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Table of Contents

Summary **1**

Abbreviations **3**

Introduction **5**

The nuclear pore complex *5*

Nucleocytoplasmic transport *7*

The Ran-GTPase system *8*

Nuclear export of RNAs *9*

Yeast tRNA export 10

Export of rRNAs 13

Export of snRNA 14

Nuclear export of mRNA 15

Cis and trans acting signals that stimulate mRNA export 15

The role of the Mex67/Mtr2 complex in mRNA export 16

The role of Yra1 and Sub2 in mRNA export 17

Nucleoporins, Gle1 and Dbp5 in mRNA export 19

The role of Gle2 in mRNA export 20

Aim of the work **22**

Results **23**

Part I: Analysis of the genetic network of *GLE2* reveals extended interactions with the NPC **23**

The gle2Δ sl screen reveals a pleiotropic network of Gle2 interactions at the NPC *23*

GLE2 genetically interacts with nucleoporins *25*

GLE2 is genetically connected to components of the protein import machinery *27*

Components of the mRNA export machinery are genetically linked to GLE2 ..... *28*

GLE2 is not required for nuclear poly(A)$^+$ RNA export. *29*

Part II: TREX is a conserved complex coupling transcription with mRNA export **31**

SUB2 is genetically linked to the THO complex, which acts in transcription elongation *31*

Table of Contents

Sub2, Yra1, Tho2, Hpr1, Mft1, Thp2 and Tex1 form the transcription-export (TREX) complex *32*

The core of the TREX complex adopts a symmetric butterfly-like structure *34*

Alternative purifications of the TREX complex *39*

Components of the TREX complex genetically interact with RRP6 and with MTR10, a member of the importin β-family that might regulate their import *41*

Discussion **44**

Is Gle2 a nucleoporin or an mRNA export factor? *44*

Coupling transcription elongation to mRNA export via the TREX complex *49*

The TREX complex at the electron microscope *52*

Publications **55**

Materials and Methods **56**

Molecular biological methods *56*

DNA manipulation 56

Cloning of plasmids 56

Genetic methods *60*

Media for yeast growth and microbiological techniques 60

Yeast transformation, plasmid selection, complementation test 61

Yeast plasmids and genomic preparation 61

Yeast strains 61

Isolation of synthetic lethal mutants starting with the gle2Δ, sub2-85, tho2Δ and thp2Δ allele 63

Biochemical methods *65*

Whole yeast protein extract 65

TAP purification 66

GST-fusion protein purification 67

Gel filtration chromatography 67

Immunological methods *67*

Western blot 67

Transfer of proteins from SDS-PAGE to nitrocellulose 67

Immunological detection of proteins immobilized on nitrocellulose filters 68

Other methods *69*

Fluorescence microscopy 69
Electron Microscopy (EM) 69
Image Processing 70

References 71

Zusammenfassung

Das Kennzeichen von eukaryontischen Zellen ist der Zellkern, welcher durch die Kernmembran vom Zytoplasma abgetrennt wird. In dieser Kernmembran sind die Kernporenkomplexe eingebettet, welche Kanäle bilden, die den Austausch von Makromolekülen regulieren. Dieser hochregulierte Import und Export von Proteinen und RNAs durch die Kernpore erfolgt über mehrere verschiedene Transportwege. Der Export von Messenger RNA (mRNA) ins Zytoplasma erfolgt als mRNA-Protein-Komplex (mRNP) nach abgeschlossener Reifung der mRNA. In Studien in der Bäckerhefe *Saccharomyces cerevisiae* wurde eine Vielzahl von Faktoren identifiziert, welche beim nukleären Export beteiligt sind, darunter Komponenten des Kernporenkomplexes, Exportrezeptoren und Proteine, welche bei der Reifung der mRNA involviert sind.

Zu Beginn meiner Dissertation führte ich einen synthetisch letalen (sl) „Screen“ durch, um die Rolle von Gle2 im mRNA-Export zu untersuchen, da vermutet wurde, dass Gle2 ein mRNA Exportfaktor ist. Daher war das Ziel, das funktionelle Verhältnis zwischen Gle2 und der Mex67-abhangigen mRNA Export Route zu verstehen. Ich konnte zeigen, dass *GLE2* synthetisch letal ist mit den mRNA Exportfaktoren Sac3 und Mex67. Desweiteren fand ich eine genetische Interaktion von *GLE2* mit Importin α und β, sowiemit mehreren Nukleoporinen, die Untereinheiten, distinkter Subkomplexe des Kernporenkomplexes sind. Dieser Teil meiner Untersuchungen deutete daraufhin, dass die Funktion von Gle2 nicht auf den nukleären Export beschränkt ist, sondern dass Gle2 eine mehr allgemeine Rolle im bifunktionellen Transport durch den Kernporenkomplex spielt.

Im zweiten Teil meiner Studien führte ich einen synthetisch letalen Screen mit *SUB2* durch, einem intranukleären Faktor, der an der mRNA Biogenese und Export beteiligt ist. Der sl-Screen deckte eine neue genetische Verküpfung zwischen *SUB2* und dem THO Komplex, der in der Transkriptionselongation involviert ist, auf. Diese Daten trugen zur Identifizierung eines neuen konservierten Komplexes bei, der TREX-Komplex (TRanskription/EXport) genannt wurde. Dieser wird durch die Exportfaktoren Sub2 und Yra1, einem bis vor kurzem unbekannten Faktor Tex1 und dem THO Komplex gebildet. Der TREX Komplex koppelt somit Transkriptionselongation mit dem mRNA Export.

Um den TREX Komplex näher zu charakterisieren, analysierte ich die genetischen Interaktionen zweier Komponenten des THO Komplexes, *THO2* und *THP2*. Auch der

Importrezeptor *MTR10* war synthetisch letal mit *SUB2* und *THP2*. Die Misslokalisation von Sub2-GFP und Thp2-GFP in *mtr10* Mutanten deutet darauf hin, dass Mtr10 als Importfaktor für Komponenten des TREX Komplexes fungiert. Zusätzlich waren *THO2* und *THP2* synthetisch letal mit *RRP6*, einem Bestandteil des Exosomkomplexes, welcher unvollständig prozessierte mRNA´s im Zellkern zurückhält und degradiert. Dies deutet auf eine Kopplung von Transkriptions-verlängerung und RNP Qualitätskontrolle hin.

Abschließend analysierte ich den TREX Komplex auf biochemischer Ebene. Der TREX Komplex wurde mit einer Vielzahl an Methoden gereinigt, darunter Tandemaffinitätsreinigung (TAP) und Gelfiltration. Unter stringenten Bedingungen konnte ich den stabilen Kern des TREX Komplexe reinigen, von dem Sub2 und Yra1 teilweise dissoziiert waren. In Zusammenarbeit mit dem Labor Böttcher (EMBL) untersuchte ich die Morphologie des Komplexes mit Elektronenmikroskopie (EM). Der Kern des TREX Komplexes zeigt eine schmetterling-ähnliche Form mit einer zweifachen Symmetrie und einer Spalte zwischen den zwei verdrehten Armen.

Zusammenfassend identifizierten meine Untersuchungen neue Verbindungen zwischen mRNA Export und Proteinimport an der Kernpore und machten deutlich, dass Transkription, Reifung und Export von RNA genetisch und physiologisch gekoppelt sind.

Summary

In the eukaryotic cell, the nuclear envelope separates the nucleoplasm from the cytoplasm. The nuclear pore complex (NPC) forms the conduit that regulates the exchange of macromolecules between these compartments. Import and export of protein and RNA through the NPCs are highly regulated and follow several different pathways. Messenger RNAs (mRNAs) are exported from the nucleus only after extensive processing and assembly into ribonucleoprotein particles (RNPs). Studies in yeast have identified many components involved in mRNA export. These include nuclear pore proteins, export receptors and components in the nucleus that couple formation of mRNPs with translocation through the pores.

When I started my PhD work, I performed a synthetic lethal (sl) screen to investigate the role of Gle2 in mRNA export. Gle2 was a proposed to be an mRNA export factor. Thus, the aim has been to gain an understanding of the relationship between Gle2 and the Mex67-mediated mRNA export pathway. I could show that *GLE2* is synthetic lethal with the mRNA export factors Sac3 and Mex67, with importins α and β, and with several nucleoporins, which are subunits of distinct subcomplexes of the NPC. This part of my studies indicated that the function of Gle2 is not restricted to nuclear export and suggested a more general role of Gle2 in bidirectional transport through the nuclear pore complexes.

To investigate the Mex67-mediated mRNA export pathway, in the second part of my studies, I performed a synthetic lethal screen with *SUB2*, an intranuclear factor which in our lab was found to act in mRNA export. Initial work suggested that Sub2 was a splicing factor. The sl screen I performed revealed a genetic link between *SUB2* and the THO complex, which is involved in transcription elongation. These data contributed to the identification of a novel conserved complex called TREX (transcription/export), formed by the export factors Sub2 and Yra1, a previously unknown factor, Tex1, and the THO complex. Thus, the TREX complex couples transcription elongation and mRNA export.

To further characterize the TREX, I analyzed the genetic interactions of two components of the THO complex, *THO2* and *THP2*. The import receptor *MTR10* was found to be synthetic lethal with *SUB2* and *THP2*. In addition, I found that Sub2-GFP and Thp2-GFP are mislocalized in *MTR10* mutants, indicating a role of Mtr10 as import factor for components of the TREX complex. Furthermore, *THO2* and *THP2* are synthetic lethal with

RRP6, a component of the exosome complex, which retains and eliminates improperly 3'-end processed mRNPs, suggesting a link between transcription elongation, and RNP quality control.

Finally, I analyzed the TREX complex at the biochemical level. The TREX complex was purified using a variety of methods, including tandem affinity purification (TAP) and by gel filtration. Under stringent conditions, I could purify a stable core of the TREX complex, in which Sub2 and Yra1 were partly dissociated. In collaboration with the Böttcher lab (EMBL), I studied the morphology of this complex by electron microscopy (EM). The core of TREX shows a butterfly-like shape, with two-fold symmetry and a cleft in between the two winged arms. Under less stringent conditions, the TREX complex contains stoichiometric amounts of Sub2 and Yra1. Nevertheless, the complex mostly retains a butterfly-like morphology at the EM level.

In conclusion, my studies identified new connections between mRNA export and protein import at the nuclear pore, and revealed that transcription, maturation and export of mRNAs are genetically and physically coupled.

Abbreviations

CBC	Cap Binding Complex
ChIP	Chromatin Immuno Precipitation
CTD	C-Terminal Domain of the largest RNAPII subunit
Da	Dalton
EM	Electron Microscopy
FG	Phenylalanine-Glycine
5-FOA	5-Fluoorotic Acid
FXFG	Phenylalanine-X-Phenylalanine-Glycine
GFP	Green Fluorescent Protein
GLEBS	Gle2 binding sequence
GLFG	Glycine-Leucine-Phenylalanine-Glycine
hnRNP	Heterogeneous Nuclear Ribonucleoproteins
kDa	Kilo Dalton
LB	Luria-Bertani
LRR	Leucine-Rich Repeat
MDa	Mega Dalton
mRNA	messenger RNA
mRNP	Ribonucleoprotein Particle
NE	Nuclear Envelope
NES	Nuclear Export Sequence
NLS	Nuclear Localization Signal
NRM	Nucleoporin RNA Binding Motif
NPC	Nuclear Pore Complex
Nup	Nucleoporins
NXF	Nuclear export factor
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PBS	Phosphate-buffered saline
PBST	PBS with 0.1 % Tween 20
POM	Integral membrane protein of the Nuclear Pore
pre-mRNA	precursor of messenger RNA

pre-rRNA	precursor of ribosomal RNA
Ran-GAP	Ran GTPase activating protein
Ran-GDP	GDP bound form of Ran
Ran-GEF	Ran GTP exchange factor
Ran-GTP	GTP bound form of Ran
REF	RNA and export factor binding proteins
RNAPII	RNA Polymerase II
rpm	rotation per minute
RRE	Rev Responsive Element
rRNA	ribosomal RNA
SDS	Sodium Dodecyl-Sulphate
SL	Synthetic Lethal
snoRNP	small nucleolar RNP
TAP	Tandem Affinity Purification
TCA	Trichloroacetic Acid
TEV	Tobacco Etch Virus
TREX	Transcription/Export complex
tRNA	transfer RNA
UsnRNA	U-rich small nuclear RNA

Introduction

The nuclear pore complex

In a eukaryotic cell, a double membrane, the nuclear envelope, separates the contents of the nucleus from the cytoplasm. This compartmentalization allows the spatial and temporal separation of DNA replication and transcription from cytoplasmic protein synthesis. This separation of function led to the evolution of selective macromolecular transport between the nucleoplasm and the cytoplasm. Bidirectional traffic occurs continuously between the cytosol and the nucleus. The proteins that function in the nucleus are selectively imported from the cytosol. These include histones, DNA and RNA polymerases, transcription factors, and RNA-processing proteins. Conversely, RNAs are synthesized in the nuclear compartment and then exported to the cytosol. Fully processed messenger RNAs (mRNAs) are exported to the cytoplasm where they are translated into protein. Transfer RNAs (tRNAs) are required for mRNA translation, the U-rich small nuclear RNAs (UsnRNAs) are exported into the cytoplasm where they are processed and matured, and ribosomal RNAs (rRNAs) exported to the cytosol as part of ribosomal subunits (reviewed by (Gorlich D and Kutay U, 1999).

The nuclear pore complex (NPC) forms the conduit for the exchange of information between the nucleus and cytoplasm. NPCs are freely permeable to small molecules (such as water and ions), but they restrict the movement of larger molecules (such as proteins and RNAs) across the nuclear envelope (NE). NPCs are large proteinaceous structures of approximately 125 MDa in vertebrates and 66 MDa in yeast, composed of about 30 proteins termed nucleoporins (Rout MP *et al.*, 2000; Cronshaw JM *et al.*, 2002) embedded in the double membrane of the NE (Gorlich D and Kutay U, 1999; Rout MP and Aitchison JD, 2001; Fahrenkrog B and Aebi U, 2002). These form repetitive substructures organized with 8-fold symmetry around an axis perpendicular to the NE, and 2-fold pseudo symmetry parallel to the NE. Built upon the symmetrical components of the NPC are asymmetric fibril structures, namely the cytoplasmic filaments and the nuclear basket (Figure 1A) (Rout MP and Aitchison JD, 2001).

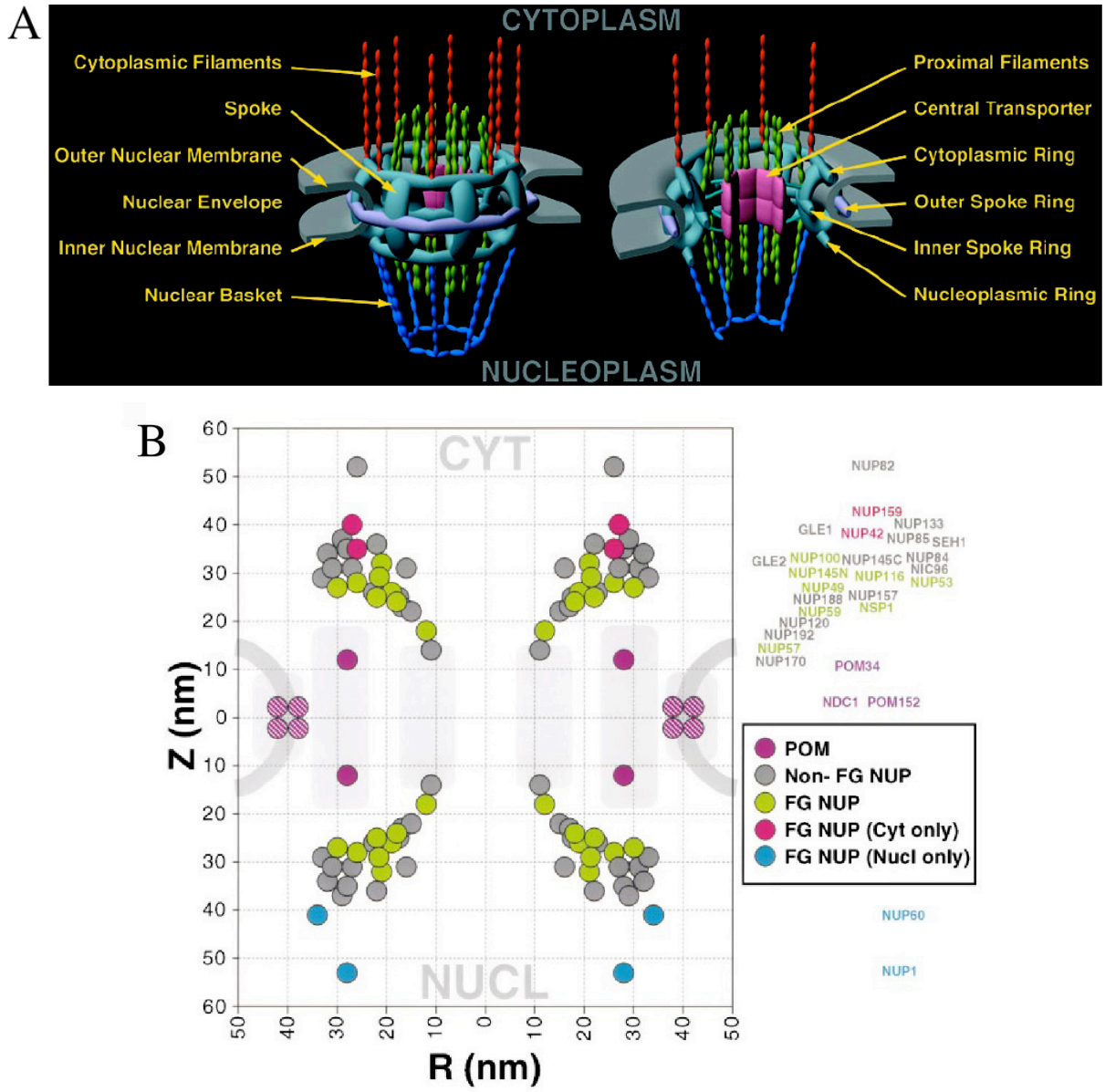

Figure 1. Structure of the nuclear pore complex and localization of the nucleoporins. (A) The NPC contains eight *spokes*, projecting radially from the wall of the pore membrane and surrounding a central tube called the *central transporter*. A *nucleoplasmic ring*, a *cytoplasmic ring*, and an *inner spoke-ring* surround the central transporter. Eight *cytoplasmic filaments* are attached at the cytoplasmic ring. Nuclear filaments originate at the nuclear ring and conjoin distally to form the *nuclear basket* (Rout MP and Aitchison JD, 2001). (B) Plot of the position of nucleoporins in the NPC. Statistical analysis of the distribution of the gold particles in each montage allows determination of the position of the proteins relative to the NPC cylindrical axis (R) and mirror plane of pseudosymmetry (Z). A mask for a crosssection of the yeast NPC and pore membrane is shown schematically to scale in light gray. FG nups are highlighted, the majority of which were found on both sides of the NPC (green), and a few that were found towards the periphery and exclusively on the nuclear side (blue) or the cytoplasmic side of the NPC (red). Most of the non-FG nups (dark gray) were found on both sides. The integral membrane protein Pom34p (purple) was close to the membrane, as were the inferred positions of Pom152p and Ndc1p (purple stripes) (Rout MP *et al.*, 2000).

Different nucleoporins have distinct roles in NPC structure and function. Nucleoporins containing multiple repeats of a Phe-Gly motif (FG, GLFG and FXFG; FG Nup in Figure 1B) localize at the entry, the exit and along the pore channel and mediate the interaction with cargoes crossing the NPC via the interaction with transport receptors (Rexach M and Blobel G, 1995; Bayliss R *et al*., 2000; Rout MP *et al*., 2000; Allen NP *et al*., 2001). The integral membrane proteins (Pom in Figure 1B) are thought to anchor the pores within the nuclear envelope (Gerace L *et al*., 1982; Hallberg E *et al*., 1993; Wozniak RW *et al*., 1994). The non-membrane nucleoporins that do not contain repeats are used as structural building blocks, which assemble in subcomplexes to constitute the overall structure of the NPC (Vasu SK and Forbes DJ, 2001). Consistent with this organization, most nucleoporins can be detected on both the cytoplasmic and nucleoplasmic faces of the NPC. Few are asymmetrically distributed, like Nup1 and Nup60 at the nuclear basket, or Nup42 and Nup159 at the cytoplasmic side (Figure 1B).

Nucleocytoplasmic transport

Nuclear import as well as nuclear export of proteins and RNA occurs through the NPC and is usually an active, carrier-mediated process. Most of the proteins that mediate the nuclear import and/or export of specific cargoes are members of the importinβ/karyopherinβ family (Gorlich D and Kutay U, 1999; Macara IG, 2001). Based on the direction in which these receptors carry their cargo, they are classified as importins (cytoplasm→nucleus) or exportins (nucleus→ cytoplasm) (Figure 2).

Members of this family interact with their cargo molecules directly or via adapter proteins, and can also bind to nucleoporins, mostly the ones containing FG repeats. The importin β family includes 14 members in yeast and at least 22 in humans. In yeast, these include the prototypic importinβ/Kap95 that mediates import of proteins containing nuclear localization signal (NLS) via the adaptor protein importinα/Kap60/Srp1 (Rexach M and Blobel G, 1995); Xpo1 (human Crm1), which serves as export receptor for proteins containing leucine-rich nuclear export signal (NES) found in many proteins (Fornerod M *et al*., 1997; Fukuda M *et al*., 1997; Stade K *et al*., 1997; Maurer P *et al*., 2001); Cse1 (human CAS), which is the importin α export factor (Kutay U *et al*., 1997; Kunzler M and Hurt EC, 1998; Solsbacher J *et al*., 1998); Los1 (human Xpo-t), which acts as tRNA export factor (Arts GJ *et al*., 1998a; Hellmuth K *et al*., 1998; Kutay U *et al*., 1998); Kap104 (human

Transportin), which recognizes import cargoes as the mRNA binding proteins Nab2, Hrp1, Nap4 (Aitchison JD *et al.*, 1996). The transport machinery is remarkably versatile, with individual karyopherins recognizing multiple cargoes and with individual cargoes binding to multiple karyopherins (see reviews, (Gorlich D and Kutay U, 1999; Macara IG, 2001).

The Ran-GTPase system

Directionality of import and export processes mediated by the importin β family is regulated by the small GTPase Ran. Its GTP-bound form (Ran-GTP) is present at high concentrations in the nucleus and at low concentrations in the cytosol. The nucleotide state of Ran in these compartments is determined by the exclusive nuclear localization of the Guanine nucleotide Exchange Factor (RanGEF) Prp20 (human RCC1) and by the cytoplasmic localization of the GTPase Activating Protein (RanGAP) Rna1 (human RanGAP1). RanGEF promotes GDP dissociation from RanGDP, thereby allowing GTP binding. When Ran leaves the nucleus, RanGAP induces GTP hydrolysis. In this way, the cytoplasmic RanGTP concentration is kept low (reviewd in (Kuersten S *et al.*, 2001). Because of the flux of macromolecules between the nucleus and the cytoplasm, Ran needs to be returned to its nuclear GTP-bound state rapidly. A transport mediator for RanGDP, NTF2, participates in Ran recycling. NTF2 binds specifically to RanGDP and mediates efficient interaction with the nuclear pore complex and translocation into the nucleus (Ribbeck K *et al.*, 1998; Smith A *et al.*, 1998).

