M, Simon-Vermot N, Polack A, Lingner J, Dalla-Favera R. 1999. Direct activation of TERT transcription by c-MYC. Nat Genet. 1999 Feb;21(2):220-4

•Wyllie FS, Jones CJ, Skinner JW, Haughton MF, Wallis C, Wynford-Thomas D, Faragher RG, Kipling D. 2000. Telomerase prevents the accelerated cell ageing of Werner syndrome fibroblasts. Nat Genet. 2000 Jan;24(1):16-7.

•Yang CP, Chen YB, Meng FL, Zhou JQ. 2006. Saccharomyces cerevisiae Est3p dimerizes in vitro and dimerization contributes to efficient telomere replication in vivo. Nucleic Acids Res. 2006 Jan 17;34(2):407-16.

•Yin H, Pruyne D, Huffaker TC, Brestcher A. 2000. Myosin-V orientates the mitotic spindle in yeast. Nature 2000 Aug 31;406(6799):1013-5.

•Yisraeli JK, Sokol S, Melton DA. 1990. A two-step model for the localization of maternal mRNA in Xenopus oocytes: involvement of microtubules and microfilaments in the translocation and anchoring of Vg1 mRNA. Development. 1990 Feb;108(2):289-98.

•Zaessinger S, Busseau I, Simonelig M. 2006. Oskar allows nanos mRNA translation in

Drosophila embryos by preventing its deadenylation by Smaug/CCR4. Development. 2006 Nov;133(22):4573-83. Epub 2006 Oct 18.

•Zalfa, F., Giorgi, M., Primerano, B., Reis, S., Oostra, B. and Bagni, C., 2003. Cell 112, pp. 317–327

•Zarnack K, Feldbrügge M. 2007. mRNA trafficking in fungi. Mol Genet Genomics. 2007 Oct;278(4):347-59. Epub 2007 Sep 1.

•Zhang, Y.Q., Bailey, A.M., Matthies, H.J.G., Renden, R.B., Smith, M.A., Speese, S.D., Rubin, G.M. and Briadie, K., 2001. Cell 107, pp. 591–603

•Zhou J, Hidaka K, Futcher B. 2000. The Est1 subunit of yeast telomerase binds the Tlc1 telomerase RNA. Mol Cell Biol. 2000 Mar;20(6):1947-55

•Zhu XD, Küster B, Mann M, Petrini JH, de Lange T. 2000. Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres. Nat Genet. 2000 Jul;25(3):347-52.

10. Summary

ASH1 mRNA translation has been shown to be required for efficient anchoring of *ASH1* mRNA to the bud-tip during the process of mRNA localization.

Khd1p, a 3-KH domain containing protein, has been shown to be involved in *ASH1* mRNA localization and has been proposed to be a translation repressor (Irie *et al.*, 2002). This thesis focuses on the role of Khd1p in *ASH1* mRNP architecture, specially in its relationship to other members of the locasome. Results presented show that Khd1p is a part of an RNA-independent protein complex that includes She3p and She2p, indicating Khd1p is a part of the locasome via protein-protein interactions. Furthermore, the observation that Khd1p and She3p interact independently of She2p indicates that another protein complex exists and that this complex could be another cargo for the SHE mRNA localization machinery. On the other hand, results presented in this work show that Khd1p floats with ER membranes, as observed for She2p (Schmid *et al.*, 2006) indicating that Khd1p is also implicated in ER-associated. The fact that Khd1p floats independently of She2p indicates that it might be involved in ER inheritance, via a direct binding to She3p. During the course of this work, it was shown that Khd1p is phosphorylated by Yck1p at the cellular periphery and upon phosphorylation, Khd1p releases the *ASH1* mRNA translation block (Paquin *et al.*, 2007) thus proving the model for Khd1p function in translation repression during *ASH1* mRNA transport and localization.

Khd1p, an hnRNP K homolog, has also been shown to play a role in telomeric silencing and be bound to subtelomeric chromatin (Denisenko *et al.*, 2002). This thesis addresses how Khd1p, an mRNA binding protein, can also play a role in this process. Results presented show that Khd1p contributes to telomericgene transcriptional silencing. Associated to loss of *KHD1* I found that Sir2p levels are downregulated, leading to loss of silencing in mutants known to be silenced (e.g. *RPD3* – Rundlett *et al.*, 1996). This loss of silencing could be partially rescued with wildtype copies of *SIR2*.

Results presented in this work suggest that *KHD1* is also involved in binding to the telomerase RNA subunit*, TLC1* or even in its biogenesis.

