EBERHARD KARLS
UNIVERSITÄT
TÜBINGEN

Interaction studies of proteins involved in mRNA export

Diplomarbeit

Dem Diplomsprüfungsausschuss für Biochemie
der Mathematisch-Naturwissenschaftlichen Fakultät der
Eberhard-Karls-Universität Tübingen

vorgelegt von

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am 14. Januar 2013

Die Diplomarbeit wurde unter der Leitung von Prof. Elena Conti im Department für zelluläre Struktubiologie am Max Planck Institut für Biochemie in Martinsried im Zeitraum vom 14. Mai 2012 bis 14. Januar 2013 durchgeführt.

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Martinsried, den 11. Januar 2013

Abstract

In yeast, recognition of mRNA for export from the nucleus occurs via the formation of Ribonucleoprotein (mRNP) particles. These mRNPs are exported by Mex67 that is recruited to the mRNPs by the adaptor proteins Yra1 and Sub2. These adaptor proteins are part of the Transcription Export (TREX) complex, an important assembly machinery in the biogenesis of mature mRNPs, and furthermore responsible for the separation of DNA and RNA strand during transcription.

My thesis focused on the study of macromolecular interactions in the mRNA export pathway. Several proteins were cloned, expressed, purified and tested in pull down assays and size exclusion chromatography.

A first project investigated the complex formation of ctYra1, ctSub2 and ctMex67, from *Chaetomium thermophilum*. As Yra1 is highly unstructured, it displays low stability, protease sensitivity and tight binding of nucleotides, disrupting the interaction with binding partners. Homologues of Yra1, Sub2 and Mex67 from a thermophilic organism were hoped to be more stable, facilitating purification. Therefore, ctYra1, ctSub2 and ctMex67 were cloned, expressed and purified to try complex formation. However, pull down experiments, gel filtration and ITC measurements indicate transient complex formation, not suitable to proceed towards crystallization.

The second project of my thesis aimed to find binding partners of the yeast TREX complex. Putative interaction partners were chosen based on literature search and computational analysis. A first candidate was Syf1, a part of the transcription elongation complex Prp19. In addition three yeast homologues of human proteins known to interact with the TREX-complex were chosen: a subunit of the cab binding complex (Sto1), a suppressor of the Transcriptional defect of Hpr1 by Overexpression (Tho1), and a protein reported to couple the histone-chaperone Spt6 with mRNA export (Iws1). Interaction studies with subunits of the TREX complex were performed *in vitro* with purified proteins, but showed no detectable binding under the conditions tested. This is possibly due to species-specific differences or indirect interactions mediated through unknown bridging factors.

As next step, purification of components of the human TREX-complex was started. Therefore, the third project dealt with the expression, purification and reconstitution of the human-specific subcomplex of Thoc5-Thoc7. As His-Thoc7 formed a soluble aggregate, complex formation failed using the separately purified proteins. Co-expression, co-lysis, as well as expression with alternative tags showed no significant improvement in stabilizing the monomer. These studies form a stable ground for further experiments, such as expression or purification with other interaction partners within the TREX-complex.

Although the studies presented in this thesis did not directly lead to a structure, they represent an important step in this direction. They deal with the purification of typical eukaryotic proteins exhibiting unstructured domains and flexible regions that are prone to aggregate.

Zusammenfassung

In Hefe erfolgt die Erkennung von mRNAs zum Export aus dem Zellkern durch Verpackung in Ribonucleoprotein-Komplexe (mRNP), welche mithilfe von Mex67 durch die Kernpore transportiert werden. Mex67 wird mittels der Adaptorproteine Yra1 und Sub2 zu den mRNP-Komplexen rekrutiert. Diese Adaptorproteine sind Teil des Transkriptions und Export Komplexes (TREX), welcher einen wichtigen Bestandteil in der Biogenese reifer mRNP-Komplexe darstellt und für die Trennung des RNA-Strangs vom DNA-Strang nach der Transkription verantwortlich ist.

Diese Arbeit beschäftigte sich mit der Untersuchung makromolarer Interaktionen im Export von mRNA. Verschiedene Proteine wurden geklont, exprimiert, aufgereinigt und mittels Pull-Down Experimenten und Gelfiltration auf Interaktionen untersucht.

Das erste Projekt beschäftigte sich mit der Komplexbildung der Adaptorproteine ctYra1, ctSub2 und ctMex67 aus *Chaetomium thermophilum*. Große unstrukturierte Bereiche in Yra1 führen zu Instabilität, Protease-Sensitivität und starke Bindung an Nukleotide, welche eine Interaktion mit Bindungspartnern verhindern. Homologe von Yra1, Sub2 und Mex67 aus einem thermophilen Organismus wurden in dieser Studie verwendet, in der Hoffnung, dass diese eine verbesserte Stabilität aufweisen, was die Aufreinigung erleichtern sollte. ctYra1, ctSub2 und ctMex67 wurden nach Klonierung, Expression und Aufreinung für Interaktionsstudien mittels Pull-Down Experimenten, Gelfiltration und ITC-Messungen verwendet. Diese zeigten jedoch eine transiente Komplexbildung auf, welche für Kristallisationsversuche nicht geeignet ist.