Members of the importin β family contain a characteristic, conserved Ran-GTP binding domain. Ran-GTP binds to these transport receptors and regulates their association with cargo. Import receptors bind their cargo in the cytosol and dissociate from it upon binding Ran-GTP in the nucleus. Conversely, export receptors bind their cargoes in the presence of Ran-GTP in the nucleus, traverse nuclear pore complexes as ternary cargo-exportin-Ran-GTP complexes, and disassemble in the cytoplasm upon hydrolysis of Ran-bound GTP. After delivering cargoes, components of the transport machinery return to the original compartment to mediate the next round of transport (Figure 2) (reviewed in (Kuersten S *et al.*, 2001).

Variations of this scheme include the binding of Ran-GTP in the nucleus, which can depend upon events occurring in the nucleus. For example, the release of the RNA binding protein Npl3 from its import receptor Mtr10 requires both Ran-GTP and RNA (Senger B *et al.*, 1998). Similarly, the import receptor Kap114 releases the TATA-binding protein (TBP) in

presence of both Ran-GTP and DNA containing TBP binding sites (Pemberton LF *et al.*, 1999). Furthermore, bi-directional transporters have recently been reported both in yeast (Yoshida K and Blobel G, 2001) and mammalian cells (Mingot JM *et al.*, 2001). There have also been reports of proteins that can enter the nucleus without the help of importins (Gorlich D and Kutay U, 1999; Hetzer M and Mattaj IW, 2000).

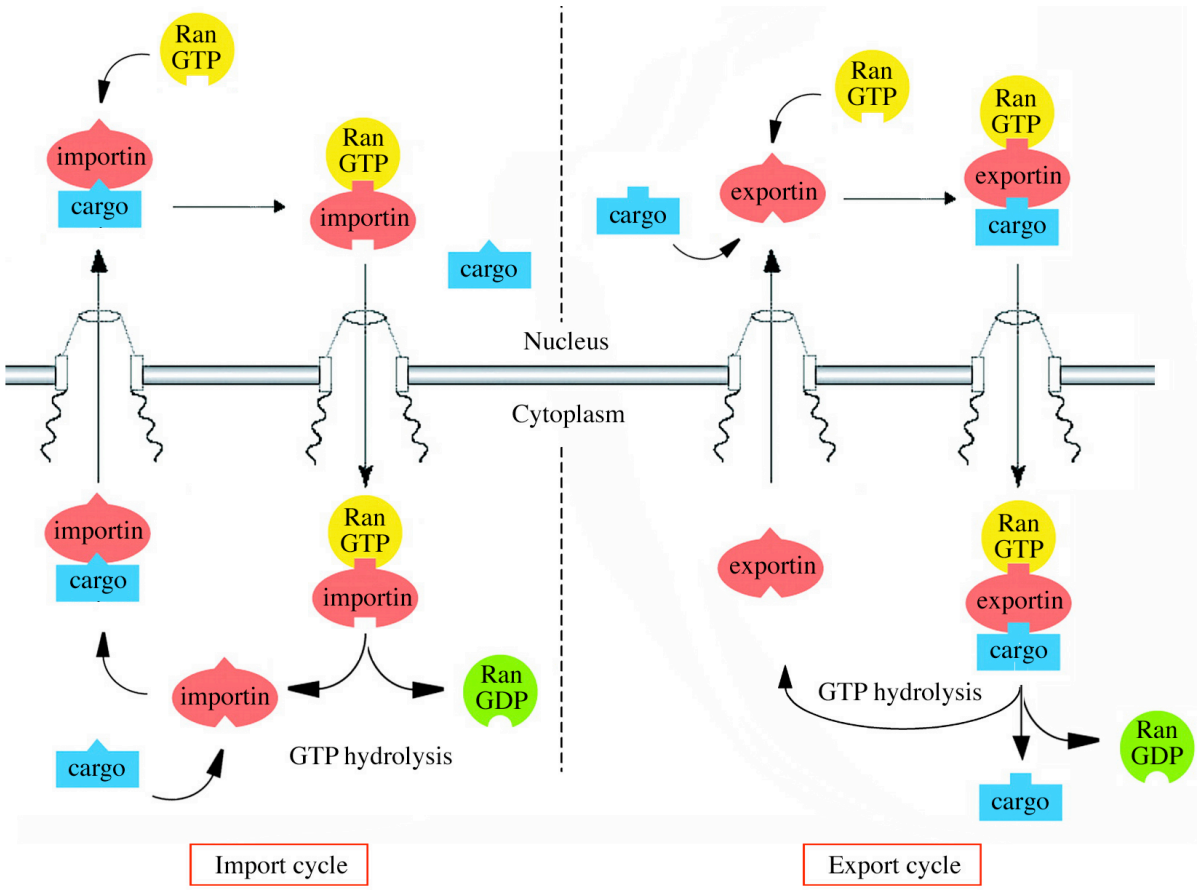

Figure 2. Import and export cycles mediated by importin-like transport receptors. Importins (left) bind to cargo molecules in the cytoplasm and mediate interactions with the nuclear pore complex to translocate the import complex into the nucleus. During an export cycle (right), the cargo is bound in the nucleus and, upon GTP hydrolysis in the cytoplasm, the exportin dissociates from the cargo (Kuersten S *et al.*, 2001).

Nuclear export of RNAs

Three different RNA polymerases synthesize different classes of RNAs and each class is exported via independent routes (Jarmolowski A *et al.*, 1994; Fischer U *et al.*, 1995). Nuclear export of tRNAs and rRNAs is mediated predominantly by transport receptors of the

importin-β family (reviewed in (Cole CN and Hammell CM, 1998; Weis K, 2002). In contrast, the mechanism of nuclear export of bulk mRNA differs from the other nucleocytoplasmic transport routes as it appears not to be directly dependent on RanGTP (Clouse KN *et al*., 2001; Reed R and Hurt E, 2002).

Yeast tRNA export

tRNAs are synthesized by RNA polymerase III as precursors that undergo maturation prior to nuclear export. Maturation includes trimming of the 5' and 3' ends, addition of three terminal CCA residues, modification of a number of nucleosides, aminoacylation and splicing, in the case of introns-containing tRNA genes (reviewed in (Wolin SL and Matera AG, 1999).

In yeast, tRNA maturation is intimately coupled with the tRNA export pathways. A nuclear export receptor for tRNA was characterized in yeast and mammalians cells. This receptor is a member of the importin β family known as Los1 (human Exportin-t/Xpo-t) (Arts GJ *et al*., 1998a; Hellmuth K *et al*., 1998; Kutay U *et al*., 1998; Sarkar S and Hopper AK, 1998). Disruption of the yeast *LOS1* gene causes accumulation of tRNA inside the nucleus (Sarkar S and Hopper AK, 1998), but does not cause a growth defect (Hurt DJ *et al*., 1987), indicating that Los1 is redundant for nuclear export of tRNA. Los1 contains an amino terminal Ran-GTP-binding motif typical of the importin β family (Gorlich D *et al*., 1997) and binds to Ran-GTP *in vivo*. In addition, Los1 binds to two FG Nups (Nsp1 and Nup2) *in vivo*, and *in vitro* it interacts with tRNA in a Ran-GTD-dependent manner (Hellmuth K *et al*., 1998). Based on this evidence, it has been proposed that Los1 interacts in the nucleus with mature tRNA and Ran-GTP. The ternary complex interacts with nucleoporins, allowing translocation through the nuclear pore, and, once in the cytoplasm, hydrolysis of GTP to GDP dissociates the complex. Los1 and Ran-GDP are then recycled back to the nucleus, while tRNAs are delivered to the translation machinery (Simos G *et al*., 2002). In addition, preferential requirement of Los1 for the export of tRNAs encoded by intron-containing genes was shown (Grosshans H *et al*., 2000). This evidence may reflect an additional role of Los1 in intranuclear transport events that are connected with splicing (Simos G *et al*., 2002).

Simos and coworkers have proposed a second tRNA export pathway in yeast that depends on aminoacylation and requires the essential protein translation factor eEF-1A. *TEF1*, the gene coding for eEF-1A, was isolated in a screen for high-copy suppressors of a *los1* mutant. tRNA aminoacylation and eEF-1A are required for efficient nuclear tRNA

export in yeast, suggesting coordination between the translation, nuclear tRNA processing and transport machineries (Figure 3) (Grosshans H *et al.*, 2000). The eEF-1A pathway is likely to be conserved. Indeed, studies in *Xenopus* oocytes have shown that tRNAs with immature 5' and 3' ends are not efficiently exported from the nucleus (Arts GJ *et al.*, 1998b) and that tRNA aminoacylation can occur inside the nucleus of *Xenopus* oocytes and facilitates nuclear tRNA export (Lund E and Dahlberg JE, 1998).

An alternative Los1-independent tRNA export pathway connecting tRNA splicing and export relies on Cca1, the essential enzyme that adds the terminal CCA residues to tRNAs. Cca1 was isolated as multicopy suppressor of the defect in tRNA nuclear export caused by *los1* null mutations. Most mutations that affect tRNA export also cause defects in pre-tRNA splicing suggesting a tight coupling of the processes. Overexpression of Cca1 in *los1* null cells rescues the nuclear export, but not the pre-tRNA-splicing defects, uncoupling pre-tRNA splicing and tRNA export. Cca1 may function as an exporter or an adapter in this pathway, as it shuttles between nucleus and cytoplasm (Feng W and Hopper AK, 2002).

Further studies recently revealed a role of the essential nucleolar protein Utp8 in tRNA export. Depletion of Utp8 blocks nuclear export of mature tRNA derived from both intronless and introns-containing pre-tRNAs. Utp8 binds tRNAs directly but it does not appear to function as a tRNA export receptor, because it does not shuttle between the nucleus and the cytoplasm. Mangroo and coworkers therefore proposed that Utp8 is a component of the tRNA export machinery that might channel tRNA to the various tRNA export pathways (Steiner-Mosonyi M *et al.*, 2003).

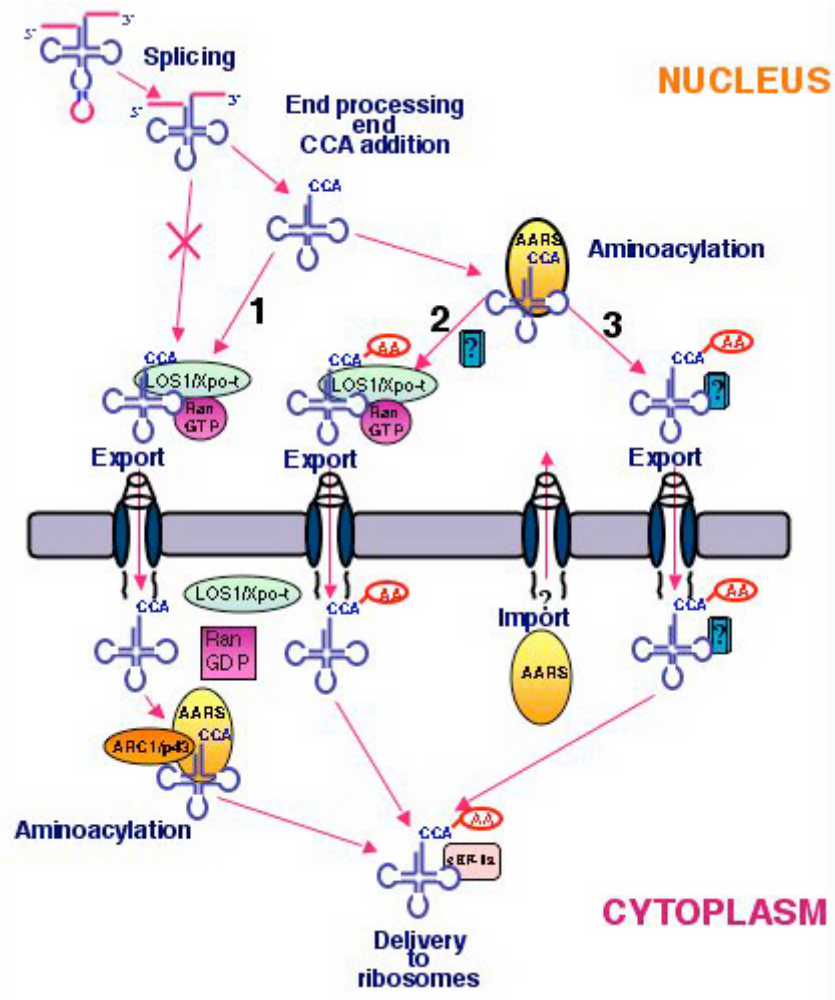

Figure 3. The tRNA biogenesis pathway. Precursor tRNAs are transcribed in the nucleus, undergo splicing if they contain introns, processing of their 5' and 3' ends, and CCA addition at the 3' end. Only such mature tRNAs bind with high affinity to the tRNA nuclear export receptor (Los1/Xpo-t) in the presence of Ran-GTP (1). The tRNA-Los1-Ran-GTP complex is translocated to the cytoplasm through the nuclear pores. On hydrolysis of GTP, the complex dissociates releasing the tRNA. Mature tRNAs can be aminoacylated inside the nucleus and delivered to the Los1-Ran-GTP complex for export (2). In this case, export of aminoacylated tRNAs is facilitated, either because these have higher affinity for Los1, or because their interaction with Los1 is stimulated by another component. Aminoacylated tRNAs may also follow an Los1-independent pathway out of the nucleus (3). In case of pathways (2) and (3), aminoacyl-tRNAs that exit the nucleus can be directly used by the protein translation machinery. Intranuclear tRNA-aminoacylation implies also that components of the aminoacylation machinery (aminoacyl-tRNA synthetasis - AARSs) and cofactors (Arc1/p43) must be imported into the nucleus, a process about which nothing is known. AA, aminoacyl-group (Simos G and Hurt E, 1999).

Export of rRNAs

The ribosomal RNA genes are transcribed to yield precursor rRNAs (pre-rRNAs), which undergo processing and covalent modification. Three of the four rRNAs are produced as a single primary transcript by the RNA polymerase I. The primary transcript is extensively processed and modified to yield the 18S, 5.8S and 25S rRNAs. About 80 ribosomal proteins associate with the primary transcript in the nucleolus to generate a pre-ribosomal particle (90S), which undergoes a series of maturation steps including splitting into a large (60S) and a small (40S) ribosomal subunit. The forth rRNA (5S) is transcribed by RNA polymerase III and recruited separately to the assembling ribosome (reviewed by (Kressler D *et al.*, 1999; Tschochner H and Hurt E, 2003).

The production of a ribosome involves numerous non-rRNA and protein factors, such as small nucleolar RNPs (snoRNPs) and non-ribosomal proteins that process and modify the pre-rRNAs (e.g. endo- and exonucleases, pseudouridine synthases or methyltransferases), mediate RNP folding/remodeling (RNA helicases, RNA chaperones) or facilitate protein association/dissociation (GTPases, AAA-ATPases) (Kressler D *et al.*, 1999; Brown JD, 2001). In contrast to the pre-60S particles, the pre-40S subunits apparently lack GTPases and AAA-type ATPases (Schafer T *et al.*, 2003). It is likely that fewer structural rearrangements occur during 40S formation in comparison with 60S biogenesis. The pre-40S and pre-60S particles are then exported to the cytoplasm separately in a RanGTPase-dependent manner, suggesting that members of the importin β family are involved (Hurt E *et al.*, 1999; Moy TI and Silver PA, 1999; Moy TI and Silver PA, 2002). Prior to nuclear exit, two nucleocytoplasmic transport factors, Nmd3 and Mtr2, associate with pre-60S particles (Ho JH *et al.*, 2000; Gadal O *et al.*, 2001; Johnson AW *et al.*, 2002; Nissan TA *et al.*, 2002). Nmd3 appears to bind to pre-60S particles through Rpl10 and serves as an adaptor for the export receptor Crm1/ Xpo1 (Ho JH *et al.*, 2000; Gadal O *et al.*, 2001). The last conversions to the mature ribosomal subunits take place in the cytoplasm. These lead to the formation of 60S subunits containing 25S, 5.8S, 5S rRNA, and 40S subunits containing the 18S rRNA (Figure 4) (see review (Tschochner H and Hurt E, 2003).

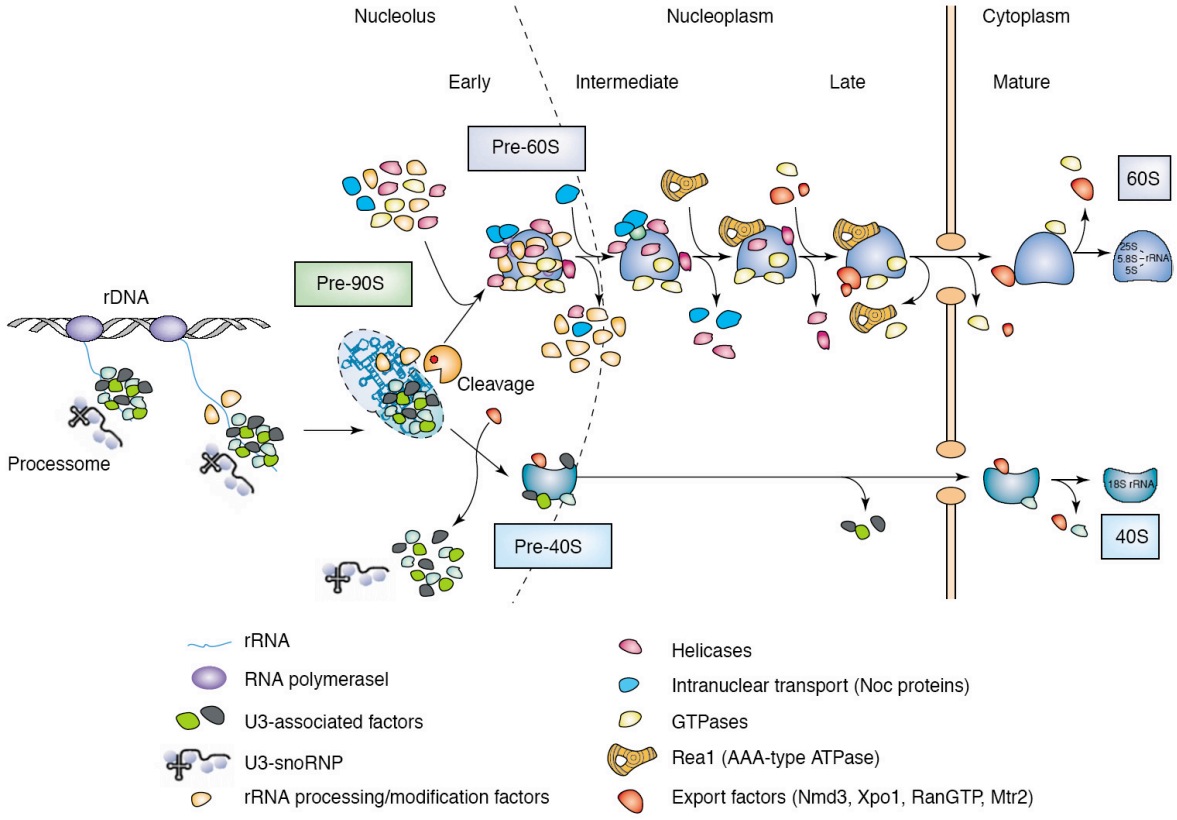

Figure 4. Current model for maturation and export of 40S and 60S ribosomal subunits. The earliest ribosomal precursor particle 90S separates into 40S and 60S pre-subunits. Many factors join the nascent subunits and many disassemble from it before nuclear exit. To gain export competence, a special subset of factors (marked in red) has to bind. The final maturation occurs after passage through the nuclear pore. Note that the export factors for 40S subunits are not known (Tschochner H and Hurt E, 2003).

Export of snRNA

Small nuclear ribonucleoproteins (snRNPs) function in the nucleus of eukaryotic cells during pre-mRNA splicing. Each snRNP contains a small nuclear RNA (snRNA) and an extremely stable core of seven Sm proteins. Biogenesis of snRNAs has been extensively studied in higher eukaryotes but little is known about them in yeast. The spliceosomal U snRNAs U1, U2, U4 and U5 are transcribed by RNA polymerase II and, like mRNA, cotranscriptionally acquire a monomethylated m^7G cap structure. They are then exported to the cytoplasm, where they are assembled into mature snRNPs, and imported back to the nucleus, where they splice pre-messenger RNAs. The mono-methyl cap serves as the essential signal for U snRNA nuclear export (Hamm J and Mattaj IW, 1990; Jarmolowski A *et al.*, 1994). The cap structure of the snRNAs is recognized by the cap-binding complex (CBC) composed of CBP20 and

CBP80. This binds to the export receptor Crm1 (Fischer U and Luhrmann R, 1990; Fischer U *et al.*, 1995; Fornerod M *et al.*, 1997) via the phosphorylated adapter protein called PHAX. Once in the cytoplasm, Ran-GTP hydrolysis and dephosphorylation of PHAX allow release of the U snRNA (Fornerod M and Ohno M, 2002). The snRNA is hypermethylated and the Sm proteins assemble. The import factor snurportin finally binds to the cap structure and to importin β (Huber J *et al.*, 1998). In yeast, no evidence has been obtained that snRNAs leave the nucleus. Thus, yeast snRNPs may assemble inside the nucleus, without export and re-import steps (Lygerou Z *et al.*, 1999).

Nuclear export of mRNA

Cis and trans acting signals that stimulate mRNA export

Messenger RNAs (mRNAs) are synthesized by RNA polymerase II as precursors that become associated with numerous heterogeneous nuclear (hn) RNP proteins/processing factors, and undergo a series of maturation steps before export to the cytoplasm. During transcription, the nascent pre-mRNA is capped at the 5' end, introns are removed by splicing and the 3' end is cleaved and polyadenylated. The export of mature mRNAs is then mediated by nuclear transport receptors that translocate mRNP cargoes across the central channel of NPCs (reviewed in (Erkmann JA and Kutay U, 2004).

What are the critical features that allow mRNA export? Pre-mRNA splicing was suggested to recruit mRNA export factors to the transcripts, as splicing has been reported to enhance export in some cases (Luo MJ and Reed R, 1999). However, the fact that intronless mRNAs are exported in all systems studied, suggests that the splicing process is not an absolute requirement for mRNA export. *Cis*-acting signals suggested to facilitate mRNA export are the monomethyl cap structure and the poly(A) tail, common to most mRNAs (Izaurralde E *et al.*, 1995; Huang Y and Carmichael GC, 1996). Injection of mRNAs in *Xenopus* oocytes demonstrated that both can enhance the mRNA export, but neither is sufficient or necessary (Jarmolowski A *et al.*, 1994). In *S. cerevisiae*, transcripts that fail to acquire a poly(A) tail are retained at or near transcription sites (Hilleren P *et al.*, 2001), whereas the presence of a cap structure seems not to be a prerequisite for mRNA export (Dower K and Rosbash M, 2002).