In addition, a loss of *KHD1* also led to increased sensitivity to DNA damaging agents (UV and MMS) of *rad52* and *xrs2* null mutants. Results presented show that *KHD1* contributes to NHEJ DNA repair efficiency and that this loss of NHEJ efficiency can be rescued with a wildtype copy of *KHD1*. In addition, a point mutant I183R, cannot rescue this NHEJ defect, thus implying that this

residue or the folding of this KH domain is essential for NHEJ function.

Results presented in this thesis establish Khd1p as a putative new cargo for the SHE machinery and also as an ER associated protein. Furthermore, results presented here also establish Khd1p as a regulator of gene transcriptional telomeric silencing, associated to a downregulation of Sir2p. In addition, we show that Khd1p contributes to NHEJ DNA repair, indicating a much wider action than just in mRNA localization.

11. Curriculum Vitae

Name:	Gonçalo Nuno Nunes da Silva Rebelo de Andrade
Address:	Estrada da Luz, 134, 3º Esq, 1600-162 Lisboa – Portugal
	Trappentreustrasse 35, 80339 Muenchen - Deutschland
Date of Birth:	31.01.1978
Nationality:	Portuguese
Marital status:	Single
Phone number:	+ 49 89 2180 76 933
Mobile:	+ 49 174 324 05 53

Education

λGymnasium completed with a score of **18** (out of 20) in Catholic School, Lisbon

λDegree in *Microbial Biology and Genetics*, from the University of Lisbon, Science Faculty, in July 2001, with a score of **16** (out of 20).

λ*I Workshop on Plant Biotechnology*, ITQB, Oeiras, September 1998.

λ*IV Practical Course on Microbial Molecular Taxonomy* – February 2001, Centre of Genetics and Molecular Biology (CGBM – Lisbon).

λ*I International Workshop on Microbial Risk Assessment and Mitigation* – Veterinary Medicine Faculty, Lisbon; École Veterinaire de Alford (France), Cornell Veterinary Medicine, April 2002.

λ*I International Workshop on Biological Sequence Analysis* – coordinators: Cheetham J., Cruz e Silva E., Carleton University / Aveiro University, Aveiro, June 2003.

Graduate School

•*III International Forensic Y-User Workshop – Y Chromosome Haplotype Databases, state of the art and future developments* – Oporto, November 2002.

•*XII Congresso Nacional de Bioquímica (XII National Biochemistry Congress)* – Lisbon, December 2002.

•*Genetic Epidemiology* – coordinator: Sousa A., UniGENe/IBMC, Oporto, December 2002.

•*The Life of an mRNA* - Speakers: Proudfoot N., Smith C., Izarraulde E., Carmo-Fonseca M., Tollervey D., Moreira A., Oporto, January 2003.

•*Advanced Course in Functional Proteomics* – coordinator: Santos M., Boucherie E., Aveiro University/Bordeaux University, Aveiro, February 2003.

•*Molecular Biology Cell Cycle* – coordinator: Sunkel C., IBMC, Oporto, February 2003.

•*RNOMICS* – coordinator: Carmo-Fonseca M., Medical Faculty, Lisbon University, Lisbon, February 2003.

•*Lymphocyte Population Dynamics* – coordinator: Rocha B., Vieira P., Freitas A., Necker Institut/Pasteur Institut, Oporto, February 2003.

•*Infection & Immunity* – coordinator: Appelberg R., IBMC, Oporto, March 2003.

•*Malaria and Tropical Diseases* – coordinator: Rosario V. & Cartwright IHMT (Tropical Medicine and Hygiene Institute) / Glasgow University, Lisbon, March 2003.

•*XII Porto Cancer Meeting* – coordinator: Sobrinho-Simões M., IPATIMUP, Oporto, March 2003.

•*T-Lymphocyte Biology* – coordinator: Arosa F. & Damjanovich S., IBMC / Hungarian Academy of Sciences, Oporto, April 2003.

•*Double Helix – 50th anniversary* - coordinator: Wigzell H & Sousa M. Karolynska Institut / IBMC, Oporto, April 2003.

•*Molecular Parasitology* – coordinator: Tomás A., IBMC, Oporto, April 2003.

•*Integrative Neuroscience: From Molecules to Mind* – coordinator: Costa R., Nicolelis M., Galhardo V., Duke University / IBMC / Medical Faculty - Oporto University, May 2003.

•*Peroxysomal biology* – coordinator: Jorge , IBMC, Oporto, May 2003.

•*Developmental Biology* – coordinator: Casares F., IBMC, Oporto, May 2003.