Das zweite Projekt umfasste die Suche nach Bindungspartnern des TREX-Komplexes aus Hefe. Literaturrecherche und Bioinformatische Analysen ergaben ein Protein des Transkriptions-Elongations-Komplexes (Syf1) als mögliche Interaktionspartner aus Hefe, sowie drei weiter Proteine deren Interaktion im Menschen bekannt ist, und welche homologe Proteine in Hefe besitzen: eine Untereinheit des 5´-Cap bindenden Komplexes (CBC) (Sto1), ein Protein, was durch Überexpression Transkriptions-defekte in Hpr1-Mutanten kompensiert (Tho1), und ein Protein was für die Verbindung des Histon-Chaperones Spt6 mit dem Export von mRNAs verantwortlich ist (Iws1). *In-vitro* Bindungsstudien von Untereinheiten des TREX-Komplexes mit den aufgereinigten Proteinen zeigten keine erkennbare Interaktion unter den getesteten Bedingungen. Ursachen hierfür können Unterschiede in den Spezies, sowie indirekte Interaktionen mittels unbekannter Adaptorproteinen sein.

Als nächsten Schritt wurde die Aufreinigung verschiedener Untereinheiten des menschlichen TREX-Komplexes versucht. Das Ziel des dritten Projektes war die Expression, Aufreinigung und Rekonstruktion des Sub-Komplexes aus Thoc5 und Thoc7, welcher spezifisch für den humanen TREX-Komplex ist. Durch die Bildung eines löslichen Aggregates von aufgereinigtem Thoc7 schlug eine Komplexbildung der einzeln aufgereinigten Komponenten jedoch fehl. Co-expression, Co-Lyse so wie die Verwendung alternativer Aufreinigungsstrategien zeigte keine signifikante Verbesserung der Ausbeute. Dennoch bilden diese Arbeiten eine solide Grundlage für weitere viel versprechende Experimente, wie die Expression und Aufreinigung mit weiteren Interaktionspartnern aus dem TREX-Komplex.

Auch wenn diese Studien nicht direkt zur Lösung der Struktur führen, so stellen sie doch einen wichtigen Schritt in diese Richtung dar. Proteinexpression und – reinigung sind wichtige Grundlagen in der Strukturbiologie, und dies Arbeit veranschaulicht Probleme in der Handhabung typischer eukaryotischer Proteine mit großteilig unstrukturierten Abschnitten und flexiblen Regionen welche zu Aggregation neigen.

Acknowledgements

It is of great importance to me, to express my sincere thanks to the people who helped me through my diploma thesis:

First and foremost, I want to thank Prof. Elena Conti for giving me the opportunity to prepare my thesis in her great lab under her friendly supervision. Thank you for allowing me to become part of your amazing lab.

Additional thanks goes to Prof. Thilo Stehle for agreeing to be the internal supervisor without hesitation.

I especially thank Rajan Prabu for all the guidance and supervision. I am really grateful for all the friendliness and patience he has shown me, and all the time he had spent on explanations and motivation. Thank you for all you have taught me and good luck for the future.

Furthermore, I want to thank Claire Basquin for ITC and Thermoflour measurements and evaluations, as well as Peter Reichelt, for never being tired of answering any stupid question on ÄKTA-handling.

A sincere thank deserve the ladies from the "chat-room" to share their office with me and for all the food and friendly non-scientific talk during incubation-times. I really appreciated especially Debora for listening to all my scientific and non-scientific problems.

Michaela Rode furthermore deserves my gratitude for always being there when needed. Thank you for believing in me and making me want to come back to this lab for my diploma thesis.

I want to thank all the "reviewers" for spending their time on reading my thesis and helping me to improve it.

I finally want to thank the entire group for integrating me, sharing their knowledge, helping me and smiling at me every day.

Table of Contents

Abbreviations

AMP-PNP	Adenosine 5′-(β,γ-imido)triphosphate lithium salt hydrate
APS	ammonuim persulfate
BSA	bovine serum albumin
CBC	cap binding complex
ChIP	chromatin Immunoprecipitation
ct	Chaetomium thermophilum
DTT	Dithiothreitol
EJC	Exon-Junction-Complex
GSH	Glutathione
GST	Glutathione-S-Transferase
His-tag	hexa histidin-tage
HL200	High load Superdex 200 gel filtration column
HL75	High load Superdex 75 gel filtration column
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITC	Isothermal titration calorimetry
K_D	Dissociation-constant
LB	Lysogeny broth
LIC	ligase-independent cloning
LRR	leucine-rich-repeat region
mRNP	ribonucleo-protein particles
NES	nuclear export sequence
NLS	nuclear localization signal
NP-40	nonyl phenoxypolyethoxylethanol
NPC	nuclear pore complex
NXF	nuclear export factor
OD_{600}	optical density at 600nm
PCR	polymerase chain reaction
PMSF	phenylmethanesulfonylfluoride
revNi	Ni-affinity to obtain protease treated protein
RNP1 + 2	consensus sequences of the RRM
RRM	RNA recognition motif
S200	Superdex200 gel filtration column
S6	Superose6 gel filtration column
S75	Superdex75 gel filtration column
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	Tetramethylethylenediamine
TEV	Tobacco Etch virus protease
THO	suppressors of the transcriptional defects of hpr-1delta by overexpression
TREX	Transcription Export-complex
Trx	Thioredoxin
UBA	Ubiquitin-associated domain