Many proteins are in dynamic association with mRNAs during their biogenesis. This led to the hypothesis that adapters might mediate mRNA export *in trans*, providing a nuclear export sequence (NES) for the mRNA export. Such mechanism is shown for the HIV Rev protein, which mediates the export of the viral mRNA. Rev binds specifically via its short leucine-rich NES to the RRE (Rev Responsive Element) of viral pre-mRNAs and to the exportin Crm1 (Fornerod M *et al.*, 1997). In yeast, Npl3, an abundant hnRNP-like protein, is an mRNA binding protein. Mutations in Npl3 cause nuclear accumulation of mRNA, indicating a role for Npl3 in the mRNA export process (Singleton DR *et al.*, 1995; Lee MS *et al.*, 1996). Npl3 shuttles between the nucleus and the cytoplasm, and its export is dependent on ongoing transcription by RNA polymerase II, but it is unclear whether this involvement is active and direct (Lee MS *et al.*, 1996).

The role of the Mex67/Mtr2 complex in mRNA export

Unlike most of the transport processes discussed above, the mechanism of nuclear export of bulk mRNA appears not to be directly dependent on Ran-GTP (Clouse KN *et al.*, 2001; Reed R and Hurt E, 2002). Furthermore, the general mRNA export receptor for mRNAs, Mex67-Mtr2 in yeast and TAP-p15 in metazoans, does not belong to the importin-β family (Conti E and Izaurralde E, 2001; Reed R and Hurt E, 2002). Mex67 was identified as synthetic lethal (sl) mutant of the *nup85Δ* allele (Segref A *et al.*, 1997). Temperature-sensitive mex67 mutants exhibit an extremely rapid accumulation of poly(A)$^+$ mRNA in the nucleus after shift to the restrictive temperature, suggesting that Mex67 is at the heart of the mRNA export machinery. *In vivo*, Mex67 requires Mtr2 to function as an mRNA export complex and for association with the nuclear pores (Santos-Rosa H *et al.*, 1998). Even though there is no human counterpart to Mtr2, p15 protein was shown to be functionally analogous. Indeed, the human TAP-p15 complex can functionally replace the Mex67-Mtr2 complex in yeast and thus performs a conserved role in nuclear mRNA export (Katahira J *et al.*, 1999). The recent resolution of the crystal structure of Mtr2 in complex with Mex67 confirmed that interactions between Mtr2 and Mex67 are similar to those seen in the human TAP–p15 structure and that the Mex67-Mtr2 heterodimer presents similar structural architecture to that of TAP–p15 (Fribourg S and Conti E, 2003).

The Mex67-Mtr2 complex gains physical contact with the nuclear pores via the interaction of Mtr2 with Nup85, while *in vitro* studies showed that Mex67 binds directly to

polyadenylated mRNA (Santos-Rosa H *et al.*, 1998). Furthermore, Mex67 and TAP can bind directly to FG repeat-containing nucleoporins (Conti E and Izaurralde E, 2001; Grant RP *et al.*, 2002). Since the Mex67-Mtr2 complex also binds to mRNA, this mRNA exporter can move the mRNP through the nuclear pore channel using the FG repeats of nucleoporins as transient docking sites (Strasser K *et al.*, 2000). Thus this complex meets the two requirements, namely binding to the nuclear pore and the transport cargo RNA, to be an mRNA exporter.

The role of Yra1 and Sub2 in mRNA export

Yra1 is the first protein identified as a bridging component between Mex67-Mtr2 and the mRNA transport cargoes (Strasser K and Hurt E, 2000). Yra1 is an essential yeast protein that belongs to the evolutionarily conserved REF (RNA and export factor binding proteins) family of hnRNP-like proteins. It was originally isolated as a protein that facilitates RNA-RNA annealing (Portman DS *et al.*, 1997), but it is still unclear whether this activity is relevant to Yra1 function *in vivo*. Later, Yra1 was shown to be involved in mRNA export. It interacts genetically and physically with Mex67, and *yra1* mutants show strong mRNA export defect (Strasser K and Hurt E, 2000; Stutz F *et al.*, 2000; Zenklusen D *et al.*, 2001). Furthermore, Yra1 displays RNA-binding activity *in vitro*, also after binding to Mex67 (Strasser K and Hurt E, 2000; Stutz F *et al.*, 2000). Thus, Yra1 could mediate targeting of Mex67 to Pol II transcripts. The mouse homologue of Yra1, Aly, interacts with the yeast Mex67 and human TAP, and is able to restore growth of the otherwise non-viable *yra1* null mutant in yeast, showing that the function of REF proteins is conserved (Strasser K and Hurt E, 2000).

Yra1 binds directly to Sub2, the product of a gene initially isolated by homology with human UAP56, a DEAD-box protein implicated in spliceosome assembly. Sub2 is required at multiple steps of spliceosome formation (Fleckner J *et al.*, 1997; Kistler AL and Guthrie C, 2001; Libri D *et al.*, 2001; Zhang M and Green MR, 2001). Sub2 and UAP56 exhibit an ATP-dependent RNA helicase activity (Wagner JD *et al.*, 1998; Wang Y *et al.*, 1998). During pre-mRNA splicing, dynamic rearrangement of RNA secondary structure within the spliceosome is crucial for intron recognition and formation of the catalytic splicing core. Therefore, splicing factors that belong to the DEAD box family are thought to have a central role in directing RNA rearrangements by unwinding RNA helices. In addition, Sub2 was shown to be essential for bulk mRNA export in *S. cerevisiae* and *D. melanogaster* (Gatfield D *et al.*,

2001; Jensen TH *et al*., 2001; Strasser K and Hurt E, 2001). Sub2 was reported to bind the domain of Yra1, which is bound by Mex67. Therefore, Sub2 and Mex67 compete for Yra1 binding (Strasser K and Hurt E, 2001). Furthermore, the interaction between Sub2 and Yra1 is conserved in higher eukaryotes. UAP56 interacts directly and highly specifically with Aly in the spliced mRNP (Luo ML *et al*., 2001).

Hence, Sub2 might associate with the mRNPs and recruit Yra1 to the mRNA. The interaction of Mex67-Mtr2 with Yra1 might displace Sub2 and targets the mRNP to the nuclear pore by direct interaction of the Mex67-Mtr2 complex with nucleoporins (Strasser K and Hurt E, 2001). This model suggests that the splicing factor Sub2/UAP56 functions in coupling splicing and export machineries (Figure 5).

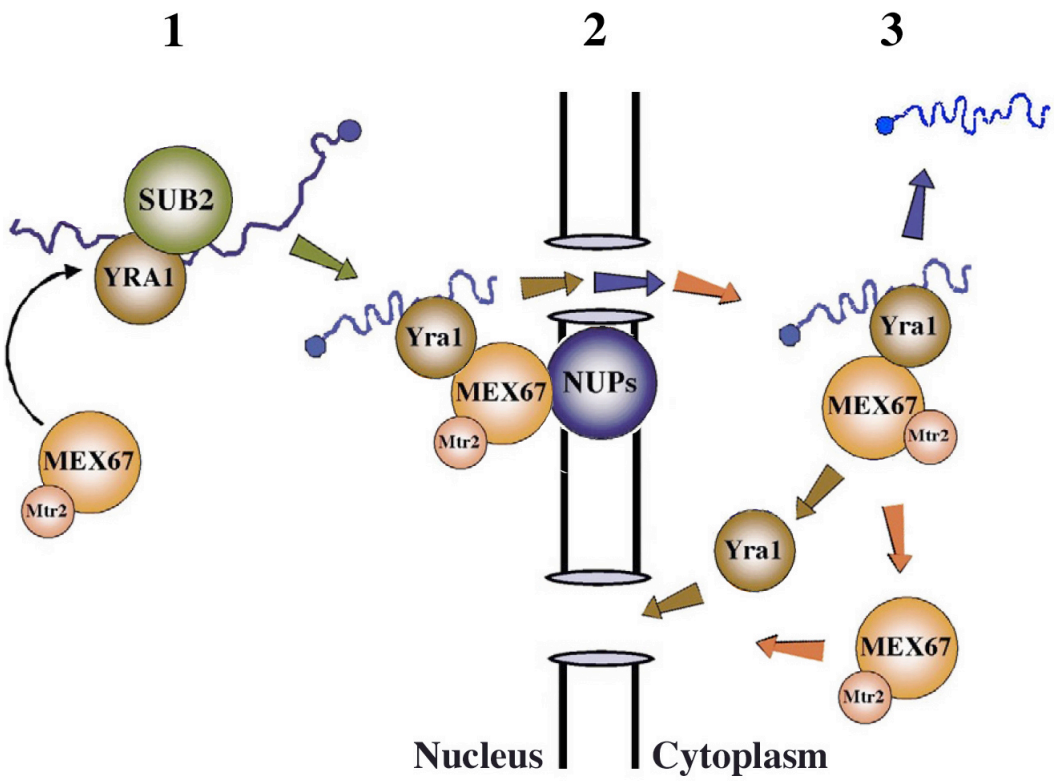

Figure 5. A model for mRNA export in yeast. Association of Sub2 with the mRNP serves to recruit Yra1. Binding of Mex67 to Yra1 displaces Sub2 (1). The interaction of Mex67 with the nucleoporins leads the translocation of the mRNP through the NPC (2). In the cytoplasm, the mRNP export complex dissociates. The mRNA is delivered to the ribosomes for translation and Mex67-Mtr2 with Yra1 are recycled to the nucleus (3).

Nucleoporins, Gle1 and Dbp5 in mRNA export

Genetic screens in yeast led to the identification of several factors that are required for mRNA export. Not unexpectedly, through these studies, nucleoporins were shown to be involved in mRNA export. The nucleoporins Nup159, Nup133, Nup116, Nup85 and Nup145 have a role in mRNA export. In higher eukaryotes, a direct involvement in mRNA export is suggested for Nup98 (yeast Nup116), Nup153, Nup 133, Nup160 and TPR (Gorlich D and Kutay U, 1999; Vasu S *et al.*, 2001). Mutations in these nucleoporins result in an mRNA export defect, suggesting that they might dock the mRNA export machinery at the NPC.

Moreover, conserved proteins like Gle1 and Dbp5 have been suggested to play a role in mRNA export. Yeast Gle1, also identified as Rss1p, is a protein essential for poly(A)$^+$RNA export (Del Priore V *et al.*, 1996; Murphy R and Wente SR, 1996), which is concentrated at the cytoplasmic fibrils of the NPC via the association with the FG nucleoporin Nup42 (Strahm Y *et al.*, 1999). Evidence for a role in mRNA export is given by the rapid block of mRNA export exhibited by *gle1* mutant strains. Gle1 contains a leucine-rich NES, which is not present in human Gle1. The human homologue hGle1 is also concentrated at the NPC by interaction with hNup155, and anti-Gle1 antibodies inhibit mRNA export when injected in HeLa cells, providing evidence for a role of Gle1 in this process (Watkins JL *et al.*, 1998; Rayala HJ *et al.*, 2004). However, to date neither human or yeast Gle1 has been shown to interact with RNA.

The translocation process through the NPC may be aided by the action of the ATP dependent DEAD-box RNA helicase Dbp5/Rat8, associated to Gle1, which localize to the cytoplasmic side of the NPC and specifically contact Nup159 (Snay-Hodge CA *et al.*, 1998; Hodge CA *et al.*, 1999; Strahm Y *et al.*, 1999). Dbp5 was shown to be required for mRNA export in yeast and humans. In *dbp5* ts mutants, rapid nuclear accumulation of poly(A)$^+$ RNA is observed when cells are shifted to the non-permissive temperature (Snay-Hodge CA *et al.*, 1998; Tseng SS *et al.*, 1998; Hodge CA *et al.*, 1999). In yeast and mammalian cells, Dbp5p is present predominantly in the cytoplasm and is highly enriched around the cytoplasmic fibrils of the NPC (Snay-Hodge CA *et al.*, 1998; Tseng SS *et al.*, 1998; Schmitt C *et al.*, 1999). The conserved nucleoporin yeast Nup159 and human Nup214 recruit Dbp5 to these fibrils (Hodge CA *et al.*, 1999; Schmitt C *et al.*, 1999; Strahm Y *et al.*, 1999). The metazoan ortholog of Dbp5 was identified based on its similarity to the yeast protein (Schmitt C *et al.*, 1999). Further studies revealed that Dbp5/hDbp5 interacts with Yra1/Aly, but the significance of this

interaction is not yet known (Schmitt C *et al*., 1999). The observation that Dbp5/hDbp5 is a conserved RNA helicase that shuttles and is located on the cytoplasmic face of the pore has led to the proposal that this protein may function in remodeling the mRNP during or shortly after translocation through the nuclear pore complex (Snay-Hodge CA *et al*., 1998; Tseng SS *et al*., 1998; Schmitt C *et al*., 1999). More recent studies using RNA interference indicate that Dbp5is not essential for mRNA export in *Drosophila* (Gatfield D *et al*., 2001).

The role of Gle2 in mRNA export

Gle2 was initially identified as genetic interactor of the GLFG repeat-containing nucleoporin Nup100 (Murphy R *et al*., 1996). Gle2 associates with nuclear pore complexes by binding Nup116. A short Gle2-binding sequence (GLEBS) within Nup116 acts as a Gle2 docking site and brings Gle2 to the nuclear pore (Bailer SM *et al*., 1998). In contrast, the GLFG repeats of Nup116 dock the heterodimeric mRNA exporter Mex67-Mtr2 to the NPC (Strasser K *et al*., 2000). Gle2 is conserved and interacts with Mex67 (Zenklusen D *et al*., 2001), as do their human counterparts Rae1 and TAP (Blevins MB *et al*., 2003).

Gle2 is dispensable for cell viability. However, a *GLE2* temperature-sensitive mutant, *gle2-1*, shows nuclear accumulation of poly(A)$^+$ RNA at the restrictive temperature (Murphy R *et al*., 1996). Gle2 homologues in other organisms were also shown to have a role in mRNA export. Temperature-sensitive mutations in *RAE1*, the *S. pombe* homologue of Gle2, also cause defective poly(A)$^+$RNA export (Brown JA *et al*., 1995). Similarly, mammalian RAE1 is a shuttling transport factor that contributes to nuclear export of mRNAs through its ability to anchor to the GLEBS-like motif of NUP98, the mammalian homolog of Nup116 (Pritchard CE *et al*., 1999). Moreover, the human RAE1 can be crosslinked to poly(A)$^+$ RNA (Kraemer D and Blobel G, 1997). The conserved interaction between Gle2 and Mex67, and the export defect observed in *GLE2* mutants led to the proposal that Gle2 might be directly involved in mRNA export (Figure 6). Definitive proof of this is however still lacking.

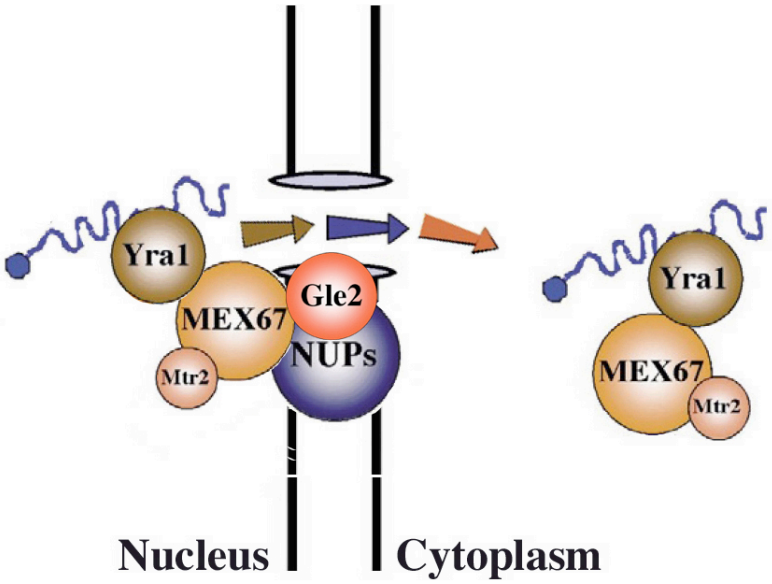

Figure 6. A proposed model for the involvement of Gle2 in mRNA export. Gle2 localizes at the NPC by association with Nup116. Through its binding to Mex67, Gle2 might favor the translocation of the mRNP through the NPC.

Aim of the work

The goal of my studies was to identify and characterize new factors involved in mRNA export. When I started my PhD work, the role of Mex67 as a general mRNA export factor was becoming established. However, the role of Gle2 in mRNA export was unclear. Therefore, despite the suggestion that it could act as a conserved mRNA export factor, one of the aims of my study has been to gain an understanding of the relationship between Gle2 and the Mex67-dependent mRNA export machinery. To this end, I took a genetic approach, based on synthetic lethality, to screen for components that functionally interact with Gle2.

In the second part of my PhD work, the objective of my investigation has been to understand the coupling between the intranuclear steps of gene expression and mRNA export. How the mRNA export machinery is connected to the machineries involved in gene expression remains a central question. In the past years, genetic studies have revealed functional links between the protein factors involved in different steps of gene expression. The factors necessary for mRNA maturation are recruited by RNA polymerase II and loaded onto the nascent transcripts. Thus, each phase of maturation is physically and functionally connected to the next. To identify novel links between transcription, mRNA processing and export, I made use of a combination of genetic, biochemical and electron microscopic approaches.

Results

Part I: Analysis of the genetic network of *GLE2* reveals extended interactions with the NPC

The *gle2Δ* sl screen reveals a pleiotropic network of Gle2 interactions at the NPC

At the beginning of my PhD work, Gle2 was considered to be an mRNA export factor (see Introduction). However, the mechanism by which Gle2 regulates mRNA export was not understood. To elucidate the genetic network around Gle2 and to gain novel insight into Gle2 function, we set up a synthetic lethal screen, making use of a *gle2Δ* strain, a deletion of the *GLE2* locus.

Genetic screens based on synthetic lethality were successfully used in the past to identify novel interactions of mRNA transport factors that act at the NPC (Doye V and Hurt EC, 1995). The non-viable combination of two viable mutations is defined as synthetic lethality, and constitutes the basis of synthetic lethal screens. The principle of this type of screen is that even if a cell can survive a mutation in a particular gene of interest, mutation in a second gene in the same pathway, in combination with the first one, might cause cell death. Lethality might reflect an indirect interaction between the two proteins encoded by the mutated genes. These proteins might be part of different steps of the same cellular process. Alternatively, lethality might be the result of the disruption of a direct interaction between the two proteins. Thus, lethality can be used as a selective criterion in this type of screens (Figure 7).

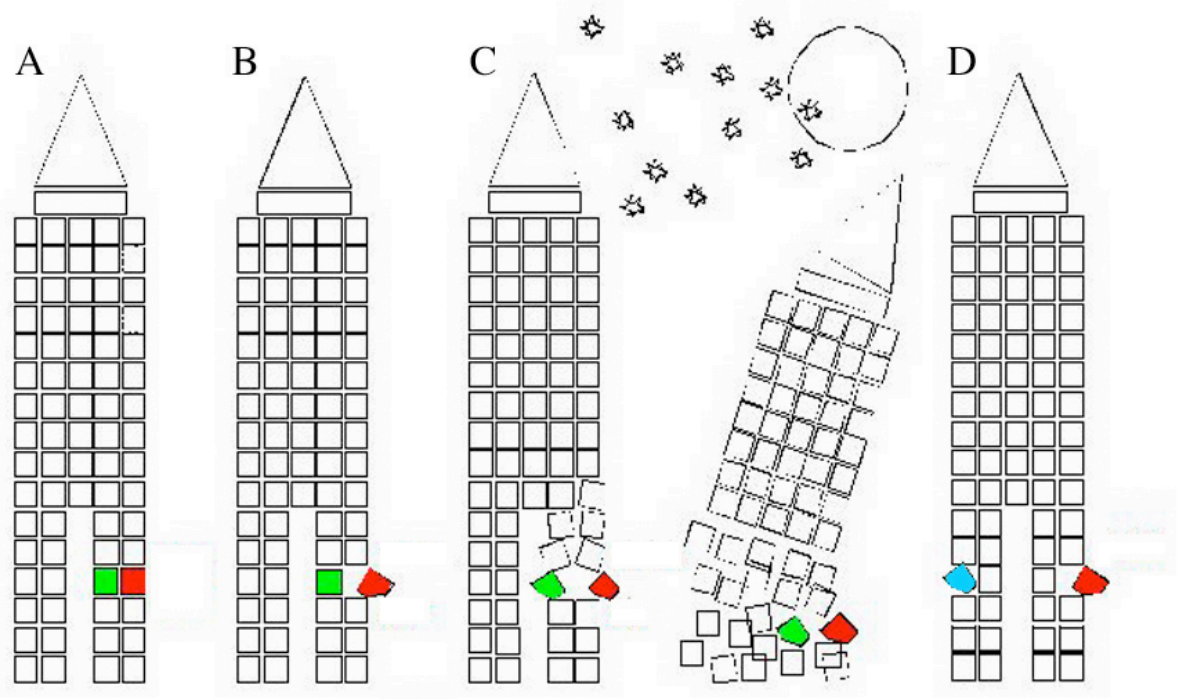

Figure 7. Synthetic lethal screens are a powerful tool to identify functionally interacting factors. (A) A viable cell is represented as a skyscraper. Protein X (red) and Y (green) act in the same cellular process, which is essential for cell viability (here depicted as a pillar). (B) Mutation in the gene encoding protein X is not sufficient to block the process of interest and therefore does not affect cell viability. (C) The combination with mutation in the gene encoding protein Y impairs the process and causes cell lethality (here drawn as a collapsing skyscraper). (D) If an unrelated gene encoding protein Z (blue) is mutated in combination with a mutant protein X, the process of interest is still functioning and cells are viable.

Synthetic lethality can be scored by detection of plasmid loss, which results in the appearance of red non-sectoring yeast colonies. The *gle2Δ*, *ade2*, *ade3* strain used for the screen shows white colour. When the strain is transformed with a plasmid carrying *ADE3* and *GLE2*, the resulting strain forms red colonies. Under non-selective conditions, a fraction of cells in a colony loses this plasmid, and these give rise to white sectors. When these cells are mutagenized, chromosomal mutations in genes that are synthetically lethal with *gle2Δ* will prevent plasmid loss and yield non-sectored colonies (see Materials and Methods).

In total, out of 10^5 colonies screened for the red non-sectoring phenotype, we isolated twenty mutants that are synthetically lethal with *gle2Δ* mutation. As Gle2 is known to interact with the nucleoporins Nup116 and Nup100, we tested whether nuclear pore proteins complement these mutants. To this end, we created a plasmid library containing all the known nucleoporins. As we were also interested in identifying mRNA export and protein import factors that interact with Gle2, we included known members of these protein classes in the

plasmid library (see Materials and Methods). The twenty mutants were transformed with this „mini-library“. Eighteen mutants were identified by complementation of the non-sectoring phenotype by a plasmid contained in the „mini-library“. The remaining two mutants were identified by transformation with a yeast genomic library (Figures 8-9). The complementing gene was identified either by restriction digest, or by sequencing of the plasmid recovered from the sectoring colonies. Five mutants were complemented by *NUP100* (Table 1), confirming the sl relationship already observed in the *Nup100Δ* screen (Murphy R *et al*., 1996). Eleven mutants were complemented by nucleoporins (Table 1). The remaining four mutants were complemented by protein import and mRNA export factors (Table 2 and 3, respectively). Thus, an extended, genetic network of interactions was unravelled.