•*Biostatistics* – coordinator: Vieira J., IBMC, Oporto, June 2003.

•*Lynphocyte Activation* – speakers: Carmo A., Davies S. , IBMC, Oporto, Oxford University, UK, June 2003.

•*Biostatistics* – coordinator: Vieira J., IBMC/Minho University, Oporto, June 2003.

Language Education

- Cambridge University First Certificate in English – Grade A.
- Cambridge University Certificate of Advanced English – Grade A.
- IELTS exam – June 2003 – band 8,5 (out of 9).
- IELTS exam – May 2008 – band 8 (out of 9).

- Currently attending German Lessons at InLingua, Muenchen.

Working Experience

- Diploma Thesis - "Molecular Epidemiology of *Mycobacterium tuberculosis* infection – Development of Fast Detection Procedures".

  In this work a RFLP and Spolygotyping analysis of isolates of *Mycobacterium tuberculosis* were performed in an attempt to elucidate relationships between isolates, strains and establish molecular connections.

  Also, a PCR-based detection method was developed, using *GyrB* gene sequences that are species-specific. Using the same approach and based on the same sequences, a FISH – Fluorescent *In Situ* Hybridisation assay, was developed.

  In order to associate MultiDrugResistant-TB with genetic determinants, antibiograms were performed in parallel with sequencing of genes known to confer resistance.

- Two-month Training Course in Food Protection and Analysis at the Sanitation Inspection Laboratory, Veterinary Medicine Faculty, Lisbon, under the supervision of Prof. Dr. Fernando Bernardo.
- Development of a FISH protocol for rapid detection of *Listeria monocytogenes* in foods in the same Laboratory.
- Three-month Training Course in Yeast Genetics and Molecular Biology, at Eukaryotic Genetics Laboratory, Aveiro University, under the supervision of Prof. Dr. Manuel Santos, EMBO Young Investigator.
- Teaching Laboratory Classes (Praticals) in the Health Science Institute – Lisbon. Subjects taught:

  Genetics

  Genetic Engineering
- PhD Thesis – "**Khd1p, a protein with multiple roles in mRNA localization and Telomeric Silencing**"

  In this work the role of a putative translation repressor, *KHD1*, was analysed in the context of mRNA localization, using the budding yeast *S. cerevisiae* as a model.

  This gene was also proposed to be involved in telomeric silencing and in nonsense mediated decay. With this work, we show that Khd1p contributes to Sir2p regulation and is involved in NHEJ regulation, a DNA repair pathway.

Posters

Portugal I., Andrade G. and Brum L. (2001). Caracterização Molecular de Isolados Clínicos de *Mycobacterium tuberculosis*. *Congresso Nacional de Microbiologia – MICRO'2001* (December 2001) Póvoa do Varzim, Portugal.

Oliveira, M.; Andrade, G.; Guerra, M. e Bernardo, F. (2002). Development of a Fluorescence *In Situ* Hybridization test for *Listeria monocytogenes* detection. *Actas Xth International Congress of Bacteriology and Applied Microbiology* (27 July-01 August). Paris, France.

Oliveira, M.; Andrade, G.; Guerra, M. e Bernardo, F. (2002). Development of a Fluorescent *In Situ* Hybridization protocol for the rapid detection of *Listeria monocytogenes*. *Actas Food Protection 2002* (22 February). Monte da Caparica, Portugal, 66.

Andrade, G. and Jansen, R.-P. (2002). How does Localization influence mRNA turnover?. *GABBA PhD Program Annual Meeting* (17^{th}–21^{st} December). Oporto, Portugal.

Guerra, M.M.; Andrade, G.R.; Saraiva Lima, M.; Bernardo, F. *Listeria monocytogenes* em ensilagens: prevalência e ecologia [*Listeria monocytogenes* in ensilages: prevalence and ecology]. Congresso da Sociedade Portuguesa de Ciências Veterinárias
(10-12 October). Taguspark-Oeiras, Portugal.

Andrade, G. and Jansen, R.-P. (2005). Translational Regulation of a Localized mRNA, an *ASH1* mRNA tale. *ELSO Meeting* (3^{rd}-6^{th} September). Dresden, Germany.

Publications

Oliveira, M.; Andrade, G.; Guerra, M. e Bernardo, F. (2003). Development of a Fluorescent *In Situ* Hybridization protocol for the rapid detection and enumeration of *Listeria monocytogenes* in milk. RPCV (2003) 98 (547) 119-124.

Andrade, G. and Jansen, R.-P. (2005). Moving with Muscleblind. *Nature Cell Biology 7 (12) 1055-6*.
News and Views.