1. Introduction

1.1. Transport across the nuclear membrane

The most important characteristic feature of eukaryotes is their nucleus and their capability for compartmentalization. This allows eukaryotes to strictly separate and regulate different processes independently, for example, replication and transcription in the nucleus and translation in the cytoplasm. Furthermore, it enables the cell to process RNAs in the nucleus in various ways. This processing has the advantage of higher stability and advanced surveillance of proper RNA synthesis. However, the functionality of the cell depends on shuttling mechanisms between the compartments for substrates, as well as for required machineries (Fig.: 1). One example for these shuttling systems is the transport of proteins across the nuclear membrane. To accommodate this, a complex system has evolved using shuttle proteins, called importins/karyopherins (Cook et al., 2007). These proteins recognize a nuclear localization signal (NLS) on the protein to be imported and are dependent on the small G-proteins RanGTP and RanGDP. For nuclear import, the karyopherins transport their cargo through the nuclear pore complex (NPC) following the RanGTP gradient and release the cargo upon GTP hydrolysis in the nucleus. A comparable system exists for the export of proteins from the nucleus to the cytoplasm, dependent on RanGDP (Cook and Conti, 2010).

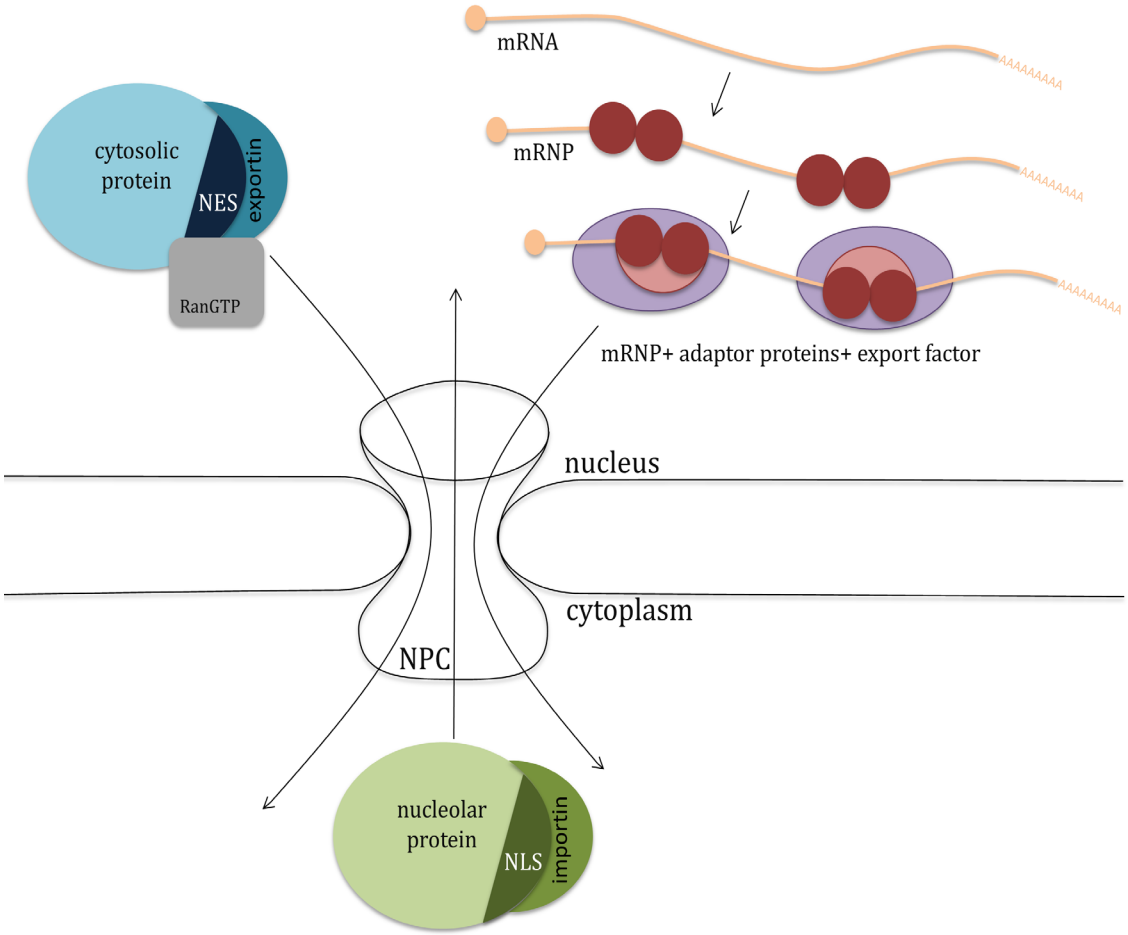


Fig.1: Schematic representation of nuclear export

As a second family of molecules dependent on transport across the nuclear membrane, RNA requires a different export system. Different types of RNA, like mRNA, tRNA, rRNA and miRNA, are transcribed in the nucleus and they have to be transported to the cytoplasm to fulfil their function. However, the different RNA families do not possess a common feature comparable to a NLS that could generalize transport of these molecules. Therefore, various possibilities are used for the different RNA families. For example, export of several small RNAs, like tRNA and miRNA, relies on a transport system comparable to protein translocation across the nuclear membrane. The processed RNA molecules are bound by a protein of the exportin family and transported through the NPC in a RanGTP-dependent manner (Cook and Conti, 2010). Recognition of the cargo is mediated through specific secondary structure elements in the RNA molecule. However, the subfamily of mRNAs varies in structure as well as in maturation. Protein-coding mRNA is processed by splicing, polyadenylation and capping, whereas, for example, Histone-mRNAs display a hairpin-structure at their 3' end, and are neither polyadenylated nor spliced (Marzluff et al., 2008). Furthermore, no sequence similarities are common among mRNAs. Therefore, export of members of this family relies on assembly of the mRNA into ribonucleo-protein (mRNP) particles. These mRNPs are furthermore bound by bridging molecules, responsible for the interaction of the RNPs with the export receptor. These receptors are general and conserved molecules, that function independently of RanGTP. This role is fulfilled by Mex67-Mtr2 in yeast, and its metazoan homologue TAP-p15. Yra1 / ALY/REF and Sub2 / Uap56 are the adaptor proteins in yeast and metazoan, respectively (Stewart, 2010). Furthermore, eukaryotes evolved a well-organized system of quality control in mRNA metabolism. One example for this is linking transcription with mRNA maturation, where the C-terminal domain of RNA polymerase II directly couples the synthesis of mRNA with capping, splicing and polyadenylation. (Hsin and Manley, 2012) This system allows the cell to stop processing of aberrant transcripts early, and hence inhibits synthesis of mutant proteins. A further checkpoint in mRNA processing is its export from the nucleus. This is coupled to mRNA processing via the Transcription Export

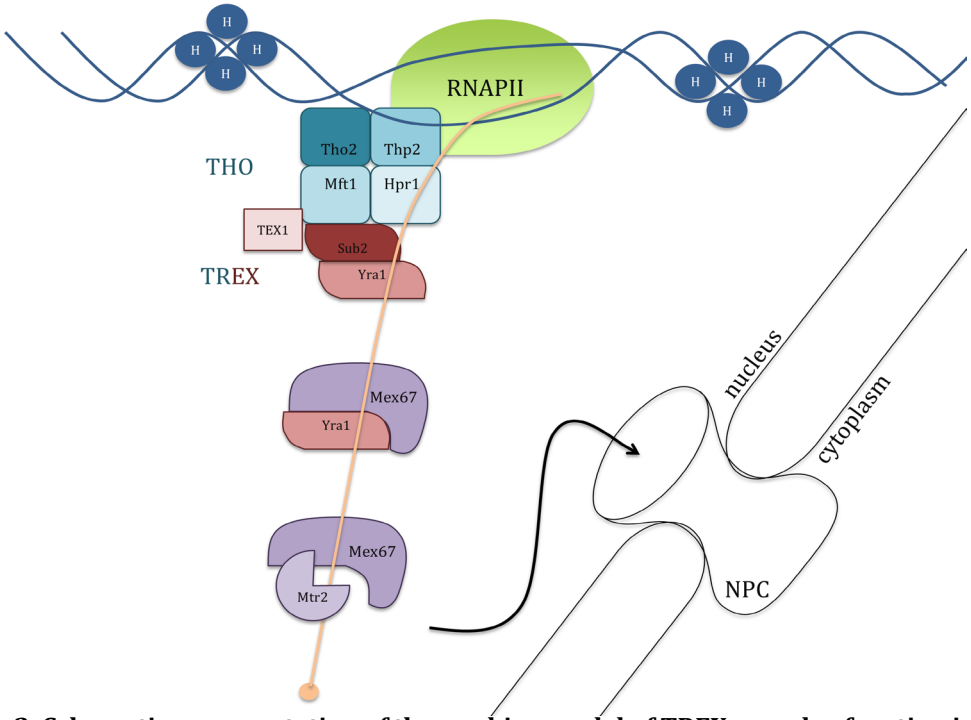


Fig.2: Schematic representation of the working model of TREX-complex function in yeast
based on (Reed and Cheng, 2005) and (Valkov et al., 2012)

(TREX) complex (Strasser et al., 2002). The TREX complex is a conserved complex from yeast to human, consisting of the THO complex (suppressors of the transcriptional defects of hpr1delta by overexpression), a complex reported to be involved in transcription elongation and mRNP biogenesis (Chavez et al., 2000), and of the adaptor proteins linking the mRNP to the export receptor. This allows the TREX complex to link mRNA synthesis and processing to export of mRNPs through the nuclear pore.