Table 1. Genetic interaction between *GLE2* and genes encoding nuclear pore proteins.

sl#	Complementing gene	Protein family	Cellular process
310, 307, 336, 584, 233	*NUP100*	FG-NUP	Protein import/mRNA export
419	*NUP170*	Non-FG NUP	Protein import/NPC structure
11, 404	*NUP188*	Non-FG NUP	Protein import/NPC structure
619, 366	*NUP1*	FG-NUP	Protein import/mRNA export
69, 595	*NSP1*	FG-NUP	Protein import/mRNA export
390, 516	*NUP49*	FG-NUP	Protein import/mRNA export
497	*NUP85*	Non-FG NUP	mRNA export/NPC structure
557	*NUP145*	FG NUP/ Non-FG-NUP	mRNA export/NPC structure

GLE2 genetically interacts with nucleoporins

Most of the nucleoporins identified in this screen are part of subcomplexes, which have role in protein import, mRNA export and structural organization of the pore. The following paragraphs provide a brief description of the each nucleoporin subcomplex identified in the *gle2Δ* screen.

Nup170 belongs to the Nup170-Nup59-Nup53 subcomplex (Marelli M *et al*., 1998). This nucleoporin subcomplex might act in protein import. In fact, Nup53 contains FG repeats

that act as binding sites for the importin Kap121/Pse1, and is required for efficient Kap121-mediated import (Marelli M *et al*., 1998).

The components of the Nup170-Nup59-Nup53 subcomplex genetically interact with another subcomplex that includes two nucleoporins, Nup188 and Nic96, and an integral membrane protein, Pom152 (Aitchison JD *et al*., 1995; Nehrbass U *et al*., 1996; Zabel U *et al*., 1996; Marelli M *et al*., 1998; Tcheperegine SE *et al*., 1999). A *nup188* disruption strain and a temperature-conditional allele of *NUP188* exhibited nuclear envelope abnormalities (Nehrbass U *et al*., 1996; Zabel U *et al*., 1996), suggesting a function of this subcomplex in the structural organization of the NPC and nuclear envelope. Furthermore, the deletion of *NUP170* and *NUP188* greatly inhanced the permeability of the yeast NE, suggesting that Nup188 and Nup170 determine the functional diameter of the NPC's central transport channel (Shulga N *et al*., 2000).

The nucleoporin Nup1 contains FXFG repeats and is asymmetrically distributed at the nuclear basket. Nup1 functions in the termination step of karyopherin-dependent import of NLS-containing cargoes. In fact Nup1 FXFG repeats domain is able to displace Srp1-Kap95 from an NLS protein (Rexach M and Blobel G, 1995; Solsbacher J *et al*., 2000). Analysis of *nup1* mutants showed also poly(A)$^+$ RNA accumulation in the nucleus (Bogerd AM *et al*., 1994; Schlaich NL and Hurt EC, 1995; Solsbacher J *et al*., 2000). The involvement of Nup1 in mRNA export was recently related to its interaction with the Sac3-Thp1 complex, which plays a role in mRNA export. The Sac3-Thp1 complex docks the mRNA exporter Mex67-Mtr2 to the nuclear side of the NPC via the interaction of Sac3 with Nup1 (Fischer T *et al*., 2002).

Nsp1 is part of two distinct NPC subcomplexes, the Nsp1-Nup49-Nup57-Nic96 complex and the Nsp1-Nup82-Nup159 complex. The Nsp1-Nup49-Nup57-Nic96 complex localizes to the nucleoplasmic and cytoplasmic face of the central gated channel and to the nuclear basket and it is involved in protein import (Grandi P *et al*., 1995b; Fahrenkrog B *et al*., 1998). Nevertheless, mutant alleles of the *NUP49*, *NSP1*, and *NIC96* exhibited also accumulation of the ribosomal protein Rpl25 in the nucleus, suggesting a direct involvement of these nucleoporins in ribosomal export (Hurt E *et al*., 1999). In addition a different mutant allele of *NUP49* accumulated poly(A)$^+$ RNA inside the nucleus (Doye V *et al*., 1994).

The Nsp1-Nup82-Nup159 complex is located asimetrically at the cytoplasmic side of the NPC and plays a role in mRNA export (Grandi P *et al*., 1995a; Hurwitz ME and Blobel G, 1995; Kraemer DM *et al*., 1995). Nevertheless, mutants of the nucleoporins forming the

Nup82–Nup159–Nsp1 complex are also affected in the nuclear export of the ribosomal subunits (Gleizes PE *et al.*, 2001), suggesting multiple roles for the subcomplex.

Finally, Nup85 and Nup145C are members of a large nucleoporin complex that include Nup145C-Nup120-Nup85-Nup84-Seh1-Sec13, which plays a dual role in nuclear mRNA export and NPC biogenesis (Siniossoglou S *et al.*, 1996; Siniossoglou S *et al.*, 2000; Lutzmann M *et al.*, 2002).

In conclusion, these results suggest that the genetic interactions of Gle2 with nuclear pore components might reflect a general role of Gle2 at the NPC.

GLE2 is genetically connected to components of the protein import machinery

In addition to nucleoporins involved in protein import, the *gle2Δ* sl screen led also to the identification of the importins Kap95 and Kap60/Srp1 (Table 2, Figure 8). The importin β family members Kap95 (importin β) and Kap60/Srp1 (importin α) are responsible for nuclear import of proteins containing a classical NLS (Rexach M and Blobel G, 1995). The cargo binds in the cytoplasm to the NLS-receptor Kap60/Srp1, which binds to Kap95 that acts as an adaptor for interaction with nucleoporins (Iovine MK and Wente SR, 1997) and references therein). Translocation through the aqueous channel of the NPC is thought to proceed by a succession of energy dependent disassociation-reassociation steps with nucleoporins. Importin α-β transport to the nucleus is mediated by the interaction of Kap95 with the Nup116's GLFG region on the cytoplasmic side of the pore (Iovine MK *et al.*, 1995), while on the nuclear side, the Nup1 FXFG repeat domain displaces Srp1-Kap95 from an NLS protein (Rexach M and Blobel G, 1995).

The interaction of Gle2 with Kap95 and Kap60/Srp1 suggests that, in addition to a general role at the NPC, Gle2 might more specifically act in conjunction with different components of the protein import machinery.

Table 2. Genetic interaction between *GLE2* and genes encoding importins.

sl#	Complementing gene	Cellular process
241	*KAP95*	Protein import
360	*KAP60/SRP1*	Protein import

Figure 8. Genetic interaction of *GLE2* with *SRP1*.
Identification of *SRP1* in the sl screen with *gle2Δ*. Strain sl 360 was transformed with the complementing plasmid recovered from the yeast genomic library (pUN100-[ChXIV 279616bp-287497bp]), with an empty plasmid (pUN100), and with plasmids harboring *GLE2* and *SRP1*. The transformants were grown for 3 days on selective plates containing 5 fluoro-orotic acid (FOA). Colony formation indicates complementation of the sl phenotype.

Components of the mRNA export machinery are genetically linked to *GLE2*

Other Gle2 genetic interactors isolated in the *gle2Δ* screen are the conserved mRNA export factors *MEX67* and *SAC3* (Table 3, Figure 9). The mRNA export factor Mex67, in complex with Mtr2, forms an essential heterodimeric mRNA exporter (Santos-Rosa H *et al.*, 1998). The Mex67-Mtr2 complex binds the mRNA via the interaction with Yra1 (Strasser K and Hurt E, 2000; Zenklusen D *et al.*, 2001), and moves the mRNP through the nuclear pore channel using the FG repeats of nucleoporins as transient docking sites (Strasser K *et al.*, 2000).

Sac3 interacts genetically with Yra1 and forms a stable complex with Thp1, which is also required for mRNA export. By interaction with the symmetrically located Nup1, the Sac3-Thp1 complex docks the mRNA exporter Mex67-Mtr2 to the nuclear entrance of the pore channel (Fischer T *et al.*, 2002).

In conclusion, in addition to its involvement in protein import, Gle2 might also be intimately connected to the mRNA export machinery.

Table 3. Genetic interaction between *GLE2* and genes encoding mRNA export factors.

sl#	Complementing gene	Cellular process
335	*MEX67*	mRNA export
660	*SAC3*	mRNA export

Figure 9. Genetic interaction of *GLE2* with *SAC3*.
Identification of *SAC3* in the sl screen with *gle2Δ*. Strain sl 660 was transformed with the complementing plasmid recovered from the yeast genomic library (pUN100-[ChIV 768184bp-779702bp]), with an empty plasmid (pUN100), and with plasmids harboring *GLE2* and *SAC3*. The transformants were grown for 3 days on selective plates containing 5 fluoro-orotic acid (FOA). Colony formation indicates complementation of the sl phenotype.

***GLE2* is not required for nuclear poly(A)$^+$ RNA export.**

The *gle2Δ* synthetic lethal screen identified a variety of proteins localized at the nuclear pore, which are not only involved in mRNA export, but also in protein import and NPC biogenesis and maintenance of its structure. These data suggest that Gle2 might have a general role at the NPC, rather then being a specific mRNA export factor as was initially hypothesized. Therefore, we decided to test whether absence of Gle2 specifically impairs mRNA export. To this end, we carried out in situ poly(A)$^+$ RNA hybridization with cells completely lacking Gle2 (*gle2Δ*), or containing a *gle2-G226E* temperature sensitive mutant allele. Mutations in the glycine residue at position 219 of Rae1, the *S. pombe* homologue of Gle2, causes nuclear poly(A)$^+$ RNA accumulation (Brown JA *et al.*, 1995). Therefore, we replaced the corresponding residue of Gle2 with a glutamate residue by site-directed mutagenesis, to yield the *gle2-G226E* allele. We expressed *gle2-G226E* in the *gle2Δ* background as a *bona fide* positive control in our experiment. By oligo(dT) *in situ* hybridization, no significant nuclear accumulation of poly(A)$^+$ RNA was observed in either *gle2Δ* or *gle2-G226E* cells at restrictive temperature (Figure 10). We conclude that Gle2, in contrast to earlier reports, is not required for nuclear mRNA export. Thus, the nuclear export function of Rae1 appears not to be conserved in Gle2, suggesting that functional homologues of *S. pombe* Rae1 other than Gle2 might be present in *S. cerevisiae*.

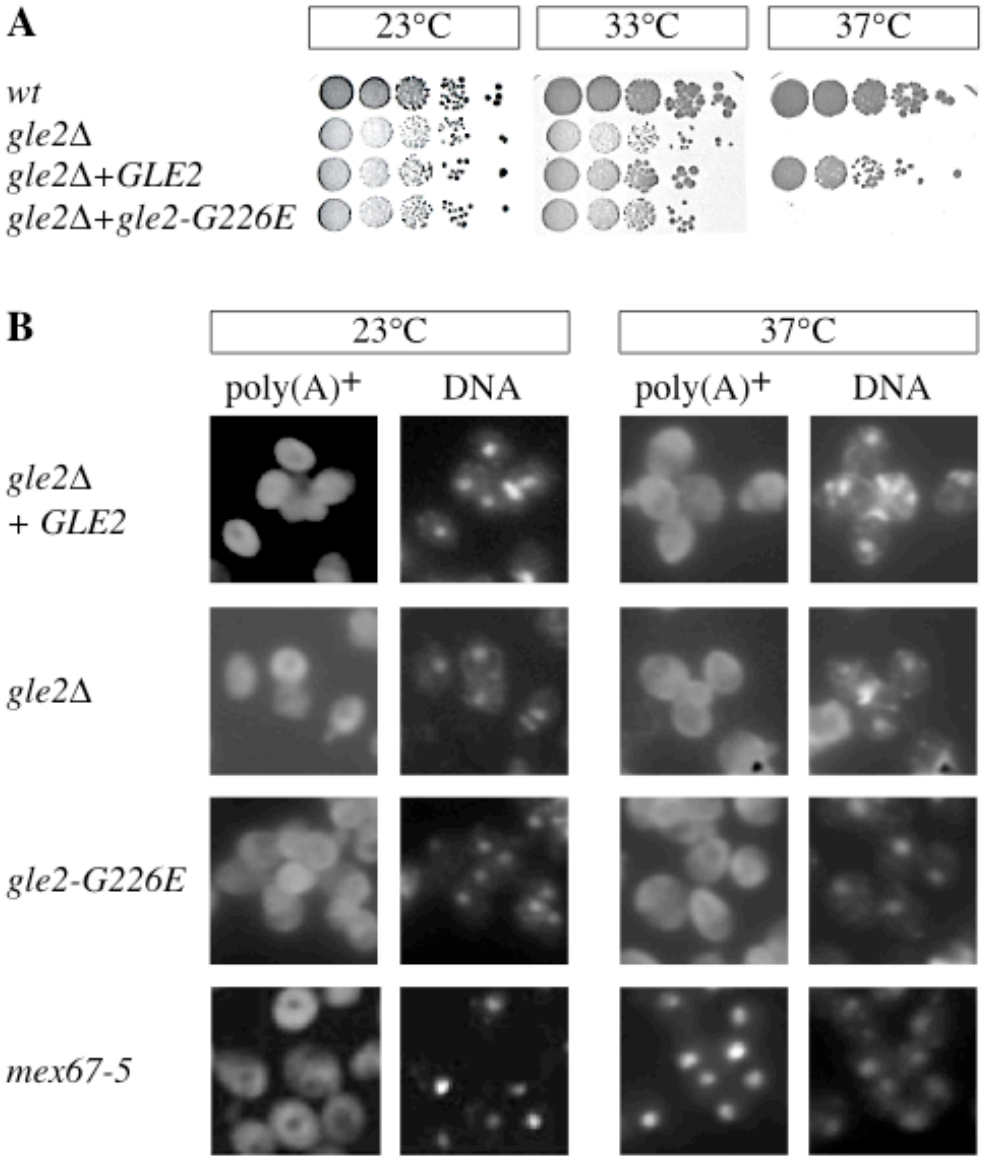

Figure 10. mRNA export is normal in *gle2* mutants. (A) *GLE2* deletion and *gle2-G226E* cause temperature sensitive phenotype. Wild-type (wt), *gle2Δ*, *gle2Δ +GLE2* and *gle2Δ +gle2-G226E* cells were spotted in 10^{-1} dilutions onto YPD plates and growth was analyzed after 3 days at the indicated temperatures. (B) Wild-type (*gle2Δ +GLE2*), *gle2Δ*, *gle2-G226E* or *mex67-5* cells were grown at the indicated temperatures and localization of poly(A)$^+$ RNA was assessed by *in situ* hybridization using FITC-labelled oligonucleotide poly(dT) probes. DNA was stained with 4', 6-diamidino-2-phenylindole (DAPI). No accumulation of poly(A)$^+$ RNA was observed, except for the control *mex67-5*, which strongly accumulates poly(A)$^+$ RNA in the nucleus upon shift to 37°C.

Part II: TREX is a conserved complex coupling transcription with mRNA export

SUB2 is genetically linked to the THO complex, which acts in transcription elongation

Sub2/UAP56 was initially identified as an essential splicing factor in yeast and metazoans (Fleckner J *et al.*, 1997; Kistler AL and Guthrie C, 2001; Libri D *et al.*, 2001; Zhang M and Green MR, 2001). Later it was shown that Sub2 is specifically required for export of mRNAs (Jensen TH *et al.*, 2001; Strasser K and Hurt E, 2001). In fact, a thermosensitive yeast mutant of *SUB2*, *sub2-85,* exhibits nuclear accumulation of poly(A)$^+$ RNAs (Strasser K and Hurt E, 2001). Moreover, excess of the human homologue of Sub2, UAP56, is a dominant negative inhibitor of mRNA export (Luo ML *et al.*, 2001). This evidence suggests that Sub2/UAP56 functions in coupling the splicing and export machineries.

To investigate whether mRNA export and other upstream events of gene expression are genetically coupled via Sub2, we set out to identify novel interactors of Sub2 by synthetic lethal screen with the *sub2-85* allele (for principle see Figure 7).

Table 4. Mutants isolated from the *sub2-85* synthetic lethal screen, and the complementing genes.

sl#	Complementing gene	Cellular process
579, 1002, 1270	*YRA1*	mRNA export
611, 1232, 1281, 1206, 3341	*HPR1*	Transcription elongation
1266, 2812	*THP2*	Transcription elongation
864, 632	*MFT1*	Transcription elongation
1166, 1212, 1362, 1925	*THO2*	Transcription elongation
886, 1210, 1392, 1698, 2759, 3205	*MTR10*	Protein import

Three independent sl mutants of *sub2-85* were complemented by an *YRA1*-containing plasmid (Table 4), confirming the reported genetic and physical interaction between *SUB2* and *YRA1* (Strasser K and Hurt E, 2001). Five mutant genes were complemented by *HPR1*, four by *THO2*, two by *MFT1* and two by *THP*2 (Table 4 and Figure 11). In addition, one mutant gene was complemented by *MTR10* coding for a nuclear import factor (Table 4). *HPR1*, *THO2*, *MFT1* or *THP2* code for members of the THO complex, which affects the incidence of

recombination associated with transcription elongation. Cells lacking *HPR1*, *THO2*, *MFT1* or *THP2* show both mitotic hyper-recombination and impaired transcription elongation (Chavez S *et al.*, 2000). Interestingly, a genetic interaction with all the components of the THO complex was also observed in our lab using an *YRA1* mutant (Strasser K *et al.*, 2002). This evidence establishes a clear connection between the mRNA export factors Yra1, Sub2 and the THO complex, which is part of transcription machinery.

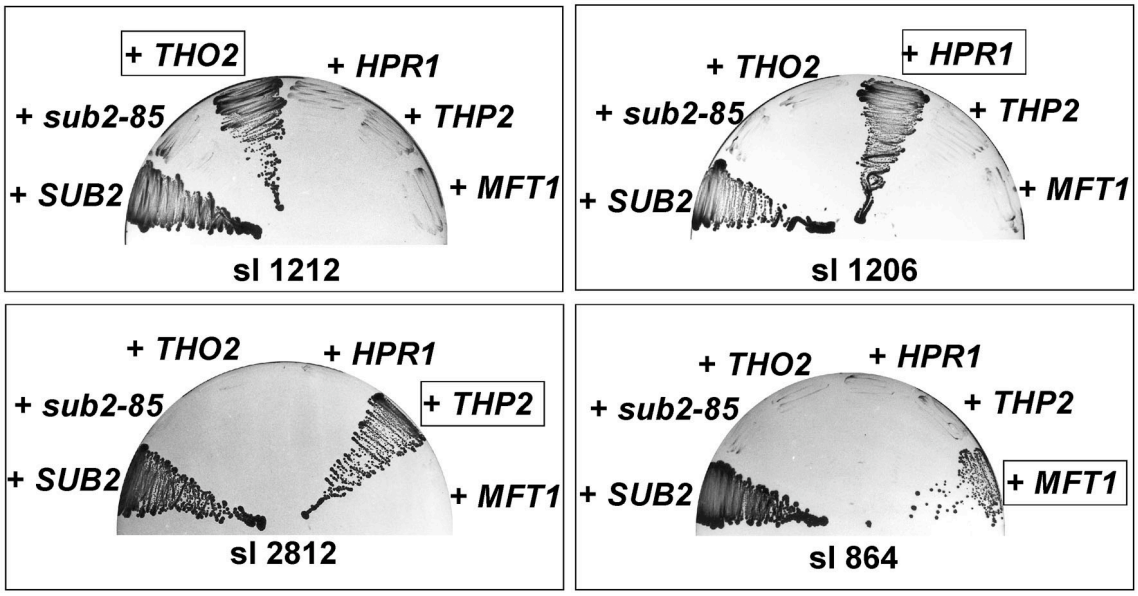

Figure 11. *SUB2* interacts genetically with the genes encoding the four components of the THO complex. Sl strains 1212 (upper left), 1206 (upper right), 2812 (lower left), and 864 (lower right) were transformed with plasmid containing *SUB2, sub2-85, THO2, HPR1, THP2* and *MFT1*. The transformants were grown for 5 days on selective plates containing 5 fluoro-orotic acid (FOA). Colony formation indicates complementation of the sl phenotype.

Sub2, Yra1, Tho2, Hpr1, Mft1, Thp2 and Tex1 form the transcription-export (TREX) complex

Consistent with the observations that the THO complex is genetically linked to mRNA export factors, an *hpr1* mutant exhibits an mRNA export defect (Schneiter R *et al.*, 1999). This defect is detectable because the *hpr1* mutant, like the other THO complex mutants, impairs but does not abolish transcription (Chavez S and Aguilera A, 1997; Piruat JI and Aguilera A, 1998; Chavez S *et al.*, 2000). In addition, Yra1 and Sub2 are specifically recruited to

transcribing genes during transcription elongation, as shown by chromatin immuno-precipitation (Lei EP *et al*., 2001; Lei EP and Silver PA, 2002; Zenklusen D *et al*., 2002).

To test whether the genetic interaction of *SUB2, YRA1* and genes coding for components of the THO complex reflects a physical association of the proteins they encode, Katja Strässer, a post-doc in the lab, showed that Sub2 co-purifies with Yra1, Tho2, Hpr1, Mft1 and Thp2, and a previously unknown protein, which was named Tex1. This complex was designated TREX, as it contains factors involved in Transcription and mRNA Export. I confirmed the physical association of the TREX components by expression of TAP tagged Mft1 and tandem affinity purification (TAP). This biochemical method involves two sequential affinity purification steps. After binding of the TAP-fusion protein to IgG beads, co-purifying proteins were eluted by Tobacco Etch Virus (TEV) protease cleavage. This eluate was further bound to Calmodulin coated beads and finally eluted with EGTA (see Materials and Methods). SDS-PAGE followed by Coomassie staining revealed that Mft1 co-purifies with Tho2, Hpr1, Thp2, Sub2, and Tex1. Two additional proteins co-purified with Mft1, which were identified by mass spectrometry analysis as the RNA binding proteins Hrb1 and Gbp2. The TREX proteins are approximately stoichiometric as concluded by the intensity of the Coomassie stained bands of the gel shown in Figure 12.

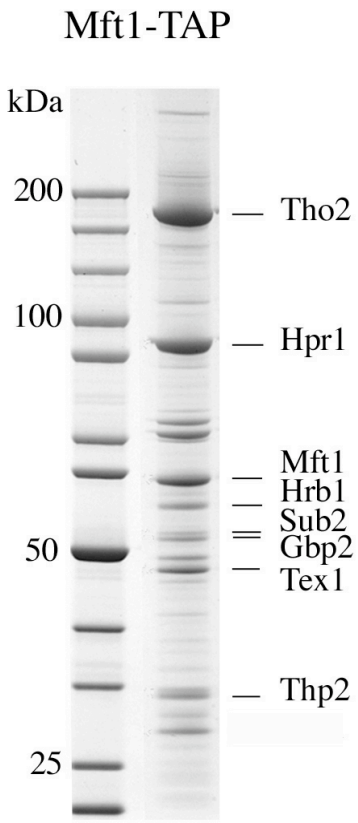

Figure 12. Mft1 interacts *in vivo* with other components of the TREX complex and Hrb1 and Gbp2. TAP purified Mft1-TAP was analyzed by SDS-PAGE and visualized by Coomassie staining. The indicated bands were identified by mass spectrometry (MS). A protein molecular weight standard is also shown.

The core of the TREX complex adopts a symmetric butterfly-like structure

In order to visualize how the components of the TREX complex are assembled and gain novel insight into its function, we decided to investigate whether we could determine the structure of the complex by electron microscopy (EM).

After TAP purification, as ribosomal proteins are still present in the final eluate (see Figure 12), the sample looks inhomogeneous at the electron microscope, thus not suitable for refined EM analysis. Therefore, we subjected the TREX complex to gel filtration chromatography. After TAP purification of the Mft1 fusion protein, the final eluate was injected on a Superose 6 gel filtration column that fractionates protein complexes according to their molecular mass (see Materials and Methods). In contrast to its predicted molecular mass of 0.5 MDa, the TREX complex eluted at a molecular mass of approximately 1MDa (Figure 13, fractions 9-11). This difference suggests that either the TREX complex adopts elongated, non globular shape, or that it contains more than one copy of each TREX component.

We separated the proteins contained in each fraction by SDS-PAGE, and performed mass spectrometry analysis of the major bands. We determined that fractions 9-11 contained Tho2, Hpr1, Mft1, Thp1 (THO complex) and Tex1 (Figure 13). Moreover, Hrb1 and Gbp2 were present. As Sub2 and Yra1 bands were not visible by Coomassie staining, we also tested the purified fractions for Sub2 and Yra1 presence by Western blotting. A strong signal was detected in the eluate applied to the gel filtration (I) for both Sub2 and Yra1. However, a weak signal for Sub2 was detected, while Yra1 was not. This suggests that these two proteins were largely dissociated under the purification conditions used in this experiment. However, a core of the TREX complex is stable *in vivo* and can be recovered by gel filtration chromatography in an essentially pure form.

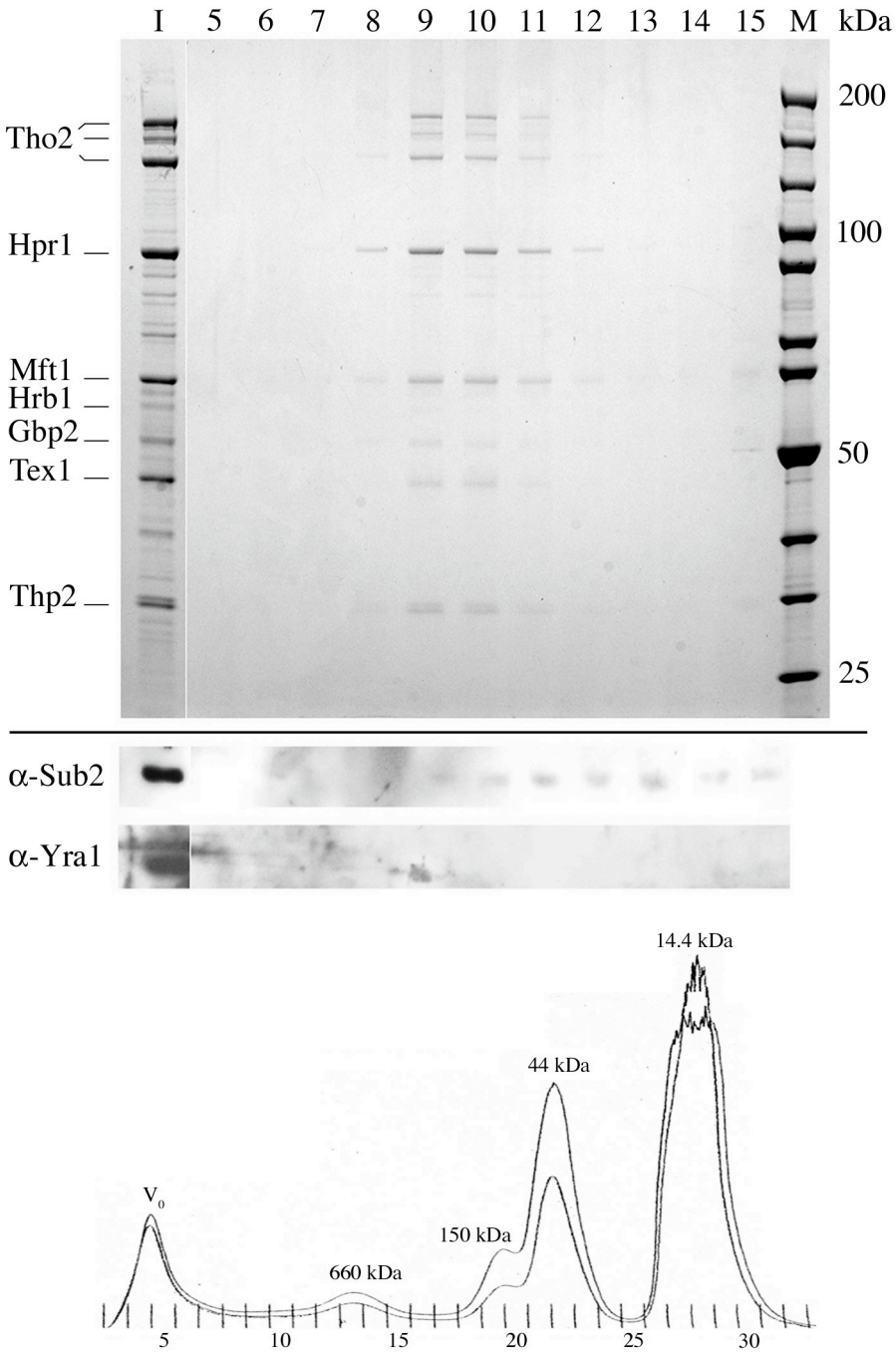

Figure 13. The stable core of the TREX complex does not contain Sub2 and Yra1. The Coomassie-stained gel (upper part) shows the Calmodulin eluate of the Mft1-TAP purification (I) and fractions 5-15 from the Superose 6 gel filtration column run. Major co-purifying proteins are indicated. A protein molecular mass standard is also shown (M). The same samples shown in the upper part of the figure were further analyzed by Western blotting (middle part) using Sub2 and Yra1 antibodies. The Superose 6 column was calibrated with molecular mass marker proteins of 660, 150, 44 and 14.4 kDa, and the calibration profile is shown (bottom part).

After affinity chromatography and gel filtration the components of the TREX complex were essentially pure, as assessed by Coomassie-stained SDS-PAGE (Figure 13 fractions 9-11). For this reason, we used these fractions to analyze the structure of the TREX core complex. The sample was negatively stained and analyzed by electron microscopy (EM) in collaboration with Bettina Böttcher (EMBL). The particles in the sample appear homogeneous, with a similar shape and size. Upon closer inspection, they display a structure with two arms of roughly similar size and a cleft in between, resembling the shape of a butterfly (Figure 14).

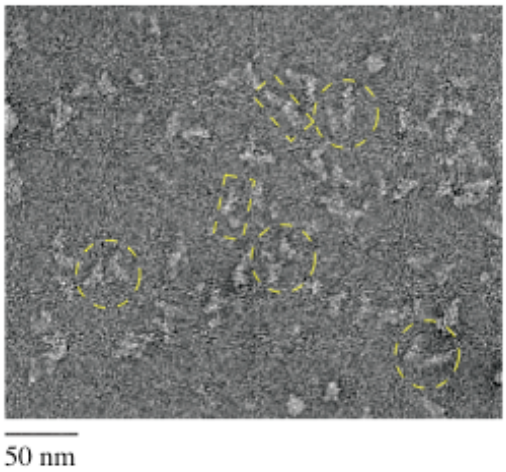

Figure 14. The core of the TREX complex displays a butterfly-like shape. Overview electron micrograph of the negatively stained preparation. Dashed yellow circles highlight the butterfly-like shapes, most likely dimeric structures. Particles that might represent monomers are indicated with dashed yellow squares.

About 800 selected particles were further analyzed. These were classified according to the shape they exhibited, which might reflect the orientation that the particles adopt upon immobilization on the support. Averaging of each particle of a class yielded high-resolution shapes of the TREX core (Figure 15, upper panel). Thirty classes of shapes were singled out representing distinct views of the complex. About half of the classes display a two-fold symmetric structure, with two arms of roughly similar size, divided by a cleft, resembling a butterfly. The cleft lies in the middle of the winged arms, along the full length of the complex. The connection between the winged arms appears to run diagonally through the cleft (Figure 15, lower panel). The whole structure is approximately 27 nm wide and the average length of each winged arm is 23 nm. The distance between the opposite edges of the wings is about 33

nm. It therefore appears that, *in vivo*, the core of the TREX complex possesses a symmetric structure.

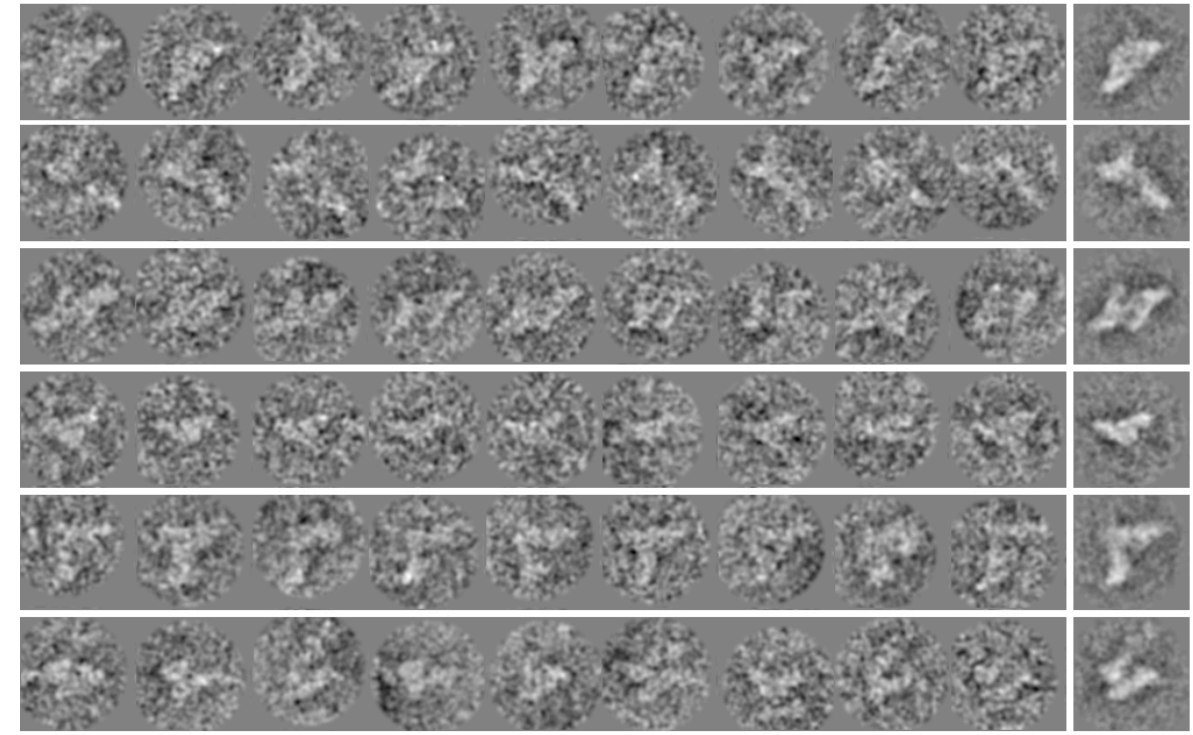

Figure 15. The core of the TREX complex possesses a two-fold symmetry. (Upper panel). Electron micrograph of selected negatively stained TREX particles. In each row, nine particles with similar appearance are shown. These were used to create an averaged image, shown in the last quadrant of each row. (Lower panel). Gallery of thirty classes of averaged particles displaying different shapes. The same six classes of the quadrants in the upper panel are shown in the first row of the lower panel. Approximately half of the classes exhibit a symmetric shape.

Alternative purifications of the TREX complex

In our previous purification of the TREX complex, Yra1 and Sub2 were largely dissociated from the complex after gel filtration chromatography (Figure 13). Therefore, the structure of the core of the TREX that we resolved (Figures 14-15) might not be that adopted by the complete TREX complex. Thus, we tested alternative purification methods in the hope of studying the complete TREX complex at the EM level. We reasoned that the conditions used to purify TREX by tandem affinity purification might be too stringent to preserve the association of Yra1 and Sub2. Therefore, we separated the TEV eluate from the first step of the TAP purification by gel filtration, without further purifying the complex with calmodulin-coated beads. We obtained a peak in the elution profile, which is similar to that observed by gel-filtering calmodulin eluates. By Western blotting, we observed that Yra1 and Sub2 were also present in the peak's fractions. However, under these conditions the TREX-containing fractions are highly enriched in ribosomal proteins. The residual presence of ribosomes made inefficient the detection of TREX particles at the electron microscope, preventing the resolution of the structure of the complete TREX (data not shown).

As an alternative attempt to purify the complete TREX complex and resolve its structure, we tagged two of its components. For this experiment, we generated a yeast strain carrying an Mft1-TAP and a Sub2-Glutathione-S-Transfrase (GST) fusion protein. The use of such strain allows substitution of the second step of the TAP purification (calmodulin), which could have caused the dissociation of Yra1 and Sub2 from the TREX complex, by GST purification. In addition, since the two tags are on two different TREX components, this procedure could yield a pure TREX complex without the need for further gel filtration chromatography (see Materials and Methods).

When tested for growth efficiency, the Mft1-TAP/Sub2-GST strain exhibited a slow growth phenotype (Figure 16, upper part). It is possible that in this strain the tagging of two different TREX components affects the formation of the complex, thus impairing growth. However, culturing at 30°C ensured enough starting material for the purification.

Lysate of Mft1-TAP/Sub2-GST strain was subjected to IgG binding and TEV proteolytic cleavage. The TEV eluate was subsequently applied to a glutathione-Sepharose (GSH) column and eluted with glutathione (GSH). SDS-PAGE analysis of the eluates, followed by Coomassie staining revealed that Mft1-TAP and Sub2-GST co-purified. Tho2, Hpr1 and Yra1 were also present in the final eluate (Figure 16, middle part). As negative

control, the Mft1-TAP strain, which does not contain any GST-tagged protein, was purified following the same procedure. As expected, no Tho2, Hpr1 and Yra1 were found in the final eluate. By Western blotting of the eluates, we further confirmed the presence of Sub2-GST and Yra1 in the Mft1-TAP/Sub2-GST eluate, whereas no signal was detected in the negative control (Figure 16, lower part).

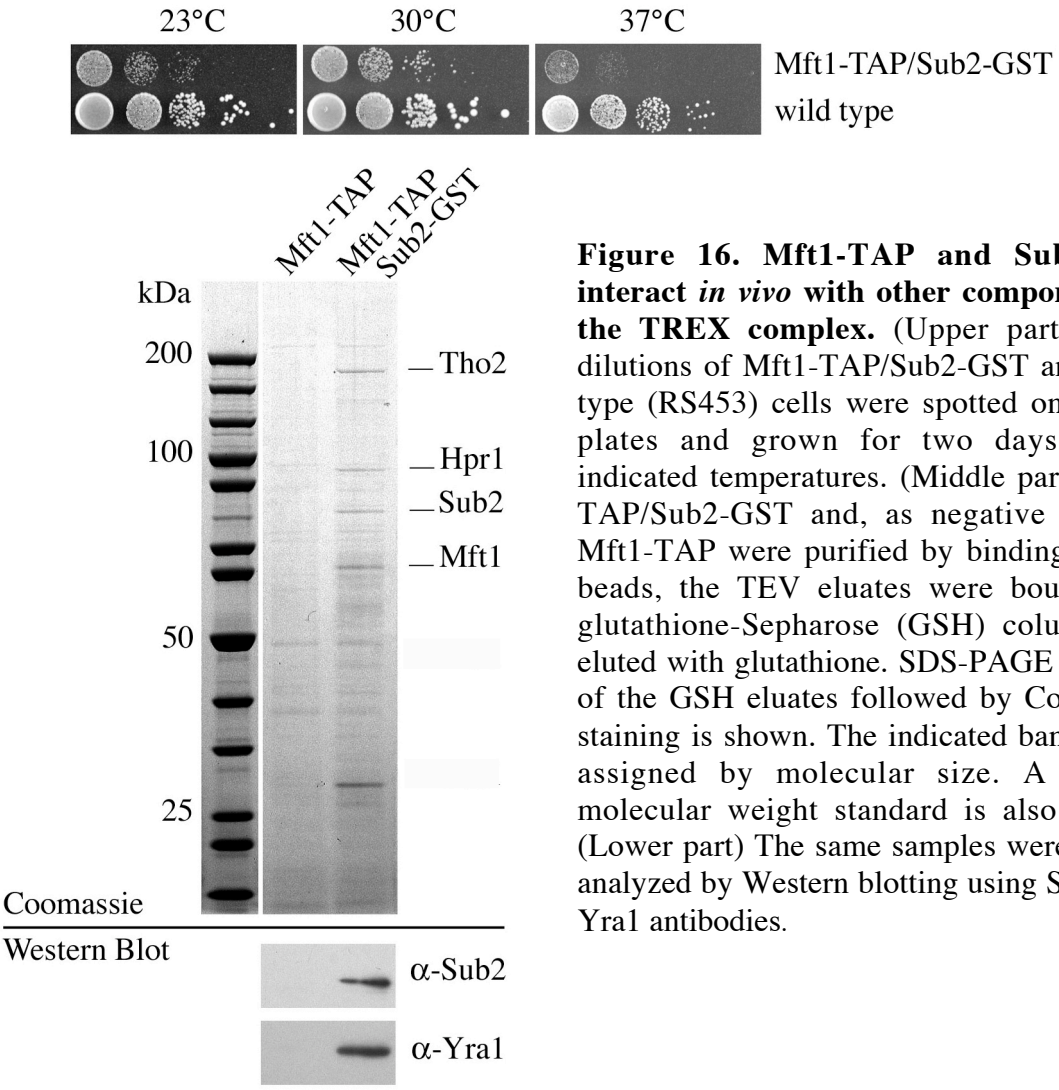

Figure 16. Mft1-TAP and Sub2-GST interact *in vivo* with other components of the TREX complex. (Upper part) Serial dilutions of Mft1-TAP/Sub2-GST and wild-type (RS453) cells were spotted onto YPD plates and grown for two days at the indicated temperatures. (Middle part) Mft1-TAP/Sub2-GST and, as negative control, Mft1-TAP were purified by binding to IgG beads, the TEV eluates were bound to a glutathione-Sepharose (GSH) column and eluted with glutathione. SDS-PAGE analysis of the GSH eluates followed by Coomassie staining is shown. The indicated bands were assigned by molecular size. A protein molecular weight standard is also shown. (Lower part) The same samples were further analyzed by Western blotting using Sub2 and Yra1 antibodies.

Although the yield of the double Mtf1-TAP/Sub2-GST purification is lower than that observed in TAP purifications of the TREX, the relatively low amount of ribosomal proteins render the sample suitable for EM analysis. Therefore, the final eluate was negatively stained and visualized under the electron microscope. Despite the fact that very few ribosomal proteins are visible by silver staining, some contaminating ribosomes were still detected at the

electron microscope. We were able to observe few butterfly-like shaped particles, with a two-fold symmetry, which appear similar to those obtained after TAP purification and gel filtration. However, too few particles were detected to allow averaging. Further analysis will be necessary to assess the shape of the TREX complex.

Taken together, these data suggest that the physical association of the TREX components, including Yra1 and Sub2, might still result in the formation of a butterfly-like, two-fold symmetric complex.

Components of the TREX complex genetically interact with *RRP6* and with *MTR10*, a member of the importin β-family that might regulate their import

The functional links between factors acting in transcription, mRNA processing and transport through the NPC, could ensure highly efficient maturation of mRNPs (reviewed in (Orphanides G and Reinberg D, 2002). To investigate whether the TREX complex is genetically connected to steps in gene expression other than transcription and mRNA export, we set up two synthetic lethal screens using *tho2Δ* and *thp2Δ* (Material and Methods). These are null alleles of the non-essential genes *THO2* and *THP2,* whose protein products are components of the THO complex. In each screen, we cloned *SUB2* and *YRA1*, confirming their genetic interaction with genes of the THO complex.

One mutant in each screen was complemented by *RRP6* (data not shown). This gene encodes a component of the nuclear exosome, which destroys RNAs that are slowly or incompletely processed (Burkard KT and Butler JS, 2000). A link of the THO complex with *RRP6* has been reported also by Jensen and coworkers (Libri D *et al.*, 2002). They showed that deletion of any component of the THO complex in yeast leads to accumulation of 3'end truncated transcripts at or near the transcription site. However, deletion of *RRP6* restores a quasinormal level of full-length transcripts and reverses this transcript sequestration phenotype (Libri D *et al.*, 2002). This evidence suggests that the nuclear exosome might be part of an mRNA surveillance system that degrades transcripts in response to defects in mRNA export.

THP2 is also sl with *MTR10,* which we previously identified by synthetic lethality with *sub2-85* (Table 4 and Figure 17). Mtr10 is a member of the importin β-family, which acts as nuclear import receptor for the SR proteins Npl3, Hrb1 and Gbp2 (Wilson SM *et al.*, 1994; Windgassen M and Krebber H, 2003; Hacker S and Krebber H, 2004). Hrb1 and Gbp2

are stably associated with the TREX complex, as they co-purify with it (Figure 13). Therefore, the sl relationship between *MTR10*, *THP2* and *SUB2* might reflect the impairment of Hrb1 and Gbp2 import, or of import of TREX components.

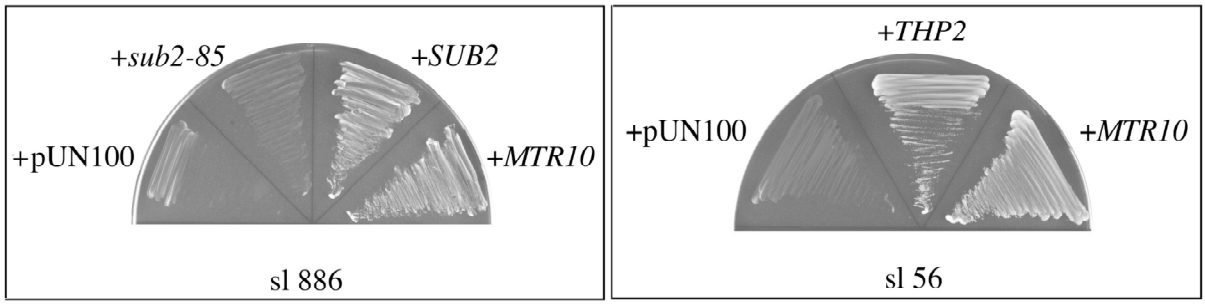

Figure 17. *MTR10* interacts genetically with *SUB2* and *THP2*. Sl strain 886 was transformed with an empty plasmid (pUN100) and plasmids containing *sub2-85, SUB2, MTR10* (left panel). Sl strain 56 was transformed with an empty plasmid (pUN100) and plasmids containing *THP2* and *MTR10* (right panel). The transformants were grown for 3 days on selective plates containing 5 fluoro-orotic acid (FOA). Colony formation indicates complementation of the sl phenotype.