1.2. The adaptor and export proteins in yeast

mRNA export via the TREX complex is initiated by the THO complex binding the mRNA. This active THO complex recruits Sub2 to the mRNP and mediates its interaction with Yra1. mRNA bound Yra1 interacts with Mex67, leading to a displacement of Sub2 from the mRNA, as interaction occurs at the same interface of Yra1(Valkov et al., 2012). It has been reported that interaction of TAP, the human homologue of Mex67, with mRNA inhibits binding to Yra1 (Hautbergue et al., 2008). This consequently leads to the displacement of Yra1 from the mRNA and its retention in the nucleus, whereas Mex67 functions as export receptor for the mRNA. (Fig.: 2)

1.2.1. Yra1

Before the discovery as a bridging factor for Mex67 binding to mRNA (Strasser and Hurt, 2000), Yra1 was known as a protein inducing cell cycle arrest in G1-Phase upon overexpression (Espinet et al., 1995), and characterized as a protein with strong single stranded RNA annealing activity (Portman et al., 1997). This RNA binding is based on the central domain of Yra1 containing a RNA recognition motif (RRM). RRM domains are distributed in all kingdoms of life, and function as tight binding of RNA or DNA. Structures of isolated RRMs in complex with their binding partner revealed high specificity for one type of nucleotides. This is achieved through 4 different residue-nucleotide interactions: two aromatic residues allow base pair-stacking with two nucleotides, one hydrophobic interaction of an aromatic residue with the sugar ring and one salt bridge between the phosphate backbone and a positively charged residue. Due to high conservation in the binding motif, these interactions are considered as general binding interface. These similarities can be seen in the two different consensus sequences found in RRMs: RNP1 with the sequence K/R - G - F/Y - G/A - F/Y - V/I/L – X - F/Y and RNP2 displaying the aminoacid chain I/V/L - F/Y - I/V/L – X – N – L . Further regions in the RRM are used for generating higher affinity to the binding partner (Maris et al., 2005). Despite the high specificity for the nucleotide type, no sequence specificity is reported.
The RRM domain of Yra1 is furthermore flanked by a N- and a C-terminal domain, predicted as mostly unstructured.
The essential role of Yra1 in nuclear export of mRNA has been discovered by synthetic lethality assays of Mex67, and further proven, as depletion or temperature sensitive mutation in Yra1 lead to a retention of polyadenylated mRNA in the nucleus (MacKellar and Greenleaf, 2011). Physical interaction with Mex67 has been shown by in-vitro complex formation in the absence and presence of RNA (Strasser and Hurt, 2000). It has been shown, that the N- and C-terminal domains of Yra1 are responsible for this interaction. Furthermore, the C- and N-terminal domains of Yra1 interact with Sub2 bound by the THO complex, leading to the formation of the TREX complex. These interactions with the THO complex and the nuclear export factor Mex67 at the same interface display the adaptor protein role of Yra1 and its function to hand over the

mRNP from the THO complex to the export factor (Strasser and Hurt, 2001).
Direct coupling of transcription to export to turnover can be seen in the interaction partners of Yra1, as binding to C-terminal domain of the Polymerase has been detected, as well as to subunits of the exosomal complex (Zenklusen et al., 2002).

1.2.2. Sub2

Sub2 belongs to the DEAD box family of RNA helicases. It is an essential pre-mRNA splicing factor critical in spliceosome assembly, as it is required for stable binding of U2 snRNP to the pre-mRNA branchpoint (Zhang and Green, 2001). Sub2 associates with the THO complex during transcription and mediated its interaction with Yra1.

1.2.3. Mex67

Mex67 is a protein of the nuclear export factor (NXF)-family and contains a RNA recognition motif, a leucine-rich repeat region at the N-terminal half, as well as a NTF2-like domain and a Ubiquitin associated domain on the C-terminal half (Fig.:3).

Fig.3: Domain architecture of Mex67

based on (Valkov et al., 2012)

Responsible for its shuttling function through the nucleus is its NTF2-like domain, closely related to the transport factor NTF2. This domain allows hetero-dimerization with Mtr2, which, together with the UBA-domain, is required for efficient interaction with the Phe-Gly-repeats.
Mex67 only possesses weak and unspecific interaction with mRNPs, requiring further adaptor proteins to enhance its binding affinity. One of these adaptor proteins is Yra1. As Yra1 is only abundant on a subset of all genomic mRNAs, further recruitment pathways exist. For example, the UBA-domain of Mex67 allows interaction of Mex67 with ubiquitinated Hpr1, and hence direct recruitment of Mex67 to the mRNP via the THO complex
Release of the Mex67:Mtr2 dimer from the mRNP after transport to the cytosol is achieved by the DEAD-box helicase Dbp5. Dbp5 is located at the cytoplasmic site of the NPC and hydrolyses ATP to remove the Mex67 Mtr2-heterodimer from the mRNA. This allows the export-factors to translocate back to the nucleus, whereas the mRNA is kept in the cytoplasm (Valkov et al., 2012).