To test whether Mtr10 is responsible for the subcellular localization of the TREX complex, we followed the *in vivo* localization of two of its components, Sub2 and Thp2 in *MTR10* mutant strains. To this end, we fused in frame a green fluorescent protein (GFP) tag to the N-terminus of Sub2 and to the C-terminus of Thp2. We expressed Sub2-GFP and Thp2-GFP in the temperature-sensitive mutants *mtr10Δ* (a deletion of *MTR10* strain), *mtr10-7* (Senger B *et al.*, 1998), and *mtr10-886* and *mtr10-56,* obtained in the *sub2Δ* and *thp2Δ* screen, respectively. In a *sub2Δ* strain, Sub2-GFP is nuclear at 23°C and 37°C. In contrast, in each *mtr10* mutant, Sub2-GFP is partially mislocalized to the cytoplasm at both temperatures, indicating a nuclear import defect (Figure 18A). GFP tagged Thp2 localizes to the nucleus in a *thp2Δ* strain. Thp2-GFP mislocalizes to the cytoplasm in *MTR10* mutant cells, albeit at a reduced rate (Figure 18B). Since Mtr10 is known to import Npl3 in the nucleus (Senger B *et al.*, 1998), we assayed Npl3-GFP localization as a positive control. As expected, Npl3-GFP is completely cytoplasmic in *mtr10-7* at both the analyzed temperatures. (Figure 18C). Taken together, the impaired nuclear localization of Sub2 and Thp2 in *MTR10* mutant cells, suggests that Mtr10 acts as an import factor for the TREX components Sub2 and Thp2.

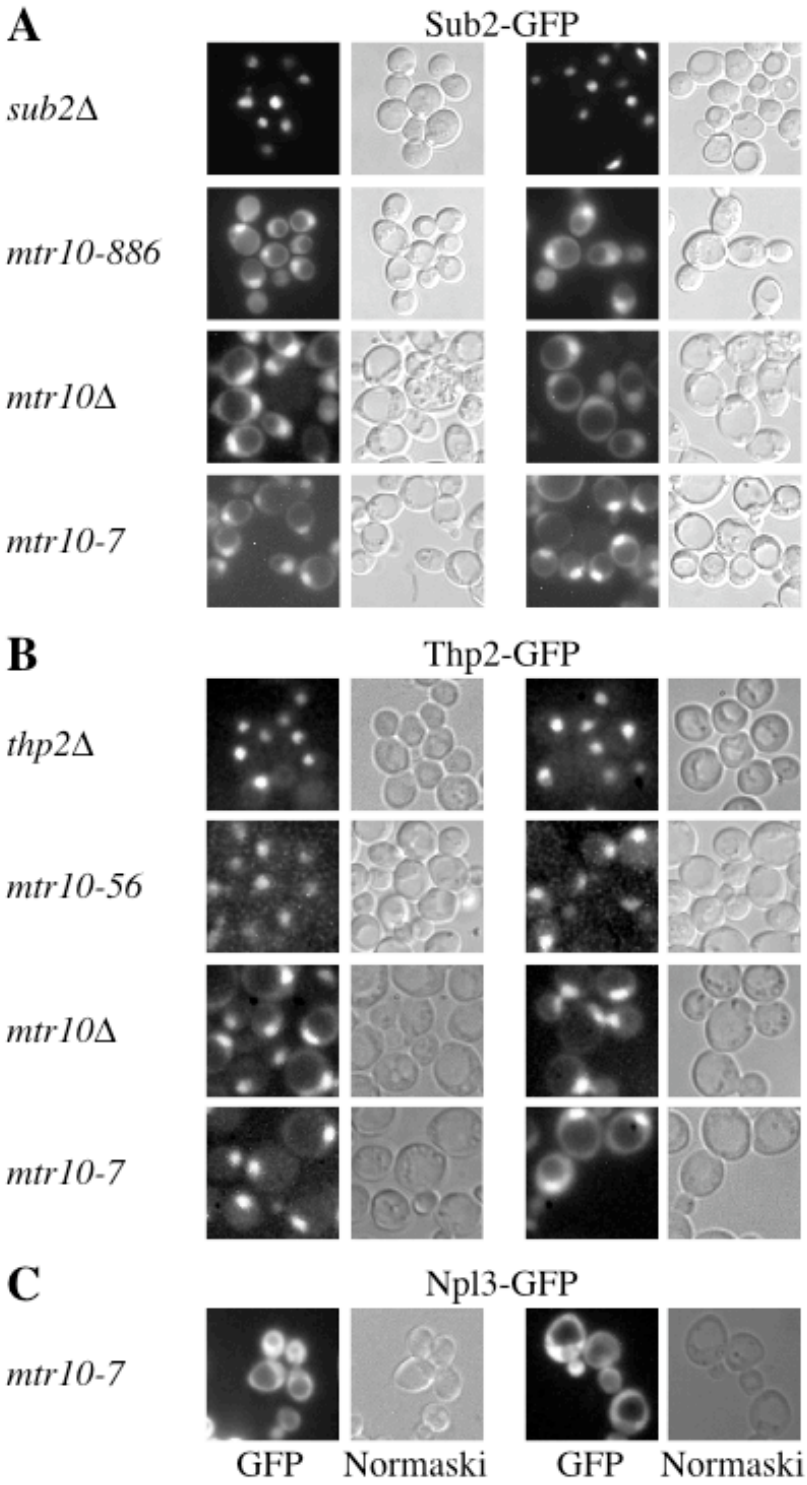

Figure 18. Mtr10 is responsible for nuclear import of the TREX components Sub2 and Thp2. Localization of Sub2-GFP (A), Thp2-GFP (B) and Npl13-GFP (C) in *MTR10* mutants was analyzed by fluorescence microscopy at permissive (23°C, left panel) and restrictive temperature (1 hour at 37°C, right panel). The localization of Sub2-GFP in *sub2Δ,* Thp2-GFP in *thp2Δ* and Npl13-GFP in *mtr10-7* were used as controls.

Discussion

Is Gle2 a nucleoporin or an mRNA export factor?

In the past, factors involved in export of mRNA were identified by genetic interaction with nucleoporins. Mex67, for instance, was identified in a genetic screen for synthetic lethality with the nucleoporin Nup85 (Segref A *et al.*, 1997). Subsequent studies have identified Mex67 as an mRNA export factor. In fact, Mex67 directly binds poly(A)$^+$ RNA (Santos-Rosa H *et al.*, 1998). The heterodimeric Mex67-Mtr2 complex directly contacts several FG repeats of nucleoporins as it carries the mRNP cargo through the nuclear pore (Strasser K *et al.*, 2000). Thermosensitive *MEX67* mutants display poly(A)$^+$ RNA accumulation in intranuclear foci, shortly after shift to the restrictive temperature (Segref A *et al.*, 1997). These characteristics, namely binding to the transport cargo RNA and to the nuclear pore, and requirement for efficient export of poly(A)$^+$ RNA, define an mRNA export factor.

Does Gle2 meet the requirements to be an mRNA export factor? Gle2 was identified in a synthetic lethal screen with the nucleoporin Nup100 (Murphy R *et al.*, 1996). Gle2 docks at the NPC by interaction with Nup116, a FG containing nucleoporin (Bailer SM *et al.*, 1998). In addition, it has been reported that mutations in *GLE2* exhibit nuclear accumulation of poly(A)$^+$ RNA (Murphy R *et al.*, 1996).

In our *gle2Δ* screen, we observed synthetic lethality with Nup100 (Figure 19A), as previously reported (Murphy R *et al.*, 1996). The Nup100 GLFG repeats interact *in vivo* and *in vitro* with both the karyopherin Kap95p (Iovine MK *et al.*, 1995; Iovine MK and Wente SR, 1997) and the mRNA export factor Mex67p (Strawn LA *et al.*, 2001). Consistent with the possibility that Gle2 acts as an mRNA transporter, we also found synthetic lethality with two other nucleoporins, Nup85 and Nup145 (Figure 19B), which are components of the Nup84 complex involved in mRNA export (Siniossoglou S *et al.*, 1996; Siniossoglou S *et al.*, 2000; Lutzmann M *et al.*, 2002). In addition, *GLE2* is synthetically lethal with *NSP1* (Figure 19C), a component of the Nsp1-Nup82-Nup159 NPC sub-complex, also required for mRNA export (Grandi P *et al.*, 1995a; Hurwitz ME and Blobel G, 1995). This sl relationship might result from the impairment of the association of Nsp1-Nup82-Nup159 with the Nup116-Gle2 complex, as these two complexes physically interact at the NPC (Bailer SM *et al.*, 2000).

Finally, *GLE2* is synthetically lethal with the mRNA export factors *MEX67* and *SAC3*, and with *NUP1* (Figure 19D-E). Mex67, in complex with Mtr2, binds and moves mRNPs through the nuclear pore channel, by step-wise docking at the repeats of different nucleoporins (Strasser K *et al*., 2000). One of these steps occurs at the nuclear basket, where Sac3, in complex with Thp1 docks the Mex67-Mtr2 by interaction with Nup1 and Mex67 (Fischer T *et al*., 2002). Gle2 uses a similar binding strategy to interact with Mex67-Mtr2. In fact, Gle2 associates with Mex67 and binds the GLEBS motif of Nup116, which is adjacent to the GLFG repeats interacting with Mex67-Mtr2 (Bailer SM *et al*., 1998; Strasser K *et al*., 2000; Zenklusen D *et al*., 2001). Considering that Nup116 is symmetrically distributed within the pore channel (Figure 1), Gle2 could function in the mRNA export pathway similarly to Sac3, but at a different step in the mRNP export process.

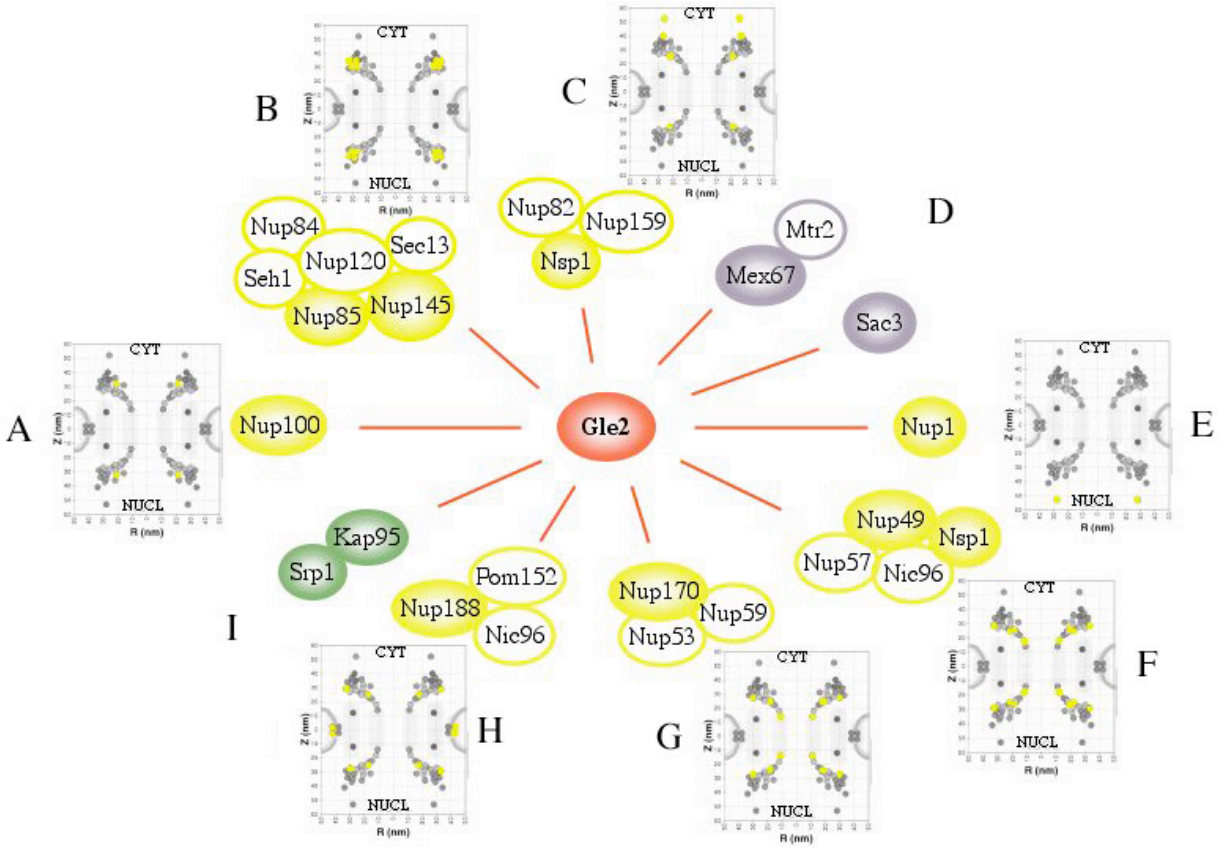

Figure 19. *GLE2* genetically interacts with several nucleoporins members of defined subcomplexes of the NPC, with importins and with mRNA export factors. *GLE2* is synthetic lethal with Nup100 (A), as previously reported (Murphy R *et al.*, 1996), with components of the Nup84 complex (B), of the Nup82 complex (C), with the export factors Mex67 and Sac3 (D), with Nup1 (E), Nsp1 and Nup49 (F), with components of the Nup170 complex (G), of the Nup188 complex (H), and with the importins Kap95 and Kap60 (I). Nucleoporins are indicated in yellow, the mRNA export factors in purple and protein import factors in green. Solid coloring indicates synthetic lethality with Gle2. The NPC localization of the nucleoporins of each complex is plotted according to (Rout MP *et al.*, 2000).

The *gle2Δ* screen uncovered also sl relationships with nucleoporins involved in protein import and maintenance of the NPC structure. These are Nsp1, Nup49, Nup170 and Nup188, which belong to distinct NPC subcomplexes (Figure 19F-H). Although Nsp1 belongs to the Nup82 complex, involved in mRNA export (see above), it interacts also with Nup49 as part of the Nsp1-Nup49-Nup57-Nic96 complex. This complex is required predominantly for protein import (Grandi P *et al.*, 1995b), even if mutants of these nucleoporins showed defect in mRNA and ribosomal export (Doye V *et al.*, 1994; Hurt E *et al.*, 1999), suggesting more than one role for the subcomplex. Nup170 belongs to the Nup170-Nup59-Nup53 subcomplex. Nup53 and Nup59 contain FG repeats that bind the importin Kap121/Pse1 (Marelli M *et al.*,

1998). The components of Nup170-Nup59-Nup53 subcomplex genetically interact with Nup188, which in turn binds to the nucleoporin Nic96, and Pom152, an integral membrane protein (Aitchison JD *et al.*, 1995; Nehrbass U *et al.*, 1996; Zabel U *et al.*, 1996; Marelli M *et al.*, 1998; Tcheperegine SE *et al.*, 1999). Nup188 and Nup170 contribute to define the functional diameter of the NPC's central transport channel (Shulga N *et al.*, 2000). The genetic interactions between the individual components of the latter two complexes and Gle2 suggest that their function is intertwined.

Furthermore, a connection with the protein import machinery was revealed by the fact that the importins α (Srp1/Kap60) and β (Kap95) are synthetically lethal with *GLE2* (Figure 19I). Cargoes to be imported in the nucleus bind to the NLS-receptor Kap60/Srp1 on the cytoplasmic side of the pore. Similarly to export of mRNPs, translocation of import cargoes through the NPC proceeds by successive docking to FG repeat-containing nucleoporins (Rexach M and Blobel G, 1995). Kap60/Srp1 binds Kap95, which acts as adaptor for interaction with Nup116 and Nup100 (Iovine MK and Wente SR, 1997). On the nuclear side, the FXFG repeats of Nup1 displace Srp1-Kap95 from the NLS-containing cargo (Solsbacher J *et al.*, 2000). It is known that Gle2 interacts physically with Kap60/Srp1 and Nup116, and genetically with Nup100 (Murphy R *et al.*, 1996; Bailer SM *et al.*, 1998). This, together with the genetic interaction of *GLE2* with *NUP1, KAP60* and *KAP95*, indicates that Gle2 could mediate an intermediate step of protein import through the NPC.

The variety of genetic interactions between Gle2 and such diverse array of nucleoporins might reflect a structural role of Gle2 at the NPC. Indeed, Gle2 sequence contains four WD repeats, which mediate the formation of macromolecular complexes with other proteins (Neer EJ *et al.*, 1994). In light of this, we cannot exclude that the mRNA export defect previously observed by Wente and coworkers (Murphy R *et al.*, 1996) is due to alteration of the nuclear pore structures, rather than a direct involvement of Gle2 in mRNA export. The fact that we did not observe a specific mRNA export defect by mutating *GLE2*, may be related to the genetic background of the strain used for the *in situ* localization of poly(A)$^+$ RNA. In fact this strain differs from the one in which poly(A)$^+$RNA accumulation in the nucleus was detected (Murphy R *et al.*, 1996).

It was recently reported that the *D. melanogaster* and mouse homologues of Gle2, named Rae1, are involved in cell cycle regulation (Babu JR *et al.*, 2003; Sitterlin D, 2004). Taken together, Gle2/Rae1 could be involved either in mRNA export and/or in cell cycle

regulation. Further analysis is required to elucidate how Gle2 might act in bidirectional transport across the nuclear pore.

In light of the broad range of *GLE2* synthetic lethal interactions with factors involved in nucleocytoplasmic transports and NPC structure formation, we propose that Gle2 itself might act as a nucleoporin. Via its symmetric NPC localization (Rout MP *et al*., 2000), Gle2 could influence both the mRNA export and protein import pathways, possibly during cargo translocation of protein and mRNP cargoes trough the NPC.

Coupling transcription elongation to mRNA export via the TREX complex

The expression of a protein-encoding gene involves several steps: transcription of the gene, extensive processing of the pre-mRNA, and export of the correctly processed mature mRNA. In yeast, our genetic and biochemical approaches revealed extensive interaction between factors involved in transcription, mRNA maturation, mRNA export and protein import. Our studies revealed that the export factors Sub2 and Yra1 are functionally connected to the components of the THO complex, which is involved in transcription elongation, defining the transcription-export (TREX) complex. In turn, the THO complex is genetically linked to the exosome component Rrp6, and the TREX components Sub2 and Thp2 to the import factor Mtr10. An overview of this extended network of genetic interactions is presented in Figure 20.

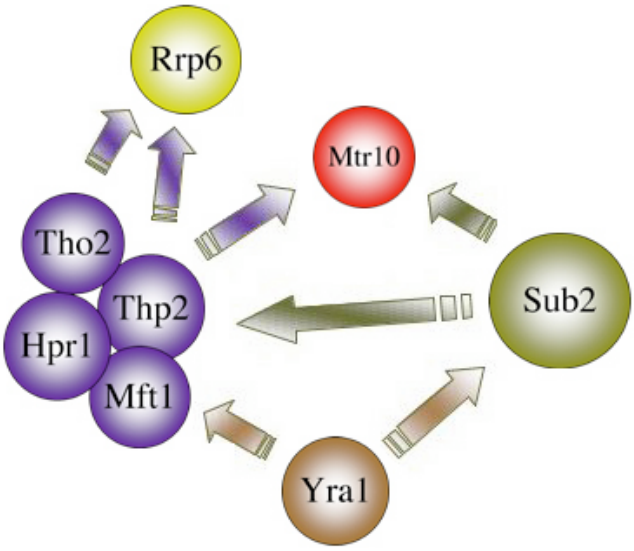

Figure 20. Genetic interactions identified among genes involved in transcription, mRNA processing and export. The emerging picture of the genetic interactions among components of the transcription elongation machinery (THO complex, purple), mRNA-export factors (Sub2 and Yra1), mRNA processing enzymes (Rrp6) and importins (Mtr10) predicts functional connection between most steps in gene expression. The genetic interactions uncovered during this study are represented as arrows starting from the gene analyzed for synthetic lethality.

We showed that the export factors Sub2 and Yra1 interact genetically with all four components of the THO complex, which acts in transcription elongation (Chavez S and Aguilera A, 1997; Piruat JI and Aguilera A, 1998; Chavez S *et al.*, 2000). These sl relationships are paralleled by physical interactions between Sub2 and Yra1, the heterotetrameric THO complex (Tho2, Hpr1, Mft1, Thp2), and Tex1. The interaction between these factors defines the TREX (transcription/export) complex. In our lab it was it was shown that the TREX complex is specifically recruited to activated genes during transcription and travels with RNA polymerase II during transcription elongation (Strasser K *et al.*, 2002).

Therefore, we propose that the TREX complex acts in co-transcriptional recruitment of factors involved in late events of gene expression, i.e. mRNA export.

Co-transcriptional recruitment might facilitate Yra1 and Sub2 binding to the nascent transcript. The mRNA exporter Mex67-Mtr2 might then be recruited. In fact Yra1 bridges Mex67-Mtr2 to intranuclear mRNA transport cargoes (Strasser K and Hurt E, 2000). *In vitro* studies have shown that Mex67-Mtr2 competes with Sub2 for the binding site on Yra1 (Strasser K and Hurt E, 2001), suggesting that Mex67-Mtr2 recruitment might displace Sub2 from the mRNP. Finally, Mex67-Mtr2 might serve to mediate mRNP contact to the nuclear pore complex and promote mRNA export (Strasser K *et al.*, 2000). A model describing the discussed role of the TREX complex in coupling transcription elongation to mRNA export is shown in Figure 21.

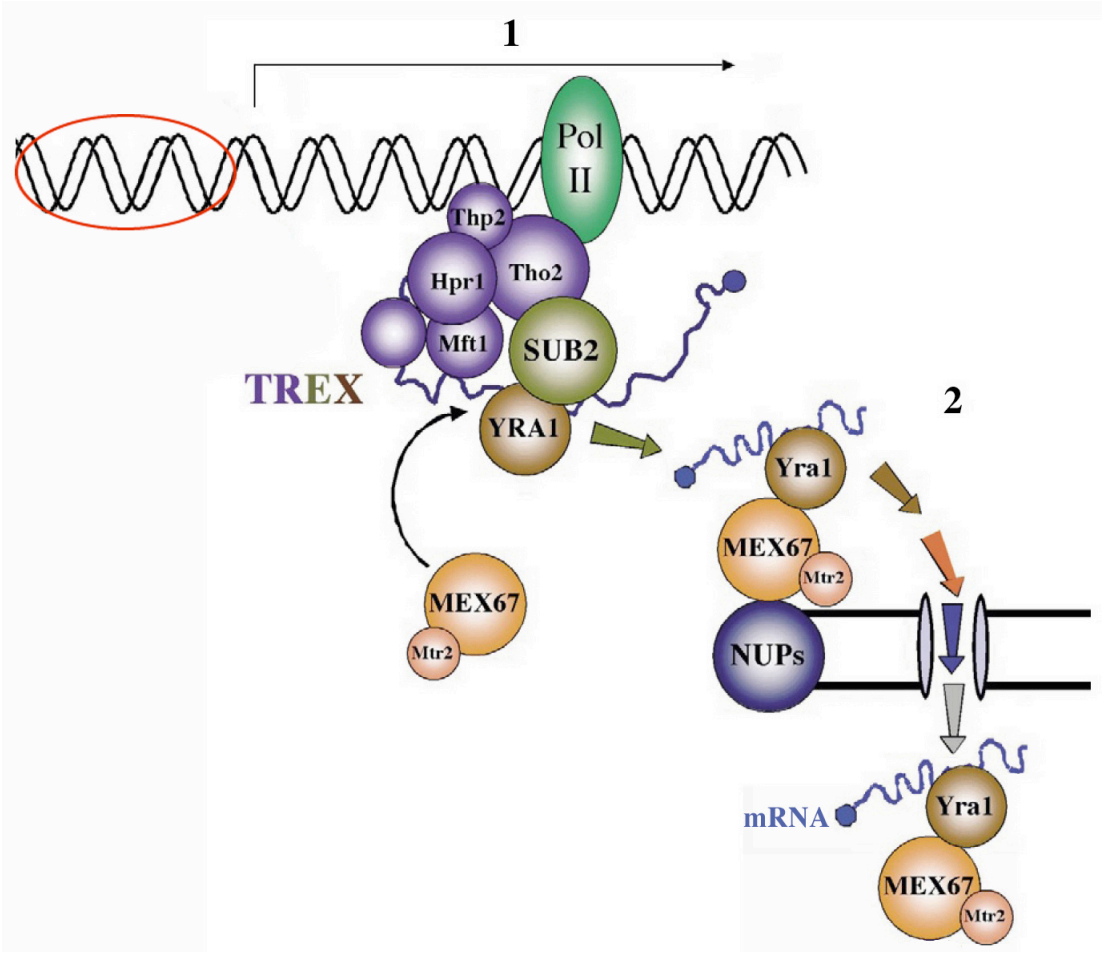

Figure 21. A model for coupling transcription elongation to mRNA export. The TREX complex is specifically recruited to activated genes during transcription and travels with RNA polymerase II during transcription elongation (1). Yra1 binds the nascent RNA and recruits Mex67 that displace Sub2 from the mRNP to promote mRNA export (2). (See text for details). The DNA double-helix is shown in black and the promoter region is highlighted with a red circle. The starting and direction of transcription is indicated with an arrow.

By TAP purification and gel filtration, we found that Hrb1 and Gbp2 are associated with the TREX complex. Hrb1 and Gbp2 are shuttling RNA-binding proteins that contain serine-arginine-rich (SR) and RNA recognition motif (RRM) domains (Windgassen M and Krebber H, 2003; Hacker S and Krebber H, 2004). The strong affinity of Gbp2 and Hrb1 for the TREX complex and their ability to bind RNAs suggest that the TREX complex might transfer Gbp2 and Hrb1 to the nascent pre-mRNA during transcription. Consistent with this, ChIP (Chromatin Immuno Precipitation) analysis recently showed association of Gbp2 and Hrb1 to the coding region of genes during transcription elongation (Hurt E *et al.*, 2004).

Gbp2 and Hrb1 are imported into the nucleus by the karyopherin Mtr10 (Windgassen M and Krebber H, 2003; Hacker S and Krebber H, 2004). We found that *MTR10* is synthetically lethal with *THP2* and *SUB2* and that GFP tagged Sub2 and Thp2, which normally localize in the nucleus, are nuclear and cytoplasmic when expressed in *mtr10* mutants. These data indicate that Mtr10 could also import TREX components into the nucleus. GFP tagged Sub2 exhibited the strongest mislocalization phenotype, suggesting that Mtr10 might primarily import Sub2. Consistent with this, Sub2 co-purifies with Mtr10-proteinA (T. Gerstberger, unpublished data).

In the synthetic lethal screen for the components of the THO/TREX complex *THO2* and *THP2,* we also cloned *RRP6*. Rrp6 is a 3'→5' exonuclease that is part of the nuclear exosome (Allmang C *et al.*, 1999; Burkard KT and Butler JS, 2000). The exosome is an RNA processing machinery present in both the nucleus and the cytoplasm, required for small nuclear RNA (snRNA), ribosomal RNA (rRNA), small nucleolar RNA (snoRNA) and pre-mRNA maturation and for nuclear RNA degradation (Mitchell P *et al.*, 1997; Hilleren P *et al.*, 2001). It was shown that heat-shock *HSP104* transcripts are 3'-end truncated and retained at the transcription site in *YRA1*, *SUB2* or *HPR1* null mutants. Consistent with our sl data, deletion of *RRP6* in these genetic backgrounds shows a strong synthetic growth phenotype. However, it leads to almost complete restoration of full-length *HSP104* transcripts and reverts the transcript sequestration phenomenon (Libri D *et al.*, 2002; Zenklusen D *et al.*, 2002). In addition to degrading aberrantly processed precursor mRNAs, Rrp6 might then control the release of the mRNA from the site of transcription. The sl relationship of the THO/TREX complex with Rrp6 that we observed suggests genetic coupling, not only of transcription and mRNA export (TREX), but also of mRNA processing. Further analysis will be required to address whether exosome components are co-transcriptionally recruited via the TREX complex.

How could the TREX be recruited to transcription sites? Jaehning and co-workers described an alternative form of the RNAPII holoenzyme containing Hpr1, a member of TREX, and components of the Paf1 complex (Chang M *et al.*, 1999). The Paf1 complex associates with RNAPII and functions in transcription elongation (Krogan NJ *et al.*, 2002; Mueller CL and Jaehning JA, 2002; Squazzo SL *et al.*, 2002; Rondon AG *et al.*, 2004). Disruption of Paf1 components *PAF1* and *CDC73* results in similar phenotypes to those of THO mutants, and *paf1Δ hpr1Δ* double mutants are lethal (Chang M *et al.*, 1999). In addition, our preliminary results confirm that components of the Paf1 complex might associate with the TREX. In fact, we were able to co-purify the Paf1 complex and Hpr1 (data not shown). Taken together, this evidence suggests that the Paf1 complex might bridge the interaction between TREX and RNAPII.

During transcription elongation, the nascent mRNA emerges from RNAPII at the base of the carboxy-terminal domain (CTD) of the largest polymerase subunit. The CTD functions as a platform for factors necessary for transcription and pre-mRNA modification. Their recruitment is regulated by serial phosphorylation of the CTD during transcription. The positioning of the RNA exit near the base of the CTD could ensure convenient coupling of transcription with subsequent steps of mRNA maturation including mRNA export from the nucleus (for reviews see (Maniatis T and Reed R, 2002; Orphanides G and Reinberg D, 2002; Buratowski S, 2003). In light of these evidences, the TREX complex could be recruited at the CTD, possibly by interaction with the Paf1 complex.

The TREX complex at the electron microscope

We purified the TREX complex to determine its structure at the EM level. By gel filtration the TREX complex eluted in a peak corresponding to a higher molecular weight then the expected one. The discrepancy between the theoretical and observed molecular mass of the TREX, and the symmetry of its structure by EM, suggest that the complex might be formed by two subunits. To further characterize the assembly of the complex, it will be interesting to determine the localization of the single components within the butterfly-like shape, by antibody-induced crosslinking. Such technique was successfully used to determine the localization of proteins within the large ribosomal subunit (Tsay YF *et al.*, 1994).

Under the experimental conditions of Tandem Affinity Purification and gel filtration using the Mft1-TAP strain, we purified a stable core of the TREX complex, enriched in THO

components (Tho2, Hpr1, Mft1, Thp2), Tex1 and the SR proteins Hrb1 and Gbp2. Sub2 and Yra1 were largely dissociated from this complex. Under different conditions, by protein-A and GST purification using the double-tagged strain Mft1-TAP/Sub2-GST, we recovered the TREX, in which Yra1 and Sub2 are present. Further investigation is required to determine the function and the biological relevance of two states of the TREX complex. However, the purified core of the TREX complex revealed a butterfly-shaped structure at the electron microscope, with two arms of roughly similar size, separated by a cleft.

How does this structure help in understanding the function of the THO/TREX complex? We proposed that the TREX complex acts in co-transcriptional recruitment of mRNA export factors. Interestingly, deletion of any component of the THO complex leads to mRNA export defect, as is the case for Sub2 and Yra1 mutants (Strasser K *et al*., 2002). Conversely, Sub2 and Yra1 mutants show gene expression defects and hyperrecombination phenotype similar to those of the THO mutants (Fan HY *et al*., 2001; Jimeno S *et al*., 2002). Such hyperrecombination phenotype was recently related to the ability of a long nascent transcript to form DNA:RNA hybrids close behind the elongating RNAPII. DNA:RNA hybrids may be responsible for the reduction in the transcription elongation efficiency caused by mutations of THO/TREX complex (Huertas P and Aguilera A, 2003). Hence, it is possible that the THO complex associates to the nascent mRNA and maintains separate the nascent transcript and the transcribed DNA, thereby ensuring elongation efficiency. We propose that symmetry of the observed structure might help to position the TREX complex between the DNA and the mRNA, as it is described in Figure 22.

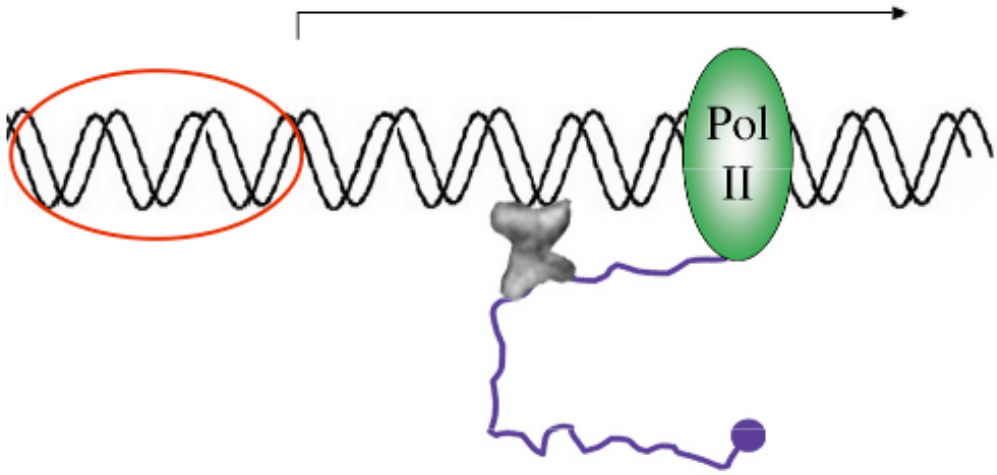

Figure 22. Model for a possible function of the TREX complex. The TREX complex might be specifically recruited during transcription elongation in order to maintain separate the nascent mRNA and the transcribed DNA. The TREX complex (gray) is the butterfly-like structure between the DNA double-helix (black) and the nascent mRNA (purple). The promoter region is highlighted with a red circle.

The THO complex recruits the mRNA export factors Sub2 and Yra1 to form the TREX complex. To date, the role of Sub2 in the TREX complex seems to be to recruit Yra1 to the nascent transcript in order to deliver the mRNP to Mex67-Mtr2 for the transport out of the nucleus. It was shown that overexpression of Sub2 is able to rescue the hyperrecombination phenotype of *hpr1Δ* mutant (Fan HY *et al.*, 2001). Although there are no evidences for that, our prediction is that Sub2 might use its helicase activity to solve formation of structures that could induce genome instability.

Publications

The following publications were derived from my PhD work:

Fischer T, Strasser K, Racz A, Rodriguez-Navarro S, Oppizzi M, Ihrig P, Lechner J, Hurt E. (2002) The mRNA export machinery requires the novel Sac3p-Thp1p complex to dock at the nucleoplasmic entrance of the nuclear pores. *EMBO J*. **21**, 5843-52.

Strasser K, Masuda S, Mason P, Pfannstiel J, Oppizzi M, Rodriguez-Navarro S, Rondon AG, Aguilera A, Struhl K, Reed R, Hurt E. (2002) TREX is a conserved complex coupling transcription with messenger RNA export. *Nature*, **417**, 304-8.

Materials and Methods

Molecular biological methods

DNA manipulation

DNA manipulations including restriction enzyme digests, fill in reactions with klenow DNA polymerase fragment, or T4 DNA polymerase, purification of DNA fragments from agarose gels, PCR amplifications and ligation reactions were done essemtially according to (Sambrook J and Russell DW, 2001).

Cloning of plasmids

The plasmids that were generated during the course of this study are listed in table 5:

Table 5. Plasmids generated during this study.

Name	*Genotype*
pHT4467Δ-*GLE2*	*CEN6mutation/ARS, URA3, ADE3, GLE2*
pHT4467Δ-*SUB2*	*CEN6mutation/ARS, URA3, ADE3, SUB2*
pHT4467Δ-*THO2*	*CEN6mutation/ARS, URA3, ADE3, THO2*
pHT4467Δ-*THP2*	*CEN6mutation/ARS, URA3, ADE3, THP2*
pRS315-*GLE2*	*CEN/ARS, LEU2, GLE2*
pRS315-*gle2-G226E*	*CEN/ARS, LEU2, gle2-G226E*
pRS315-*MFT1*	*CEN/ARS, LEU2, MFT1*
pRS315-*NPL3-eGFP*	*CEN/ARS, LEU2NPL3, eGFP*
pRS315-*THP2*	*CEN/ARS, LEU2, THP2*
pRS315-*THP2-eGFP*	*CEN/ARS, LEU2, THP2, eGFP*

The plasmids that were used in this work and were previously described, are listed in table 6:

Table 6. Plasmids used in this study.

Name	*Genotype*	Source
pFA6a-GST-His3MX6		(Longtine MS *et al*., 1998)
pGAD424-*SRP1*	2μ, *LEU2, SRP1*	(Kunzler M and Hurt EC, 1998)
pHT4467Δ	*CEN6mutation/ARS, URA3, ADE3*	(Bassler J *et al*., 2001)
pNOPPATAM1-*KAP95*	*KAP95*	Lab library (Senger, B)
pRS314	*CEN/ARS, TRP1*	(Sikorski RS and Hieter P, 1989)
pRS314-*mtr10-ts7*	*CEN/ARS, TRP1, mtr10-ts7*	(Senger B *et al*., 1998)
pRS314-*sub2-85*	*CEN/ARS, TRP1, sub2-85*	(Strasser K and Hurt E, 2001)
pRS315	*CEN/ARS, LEU2*	(Sikorski RS and Hieter P, 1989)
pRS315-*NUP2*	*CEN/ARS, LEU2, NUP2*	(Loeb JD *et al*., 1993)
pRS315-*NUP53*	*CEN/ARS, LEU2, NUP53*	(Marelli M *et al*., 1998)
pRS315-*NUP57*	*CEN/ARS, LEU2, NUP57*	(Schlaich NL *et al*., 1997)
pRS315-*NUP59*	*CEN/ARS, LEU2, NUP59*	(Marelli M *et al*., 1998)
pRS315-*NUP120*	*CEN/ARS, LEU2, NUP120*	(Siniossoglou S *et al*., 1996)
pRS315-*NUP159*	*CEN/ARS, LEU2, NUP159*	(Gorsch LC *et al*., 1995)
pRS315-P_{NOP1}-*GFP*	*CEN/ARS, LEU2, GFP*	(Hellmuth K *et al*., 1998)
pRS315-P_{NOP1}-*GFP-SUB2*	*CEN/ARS, LEU2, SUB2,GFP*	(Strasser K and Hurt E, 2001)
pRS315-*RRP6*	*CEN/ARS, LEU2, RRP6*	Lab library (Strasser, K)
pRS315-*SAC3*	*CEN/ARS, LEU2, SAC3*	(Fischer T *et al*., 2002)
pRS315-*sub2-85*	*CEN/ARS, LEU2, sub2-85*	(Strasser K and Hurt E, 2001)
pRS315-*THO2*	*CEN/ARS, LEU2, THO2*	Provided by A. Aguilera
pUN100	*CEN/ARS, LEU2*	(Elledge SJ and Davis RW, 1988)
pUN100-*HPR1*	*CEN/ARS, LEU2, HPR1*	Lab library (Bassler, J)
pUN100-*NIC96*	*CEN/ARS, LEU2, NIC96*	(Grandi P *et al*., 1995b)
pUN100-*MTR10*	*CEN/ARS, LEU2, MTR10*	(Senger B *et al*., 1998)
pUN100-*NUP49*	*CEN/ARS, LEU2, NUP49*	Lab library (Doye, V)
pUN100-*NUP84*	*CEN/ARS, LEU2, NUP84*	Provided by C. Wimmer
pUN100-*NUP133*	*CEN/ARS, LEU2, NUP133*	(Doye V *et al*., 1994)
pUN100-*NUP145*	*CEN/ARS, LEU2, NUP145*	(Fabre E *et al*., 1994)
pUN100-*NUP170*	*CEN/ARS, LEU2, NUP170*	Lab library (Santos-Rosa, E)
pUN100-*NUP188*	*CEN/ARS, LEU2, NUP188*	(Zabel U *et al*., 1996)
pUN100-*NUP192*	*CEN/ARS, LEU2, NUP192*	(Kosova B *et al*., 1999)
pUN100-protA-*NUP82*	*CEN/ARS, LEU2, protA, NUP82*	(Grandi P *et al*., 1995a)
pUN100-protA-*NUP85*	*CEN/ARS, LEU2, protA, NUP85*	Lab library (Tekotte, H)
pUN100-*SUB2*	*CEN/ARS, LEU2, SUB2*	(Strasser K and Hurt E, 2001)
pUN100-*YRA1*	*CEN/ARS, LEU2, YRA1*	(Strasser K and Hurt E, 2000)
Yep13-*NSP1*	2μ, *LEU2, NSP1*	Lab library (Hurt, E)

The construction of these plasmids was done as described bellow:

Cloning of pHT4467Δ-GLE2

The *GLE2* gene was cut from the plasmid pUN100-Gle2 (Siniossoglou, lab library) with *Sal*I (subsequently blunt ended) and *Sac*I. This fragment was subcloned into the vector ptf28 (provided by T. Fiedler) previously digested with *BamH*I (then blunt ended) and *Sac*I.

Cloning of pHT4467Δ-SUB2

The *SUB2* gene was excised from the plasmid pRS314-SUB2 with *Sac*I and *Xho*I (subsequently blunt ended). This fragment was subcloned into the plasmid pHT4467delta digested with *Xba*I (subsequently blunt ended) and *Sac*I.

Cloning of pHT4467Δ-THO2

The *THO2* gene was PCR amplified using yeast genomic DNA as template and as primers NotI-site containing oligonucleotide and SacI-site containing oligonucleotide that hybridized 334 nucleotides upstream and 323 nucleotides downstream of the ORF, respectively. This fragment was subcloned into the plasmid pHT4467delta digested with *Not*I and *Sac*I.

Cloning of pHT4467Δ-THP2

The *THP2* gene was excised from the plasmid pRS315-THP2 with *Sac*I and *Sma*I. This fragment was subcloned into the plasmid pHT4467delta digested with *Xba*I (subsequently blunt ended) and *Sac*I.

Cloning of pRS315-GLE2

The *GLE2* gene was PCR amplified using the plasmid pUN100-Gle2 (Siniossoglou, lab library) as template and primers that contained a *Sac*I restriction site and *Apa*I restriction site that hybridized 386 nucleotides upstream and 357 nucleotides downstream of the ORF, respectively. This PCR fragment was subcloned into the pRS315 vector creating plasmid pRS315-GLE2.

Cloning of pRS315-*gle2-G226E*

To engineer the G226E mutation in *GLE2*, the gene cassette was amplified by PCR in two halves using the plasmid pUN100-Gle2 (Siniossoglou, lab library) as template. For the first

half, a *Pst*I 5' primer and a 3' primer containing the G226E mutation were used. For the second half, a 5' primer containing the G226E mutation and a 3' *Xba*I primer were used. The two PCR products were mixed and used as template for a second PCR using the primers containing the *PstI* and the *XbaI* restriction sites. This PCR fragment was subcloned *PstI*/*XbaI* into the pRS315-GLE2 vector to yield pRS315-*gle2-G226E*. The presence of the mutation was checked by restriction digest with *HinfI* enzyme.

Cloning of pRS315-MFT1

The *MFT1* gene was PCR amplified using using yeast genomic DNA as template and as primers *Sal*I-site containing oligonucleotide and *BamH*I-site containing oligonucleotide that hybridized about 500 nucleotides upstream and about 500 nucleotides downstream of the ORF, respectively. This PCR fragment was subcloned into the pRS315 vector creating plasmid pRS315-MFT1.

Cloning of pRS315-NPL3-eGFP

The *NPL3* gene was PCR amplified using yeast genomic DNA as template and as primers *Sac*I-site containing oligonucleotide and *BamH*I-site containing oligonucleotide that hybridized 456 nucleotides upstream the ORF and at the very end of the ORF, respectively. This PCR fragment was subcloned into the pRS315 RIX24 eGFP vector, from which RIX24 was excised with *Sac*I and *BamH*I

Cloning of pRS315-THP2

The *THP2* gene was excised from the plasmid pUN100-211 (plasmid from the pUN100-genimic library) with *Nhe*I and *Sac*I. This fragment was subcloned into the plasmid pRS315 digested with *Xba*I and *Sac*I.

Cloning of pRS315-THP2-eGFP

The *THP2* gene was PCR amplified using yeast genomic DNA as template and as primers *Sac*I-site containing oligonucleotide and *BamH*I-site containing oligonucleotide that hybridized 447 nucleotides upstream the ORF and at the very end of the ORF, respectively. This PCR fragment was subcloned into the pRS315 RIX24 eGFP vector, from which RIX24 was excised with *Sac*I and *BamH*I.

The oligonucleotides that were used as primers for the PCR reactions were ordered from Interactiva (Freiburg). Table 7 contains the list of oligos used in the various cloning procedures described above:

Table 7. Oligoes used in this study.

Name	Sequence	Restriction site	Direction
F-SacI-Gle2	TCTGAGCTCATTGAAGTAGCTTGGCA	SacI	5' to 3'
R-ApaI-Gle2	TCTGGGCCCGCACGAAAGGTTCTAAA	ApaI	3' to 5'
F-PstI-Gle2	AAAACTGCAGTCTTTTTTTTAATCGATC	PstI	5' to 3'
R-gle2-G226E	CTTCCACTGATTCAATCGCATATCC		3' to 5'
F-gle2-G226E	ATGCGATTGAATCAGTGGAAGGTAG		5' to 3'
R-Xba-Gle2	TTTTCTAGATGAAATTCGTTCTTCGGATAG	Xba	3' to 5'
F-Not1-Tho2	TCCGCGGCCGCTAGAACGTAACATACC	Not1	5' to 3'
T-SacI-Tho2	TCCGAGCTCGTGGACGAAGTAGTCAC	SacI	3' to 5'
F-SacI-Mft1	TCTGAGCTCGTCAAATTCTCCAGCAC	SacI	5' to 3'
R-BamHI-Mft1	TCCGGATCCTTTTACTTCTTCAACAG	BamHI	3' to 5'
F-SacI-Npl3	TCTGAGCTCATCTACCGCAGTGAGTC	SacI	5' to 3'
R-BamHI-Npl3	CTTGGATCCCCTGGTTGGTGATCTTT	BamHI	3' to 5'
F-SacI-Thp2	TCTGAGCTCAACATCAATAGCTTCTG	SacI	5' to 3'
R-BamHI-Thp2	TCCGGATCCTTCTAAAACTATGTCAC	BamHI	3' to 5'
F2-Sub2	CGATGTCAAAATCGCTGAATTCCCAGAA GAAGGCATTGATCCGTCCACTTATTTGA ATAATCGGATCCCCGGGTTAATTAA		5' to 3'
R1- Sub2	TTCGCTATAACTGCTAATTTTTTTCCACTT CCCCTTTTTTGTTTGTTTTGTTCGTTTGAAT GAATTCGAGCTCGTTTAAAC		3' to 5'

Genetic methods

Media for yeast growth and microbiological techniques

Yeast cells were grown in rich YPD medium (1% yeast extract, 2% bacto-peptone, 2% glucose) or in synthetic medium (2% glucose. 0.67% yeast nitrogen base complemented with

all the necessary amino acids except of the one(s) required for the selection). Final concentrations of the amino acids and preparation of the media were according to Sherman, 1990. For counter selection of cells containing *URA3* plasmids, 5-fluoro-orotic acid (FOA) was used at 1 mg/ml.