1.3. TREX complex in yeast

In yeast the TREX complex is composed of the four proteins forming the THO complex (Hpr1, Thp2, Mft2, Tho2), Sub2 and Yra1, as well as Tex1. This leads to the current model, of the THO complex traveling along the template DNA with the polymerase, separating the DNA and the RNA strand. This separation is reported to be important for mRNA processing, as well as DNA

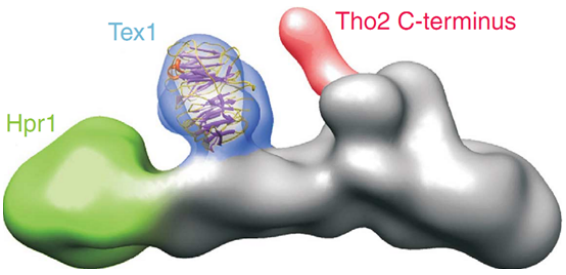


Fig.4: EM reconstruction of the THO complex
from (Pena et al., 2012)

stability, as depletion of THO complex components leads to accumulation of mRNA in the nucleus and to enhanced transcription-coupled hyper-recombination and defective transcription elongation due to R-loop formation (Katahira and Yoneda, 2009). The THO complex recruits Sub2 and Yra1 to the nascent mRNA strand in a stepwise manner to form the TREX complex. Previous structural studies with electron microscopy revealed the overall architecture of the THO complex, displayed in Fig.:4 (Pena et al., 2012). In this publication it is furthermore shown, that the C-terminal domain of Tho2 is responsible for nucleotide interaction, and that truncation of Tho2 interferes with gene expression.

1.4. TREX complex in humans

Generally, in eukaryotes, mRNA is further processed during and after replication, by capping, polyadenylation and splicing. However, intron-containing genes are less abundant in yeast than in metazoans. To ensure that only properly processed mRNA enters the cytosol, the export machinery of metazoans is consequently recruited in a splicing dependent manner (Masuda et al., 2005). Further differences between yeast and metazoan mRNA export can also be seen in the general assembly of the TREX complex. Whereas homologues of Hpr1 and Tho2 from yeast have been found in human and drosophila (Thoc1 and Thoc2), as well as of Yra1 and Sub2 (Aly/REF and Uap56), no homologues of Mft1 and Thp2 in humans or drosophila are known. The metazoan TREX complex, on the other side, consists of three proteins Thoc5, 6 and 7, not found in yeast, but conserved from drosophila to human. (Fig.5)

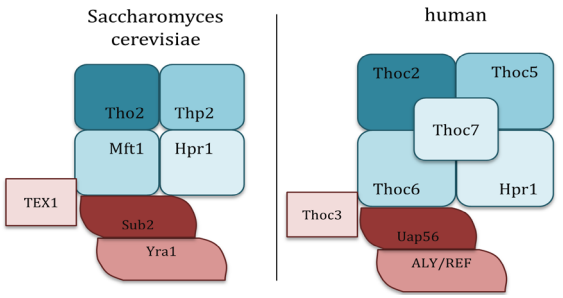


Fig.5: Schematic drawings of yeast and human TREX complex

1.5. TREX complex interacting proteins

1.5.1. Sto1

In humans, TREX complex was shown to mediate export in a splicing dependent manner, rather than co-transcriptionally, as in yeast. This is explained, by the high abundance of spliced mRNA, whereas most of yeast mRNA is intronless. However, in humans also unspliced mRNA exists, requesting an export pathway independent of the exon-junction complex (EJC), i.e. splicing. One transport pathway has been shown to function via an interaction of the export receptor with the

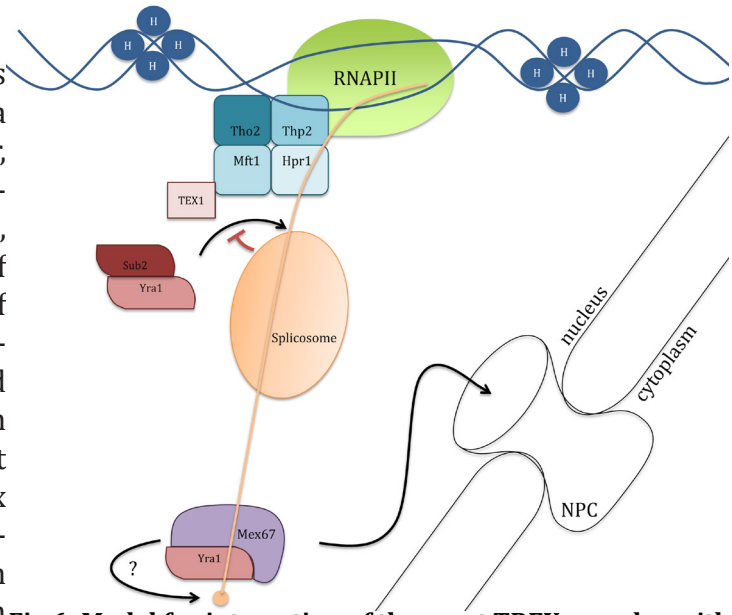


Fig.6: Model for interaction of the yeast TREX complex with the CBP

based on (Abruzzi et al., 2004)