Basic yeast microbiological and genetic techniques including crossing of haploid strains, sporulation of diploids, dissection of asci and tetrad analysis was done according to (Sherman F, 1990).

Growth of yeast strains in liquid medium was monitored by light absorption at 600nm (OD_{600}).

Yeast transformation, plasmid selection, complementation test

Transformation of plasmid DNA into *S. cerevisiae* was performed as described in (Ito H *et al.*, 1983). After transformation, yeast cells were plated in dropout plates in order to select for those that have uptaken the plasmid DNA.

Yeast plasmids and genomic preparation

Plasmid recovery from yeast cells was done according to (Robzyk K and Kassir Y, 1992). For genomic DNA preparation, a 5ml stationary yeast culture was harvested by centrifugation, washed once with H_2O and the pellet was resuspended in 0.5 ml of water, 0.2 ml of 2% EDTA, 0.3 ml of a 25:24:1 Phenol/cloroform/isoamyl alcohol mix and about 0.2 ml of 45 mm glass beads. This suspension was vortexed for 3 min and spun at 12000 rpm for 15 min in a Eppendorf minifuge. The aequous phase was precipitated with 1.2 ml 100% ethanol, centrifuged for 30 min at 4°C, and the dried pellet resuspended in 0.4 ml of TE buffer and digested with RNAse (20 μl of a 10 mg/ml stock) for 45 min at 37°C. After digestion, the DNA was again precipitated as before and the final pellet resuspended in 30 μl TE buffer.

Yeast strains

On Table 8 are listed the yeast strains used during the course of my study. This list includes both the strains that have been already described, as well as those that have been generated now.

Table 8. Strains used during the course of this study.

Name	Genotype	Source
RS453 (autodiploid)	*Mat α, ade 2–1 trp 1–1 leu 2–3 his 3–11,15 ura 3–52 can 1–100*	(Wimmer C *et al*., 1992)
Mft1-TAP	*Mat α, ade 2–1 trp 1–1 leu 2–3 his 3–11,15 ura 3–52 can 1–100 MFT1-CBP-TEV-protA::TRP1-KL*	(Strasser K *et al*., 2002)
Y950 (*gle2Δ)*	*Mat a, ura3, trp1, leu2, ade2, his3, gle2::HIS3*	
CH1462	*Mat α, ade2, ade3, his3, leu2, ura3*	(Kranz JE and Holm C, 1990)
gle2Δ, ade2, ade3	*Mat a, ade2, ade3, ura3, trp1, leu2, his3, gle2::HIS3*	This study
sub2Δ +pURA-SUB2	*Mat α, ura3-52, his3Δ200, leu2Δ1, trp1Δ63, sub2::KAN+pYCG-SUB2*	(Strasser K and Hurt E, 2001)
White JB a	Mat a, trp1, ura3, ade2, ade3, leu2 , his3 + pRS316-MTR2	
White JB α	*Mat α, TRP1, ura3, ade2, ade3, leu2, his3*	
sub2Δ, ade2, ade3 +pHT4467Δ-SUB2	*Mat α, ade2, ade3, sub2::kanMX4 +pHT4467Δ-SUB2*	(Strasser K *et al*., 2002)
tho2Δ	*MAT a, ura3-1 leu2-3,112 trp1-1 his3-11,5 ade2-1 can1-100, tho2::KAN1*	(Piruat JI and Aguilera A, 1998)
tho2Δ, ade2, ade3	*Mat a, ade2, ade3, TRP1+, ura3, leu2, his3, tho2::KAN1*	This study
thp2Δ	Mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, thp2::KAN1	(Chavez S *et al*., 2000)
thp2Δ, ade2, ade3	*Mat a, ade2, ade3, trp1, ura3, leu2, his3, thp2::KAN1*	This study
Mft1-TAP/Sub2-GST	*Mat α, ade 2–1 trp 1–1 leu 2–3 his 3–11,15 ura 3–52 can 1–100, MFT1-CBP-TEV-protA::TRP1-KL SUB2- GST::HIS5*	This study
mtr10Δ	*Mat α ade2-1 his3-11,15 ura3-52 leu2-3,112 trp1-1 can1-100 mtr10::HIS3*	(Senger B *et al*., 1998)

The strains created in this study listed in Table were constructed as follow :

gle2Δ, ade2, ade3

The strain was generated by mating CH1462 (Kranz JE and Holm C, 1990) with gle2::HIS3, followed by tetrad analysis.

tho2Δ, ade2, ade3

The strain was generated by mating tho2::KAN1 with the white strain JB a, followed by tetrad analysis.

thp2Δ, ade2, ade3

The strain was generated by mating thp2::KAN1 with the white strain JB alpha, followed by tetrad analysis.

Mft1-TAP/Sub2-GST

The GST cassette was amplified by PCR from plasmid pFA6a-GST-His3MX6 (Longtine MS *et al.*, 1998) using 80 nucleotide long primers that had 5´-ends (60 nt) annealing to the upstream or downstream regions of the *SUB2* ORF and 3´-ends (20 nt) annealing to the marker gene. The products of the PCR reaction was used to transform the haploid strain Mft1-TAP and select for His^+ transformants. The integration of the *GST::HIS5* in the correct loci and generation of *SUB2- GST::HIS5* was confirmed by PCR and by Western blot.

Isolation of synthetic lethal mutants starting with the* gle2Δ, sub2-85, tho2Δ *and* thp2Δ *allele

Mutations that are synthetic lethal in combination with a known mutation can be isolated by a systematic approach that relies on the ability to detect plasmid loss. Following this method, the yeast strain bearing the *gle2Δ* mutation, as well as mutation in the *ADE2* and the *ADE3* (two genes involved in adenine synthesis) is transformed with a plasmid carrying *ADE3* and *GLE2*. The resulting strain forms red colonies because the *ade2* block causes the accumulation of a pathway intermediate, leading to synthesis of a pigment. Under non-selective conditions, a small fraction of cells in a colony lose this plasmid, and these give rise to white sectors, because the *ade2 ade3* strain does not synthesize the pigment. When cells carrying the *ADE3*- and *GLE2*-containing plasmid are mutagenized, chromosomal mutations in genes that are synthetically lethal with *gle2Δ* will prevent plasmid loss and yield non-sectored colonies (Guarente L, 1993). To confirm the sl relationship, the *URA3* gene is also part of the reporter plasmid used. Sl candidates that cannot lose the reporter plasmid also die when plated on 5-FOA-containing medium, since 5-FOA is toxic for cells that express *URA3* (Figure 23).

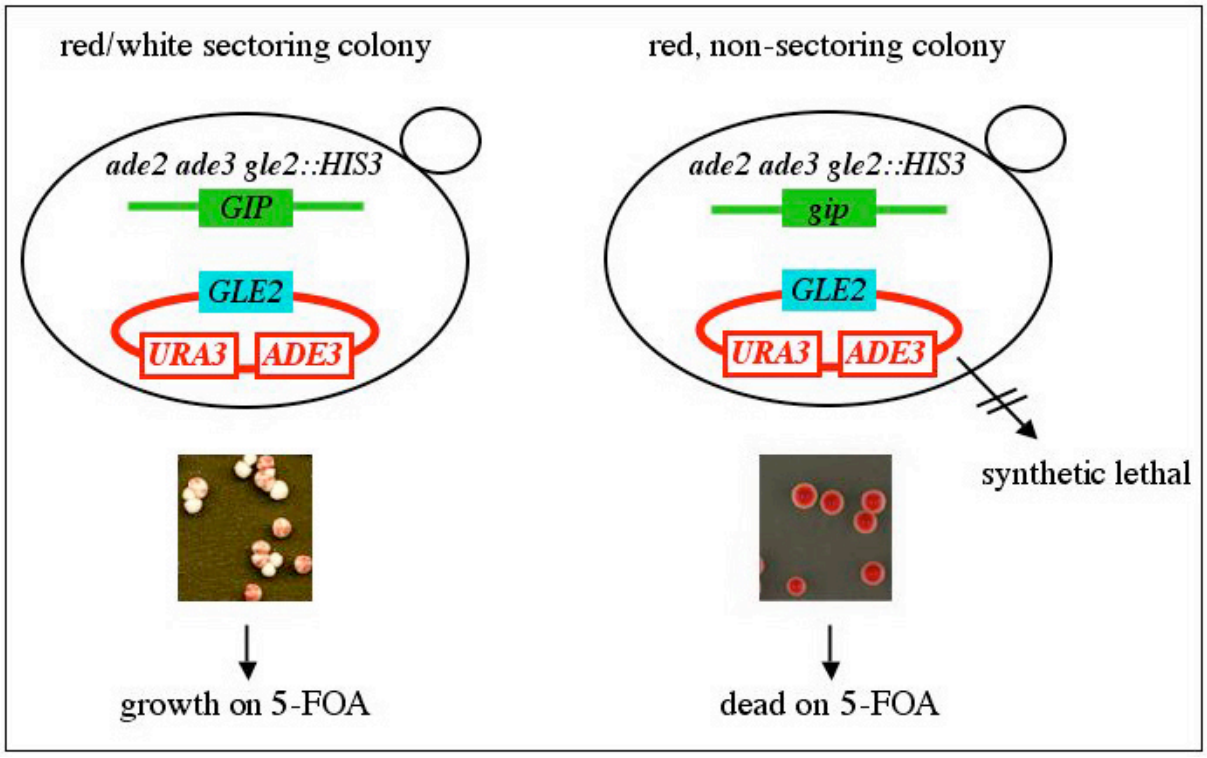

Figure 23. Scheme of a synthetic lethal screen, for instance with *gle2Δ*. The screening strain is white (*ade2*, *ade3*), has a disrupted chromosomal *gle2::HIS3* and contains the red colour indicating plasmid carrying the wt *GLE2* gene. The cells tolerate a mutation in a single gene (*gle2::HIS3*), the colonies show red/white sectoring colour and can grow on 5-FOA. When the first mutation is combined with a mutation in another gene related to it (*GIP* for Gle2 interacting protein), the cells can not lose the red colour indicating plasmid, therefore the colonies show red, non-sectoring colour and are dead on 5-FOA would lead to cell death.

For the synthetic lethal screen with *gle2Δ* null mutant, a screening strain was constructed for the red/white colony sectoring assay (Wimmer C *et al.*, 1992). The *gle2::HIS3, ade2, ade3* has a white color on a YPD plate. This strain was transformed with the plasmid pHT4467Δ-GLE2, and the sectoring of different transformants was followed on YPD plates containing 4% glucose (YPD4%). One candidate (spore 11A) showed optimal sectoring at 23°C and therefore was selected as starting strain for the mutagenesis. This strain was streaked from a SDC-Ura plate to 1 ml of liquid YPD (final concentration: 0.5 D_{600} per ml). From this stock, a 1:100 dilution was made in liquid YPD, and from this dilution, aliquots of 100 µl were plated on YPD 4% plates. After 20 minutes each of these plates was subjected to UV light (254 nm) for 30 sec. Plates were then incubated at 23°C in dark, and then screened for non sectoring colonies. Out of 10^5 colonies screened, 20 mutants were not growing on 5-FOA containing plates, and were transformed with a pRS315-GLE2 plasmid to test for re-sectoring and

growth on 5-FOA plates. The 20 mutants were initially transformed with a mini-library composed of nucleoporins containing plasmids: Nup120, Nup2, Nup133, Nup145, Nup170, Nup188, Nup192, Nup49, Nup57, Nup53, Nup59, Nup82, Nup84, Nup85, Kap95, Nsp1, Nic96, Mtr10, Nup159, Nup1, Nup100, Nup116, Mtr2, Mex67. Of the 20 sl mutants, 18 were complemented by the mini-library. The remaining 2 candidates, sl360 and sl660 were transformed with a yeast genomic library inserted into pUN100–LEU2 as described earlier (Grandi P *et al.*, 1995b), and the complementing plasmid was recovered from transformants which regained both a red/white colony sectoring phenotype and growth on 5-FOA.

For the synthetic lethal screen performed with the *sub2-85* allele, the screening strain *sub2::KAN, ade2, ade3* containing the plasmid pRS314-*sub2-85*, was transformed with the plasmid pHT4467Δ-*SUB2*. The screen was then performed with the same procedure described for *gle2Δ*, except that the cells were plated on SDC-Trp with 4% glucose for the UV irradiation.

For the synthetic lethal screen performed with *tho2Δ* and *thp2Δ* alleles, the screening strain *tho2::KAN, ade2, ade3* (constructed as described in section 5.2.4) was transformed with the plasmid pHT4467Δ-THO2, and *thp2::KAN, ade2, ade3* (constructed as described in section 5.2.4) was transformed with the plasmid pHT4467Δ-*THP2*. The screen was then performed with the same procedure described for *gle2Δ*, except that the cells were grown at 30°C. From the *thp2Δ* screen, sl 56 was transformed with a yeast genomic library inserted into pUN100–*LEU2* as described earlier (Grandi P *et al.*, 1995b), and the complementing plasmid was recovered from transformants which regained both a red/white colony sectoring phenotype and growth on 5-FOA.

Biochemical methods

Whole yeast protein extract

50 ml of yeast culture were grown overnight on YPD or on synthetic medium when plasmid selection was required, at the appropriate incubation temperature. 10 OD600 of the yeast culture were harvested by centrifugation at 3000 rpm for 3 min. The pellet was resuspended in approximately 1 ml of H_2O and transferred into microfuge tubes. Cells were spun down for 30 sec, and supernatant was poured off. 200 μl of 2x sample buffer and almost an equal amount of glass beads were added to the pellet which was incubated for two rounds at 95°C. Each

round of incubation was followed by 1 min of vortexing. The glass beads and the cell debris were removed by short centrifugation and the supernatant was transferred to new tubes. In each case, 5 μl were loaded on SDS page gel and western blot analysis followed.
The 4 x sample buffer contained 250 mM tris-Hcl pH 8, 9.2 % SDS, 40 % glycerol, 0.2 % (w/v) Bromophenol blue, 100 mM DTT.

TAP purification

Affinity purification of TAP tagged Mft1 was performed using 2 to 8 lt of yeast culture. Yeast cell extracts from 2 lt culture were first incubated with 500 μl dry IgG Sepharose bead suspension (IgG Sepharose™ 6 Fast Flow, Amersham Biosciences) for 1h at 4˚C. The beads were previously washed three times with NB buffer (150 mM NaCl, 50 mM Kac, 20 mM Tris pH 7.5, 2 mM MgAc, 0.01% NP-40) without DTT and protease inhibitors. Next, the beads were collected in a 0.8 ml Mobicol™ minispin column (MoBiTec, Germany) and washed once with 10 ml NB buffer. The bottom of the column was closed and the IgG agarose beads were resuspended in 150 μl TEV cleavage buffer (NB buffer with 0.5 mM DTT) and 10 μl TEV enzyme (equals approximately to 100 units of Gibco TEV) was then added. The top of the column was closed and the columns were transferred into a shaker for 1 hour at 16˚C.

The top and bottom plugs of the column were removed and the TEV eluate was eluted with a syringe. The eluate was transferred into the column containing 600 μl dry Calmodulin beads (Calmodulin Affinity Resin, Stratagene) in 200 μl CBP binding buffer (NB buffer with 4 mM $CaCl_2$ and 1 mM DTT). The Calmodulin beads were previously washed three times with CBP wash buffer (NB buffer with 2 mM $CaCl_2$ and 1 mM DTT). The column was closed and left to rotate for 1 hour at 4˚C. After binding the column was allowed to drain by gravity flow and was subsequently washed once with CBP wash buffer. The calmodulin eluate was eluted with 600 μl elution buffer (NB buffer with 5mM EGTA) for 5 min at 37˚C. The liquid was saved with help of a syringe in a siliconized Eppi-tube. The sample obtained by this method was loaded on SDS-PAGE later stained with Coomassie, or concentrated by ultrafiltration (Microcon YM-50, Millipore) and injected on gel filtration column.

GST-fusion protein purification

Purification of Mft1-TAP/Sub2-GST was performed using 8 lt of yeast culture. Yeast cell extracts from 2 lt culture were first incubated with IgG agarose bead suspension as described for the TAP purification. The TEV eluate was transferred into the column containing 300 μl dry GSH beads (Glutathione Sepharose^{TM} 4B, Amersham Biosciences) in 200 μl NB buffer. The GSH beads were previously washed with 10 ml NB buffer. The column was closed and left to rotate for 1 hour at 4˚C. After binding the column was allowed to drain by gravity flow and was subsequently washed once with 10 ml NB buffer. The GSH eluate was eluted twice with 300 μl elution buffer (NB buffer with 10mM GSH) for 15 min at RT. The liquid was saved with help of a syringe in a siliconized Eppi-tube. The sample obtained by this method was loaded on SDS-PAGE later stained with Coomassie, or concentrated by ultrafiltration (Microcon YM-50, Millipore) and used for EM analysis.

Gel filtration chromatography

The native protein complex obtained as described in the previous section was concentrated by filtration and then injected in a Superose 6 gel filtration column connected to a FPLC system (Pharmacia). The flow rate was 0.050 ml/min and 50µl fractions were collected. Each fraction was TCA precipitated and resuspended in 20µl. 15µl were analysed by SDS-PAGE and stained with Coomassie Blue, and 5µl were analysed by SDS-PAGE and western blot.

Immunological methods

Western blot

Transfer of proteins from SDS-PAGE to nitrocellulose

Transfer of proteins from SDS-PAGE to nitrocellulose membrane (Western Blot) was performed as described by (Sambrook J and Russell DW, 2001) using the Semi-Dry blot system. In any case proteins were transferred for 45 min at 10V. Upon blotting, membranes were stained with Ponceau Red to visualize molecular weight markers and to assess the

quality of the transfer.

Immunological detection of proteins immobilized on nitrocellulose filters

Immunological detection of proteins immobilized on nitrocellulose filters was performed based on (Sambrook J and Russell DW, 2001). Ponceau Red-stained nitrocellulose membranes were briefly incubated in blocking buffer to remove the dye. Fresh blocking buffer was added and the membranes were incubated for approximately 1 hr.

Incubation with the anti-ProtA ab (PAP, diluted in fresh blocking buffer 1:1000) was done at RT for 1 hr with gentle rocking, followed by a extensive washing with fresh blocking buffer 3 times for 10 min each. Since PAP antibody is already conjugated with the horseradish peroxidase, there is no need of incubating with the secondary antibody. The nitrocellulose membrane was further washed with PBST for 3 times for 10 min each. ECL reaction was performed directly after washing, using the ECL detection kit (Amersham Bioscience). Signals were detected using Kodak X-OMAT AR films.

Incubation with the anti-Yra1 or anti-Sub2 ab (diluted in fresh blocking buffer 1:2000) was done at 4°C, over night. The membrane was washed with blocking buffer 3 times for 10 min each at RT and it was then incubated for 1 hr with peroxidase-conjugated secondary antibody dissolved in fresh blocking buffer (1:1000) at RT. Finally, the membrane was washed 3 times for 10 min each with blocking buffer and 3 times for 10 min each with PBST at RT.

ECL reaction was performed directly after washing, using the ECL detection kit (Amersham Bioscience). Signals were detected using Kodak X-OMAT AR films. The blocking buffer consisted of 5 % non-fat dry milk in 1 x PBST (PBS with 0.1 % Tween 20).

Table 9. Antibodies used during this study.

Primary antibody	Type	Source/Reference	Dilution	Secondary antobody	Dilution
ProtA (PAP)	Rabbit-Horseradish Peroxidase conjugated	DAKO	1:1000		
Sub2	rabbit (serum) raised against HIS6-Sub2	(Strasser K *et al.*, 2002)	1:2000	Goat anti-rabbit	1:3000
Yra1	rabbit (serum) raised against GST-Yra1	(Strasser K and Hurt E, 2001)	1:2000	Goat anti-rabbit	1:3000
Goat anti-rabbit	Horseradish Peroxidase conjugated affinity purified	BIO RAD	1:3000		

Other methods

Fluorescence microscopy

The localization of the GFP-tagged proteins in living yeast cells was examined in the fluorescein channel of a Zeiss Axioskop fluorescence microscope. Pictures were obtained with a Xillix Microimager CCD camera and processed with Improvison Openlab 1.7 and Adobe-Photoshop

Electron Microscopy (EM)

Carbon coated 400 mesh copper grids were glow discharged in air for 1 min and used within one hour for sample preparation. The protein was applied to the grids. After about 30 s incubation the sample was removed by blotting the edge of the grid with filter paper (Whatman No 5). Then the grid was washed once or twice with water and three times with 2% uranyl acetate or 2% uranyl formate by applying a drop of solution to the grid and then removing it with filter paper. The last drop was incubated for more than 2 minutes to improve the staining. Occasionally, TMV was added to improve the staining. In this case, first 2 µl of

TMV solution (0.1 mg/ml) were applied to the grid. Then the TMV-solution was removed with filter paper and a drop of complex-solution was applied. Washing and staining were done as before.

Data were collected on a Philips CM120 Biotwin electron microscope operating at 100 kV and equipped with a LaB_6-filament. For processing we chose areas of the grid, where the proteic particles were embedded deeply in the stain. The images were taken with a defocus of 500-700 nm under low dose conditions. Micrographs were recorded on Kodak SO-163 film, which was developed for 10 min in Kodak D-19 developer at room temperature. The magnification of the microscope was calibrated with catalase (Wrigley NG, 1968) and TMV and was 50.000.

Image Processing

Electron micrographs were scanned with 21 μm/pixel on a Zeiss SCAI scanner corresponding to a pixel size of 0.42 nm at the specimen level. Particle images of the TREX-complex were selected and boxed off from the micrographs using the MRC software package (Crowther RA *et al*., 1996). All subsequent image analysis steps were performed within the IMAGIC 5 software package (van Heel M *et al*., 1996). In brief: After band-pass filtering, the images of the particles were normalized in their grey value distribution, mass-centred and then classified according to their similarity. Well-defined class averages were aligned in respect to each other and used as a set of new references in a multi-reference alignment followed by classification. The whole process was repeated several times until class-averages did not change significantly.

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