cap-binding complex (CBC) (Fig.6) (Katahira, 2012), (Nojima et al., 2007). As export of yeast mRNA is independent of splicing, an interaction between Sto1, a subunit of the yeast CBC (Katahira, 2012), and the TREX complex would be possible, and would show further homologies between mRNA export in yeast and human.

1.5.2. Syf1

As an additional putative interaction partner, Syf1 was reported (Katahira, 2012).This protein is part of the Prp19 complex, a transcription elongation factor. Furthermore, depletion of Syf1 in yeast reduces the occupancy of TREX complex. Synthetic lethality and ChIP analysis showed a requirement of Syf1 for the occupancy of the TREX complex on mRNA in yeast (Chanarat et al., 2011). The current recruitment model is displayed in Fig.:7 .

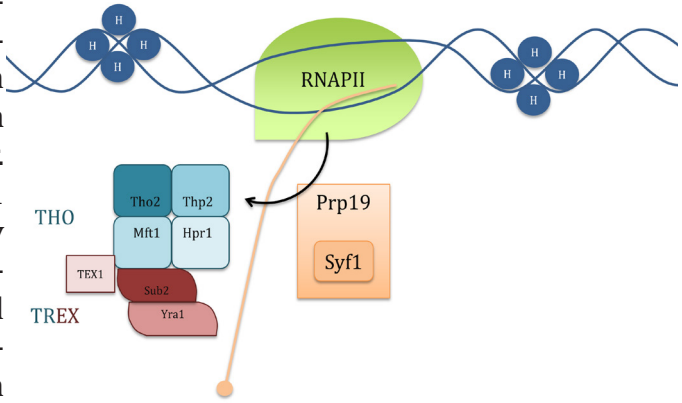


Fig.7: Recruitment model of the TREX complex dependent on Prp19 and Syf1
based on (Chanarat et al., 2011)

1.5.3. Tho1

Another potential interaction partner is Tho1 (Katahira, 2012). The formation of a stable complex between Aly, Uap56 and CIP29, the human homologue of Tho1, was reported under addition of ATP (Dufu et al., 2010). Complex formation was confirmed by Co-Immunoprecipitation assays as well as gel-filtration in the presence but not in the absence of ATP. Complex reconstitution after ATP addition was shown to be successful. Functional involvement of CIP29 in mRNA export was furthermore shown by fluorescence-in-situ-hybridization, as well as reporter gene assays. (Dufu et al., 2010) Furthermore, Tho1 was shown to be a multi-copy suppressor of Hpr1-deletion in yeast (Piruat and Aguilera, 1998).

1.5.4. Iws1

Iws1 from human was shown to couple the histone-chaperone Spt6 with mRNA export, by a direct interaction with ALY/REF (Yoh et al., 2007). This interaction was shown by Co-Immunoprecipitation, as well as pull down experiments. Furthermore, the relevance of Iws1 for mRNA export was shown by RNA-fluorescence-in-situ-hybridization and immunofluorescence of Iws1 depleted and wild type HeLa cells. The exact role of Iws1 in mRNA export, however, is not clear.

2. Aim of the thesis

Crystallization trials performed by Rajan Prabu PhD of the yeast TREX complex have up to now only yielded badly diffracting crystals. On-going efforts in optimization of crystallization conditions or construct design have not shown further improvements. The aim of the thesis was to test alternative strategies, that might at the end improve crystal formation.

2.1. Reconstitution of the Yra1-Sub2 and Yra1-Mex67 complex from *Chaetomium thermophilum*

A first project investigated in the adaptor proteins ctYra1, ctSub2 and ctMex67 from *Chaetomium thermophilum*. Previous studies revealed problems in the purification of Yra1 due to low stability, protease sensitivity and tight binding of nucleotides, disrupting the interaction with binding partners. As proteins of the thermophilic yeast Chaetomium thermophilum are expected to be more stable, ctYra1, ctSub2 and ctMex67 were hoped to enhance protein yield and facilitate purification.

2.2. Putative TREX-complex interacting proteins in yeast

The aim of the second project was to find interaction partners of THO and TREX complex, to stabilize the crystal and the crystal contacts. Therefore, Sto1, Syf1, Tho1 and Iws1 have been chosen to be tested as interaction partners, based on their predicted degree of disorder (Fig.8).

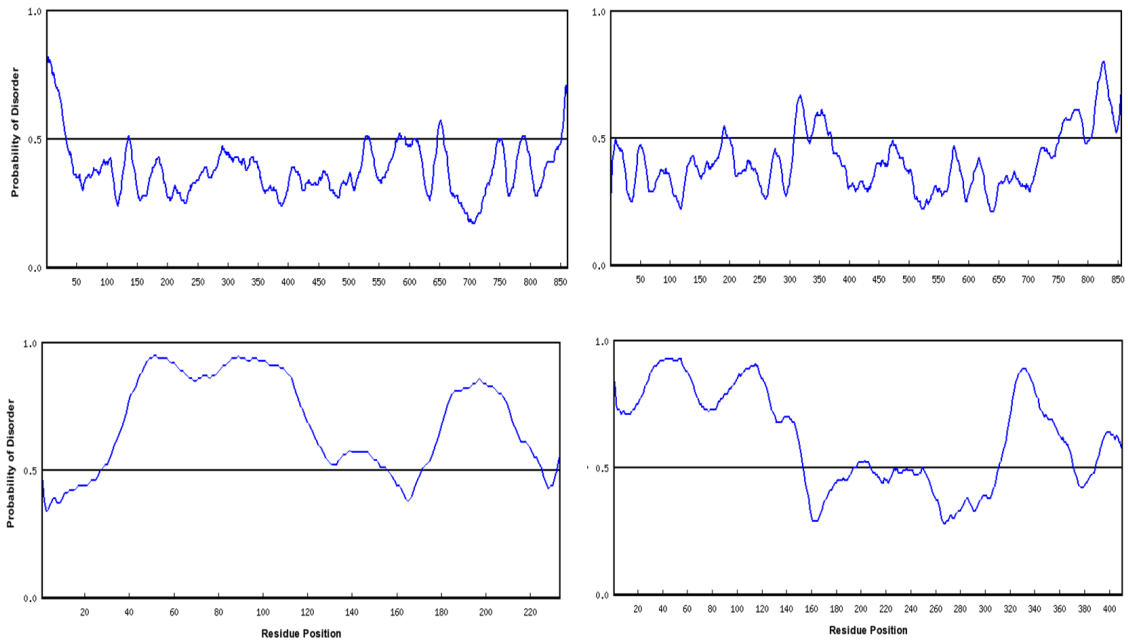


Fig.8: Prediction of Disorder for Sto1 (top left), Syf1 (top right), Tho1 (bottom left) and IWS1 (bottom right)

based on (Chanarat et al., 2011)

2.3. Reconstitution of the subcomplex of Thoc5 and Thoc7

As crystallization improvements of yeast TREX complex remained unsuccessful, reconstitution of the human TREX complex for structural studies was tested. Therefore, a third project of this thesis was the expression and purification of the human-specific subcomplex of Thoc5 and Thoc7. Thoc5 and Thoc7 were shown in previous experiments performed by Rajan Prabu PhD to form a stable subcomplex, however expression and purification required optimization.

3. Materials and Methods

3.1. Materials

All chemicals and reagents were purchased from Sigma-Aldrich and Fluka, enzymes were bought from Fermentas of New-England Biolabs. Columns and resigns were purchased from GE-healthcare, Sigma and Clontech. Synthetic genes were ordered from GeneArt by life technologies. As SDS-PAGE marker the unstained protein molecular weight marker from Fermentas was used, displaying lanes in 116,0 kDa, 66.2 kDa, 45.0 kDa, 35.0 kDa, 25.0 kDa, 18.4 kDa, 14.4 kDa.
Abbreviations of the buffers used in for the experiments are displayed in table 1. Numbers listed in the index display the concentration in mM.

Table 1: Abbreviations of buffers

Abbreviation	full Name
T	Tris(hydroxymethyl)-aminomethan
H	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
N	Sodium Chloride
I	Imidazole
β	2-Mercaptoethansulfonat-Natrium
DTT	Dithiothreitol
Glu	Glutathione
Gly	Glycerol

3.1. Cloning

The desired gene sequences were cloned into pEC vectors containing a Kanamycin or Ampicilin resistance, a N-terminal tag (His, His-GST, His-Z, His-TRX or Sumo), a LacO site for regulation of transcription via IPTG, and a T7 promoter. The vectors furthermore contained a protease cleavage site for TEV, precission3C or Sumo protease between the tag and the gene of interest. Vector stocks processed for LIC-cloning (Haun et al., 1992) were available in the lab. The LIC system was established in such a way that the gene of interest can be cloned in many vectors containing different tags in parallel.

Table 2: List of Primers used for cloning

	species	Forward primer	Reverse primer
Sto1	S.c.	ccagggagcagcctcgatgGACTTCGATGAAGATGAAAATTACCGTGATTTTAG	gcaaagcaccggcctcgttaAACTTCCTTTGTTTCTTGAATCCAATTCGAAATTGTTC
Syf1	S.c.	ccagggagcagcctcgatgATGTCAGCATACATCGCAATGAAAGGAGTAATAAC	gcaaagcaccggcctcgttaAATATCTAGTTCTATTTCATCTGGATTAATCGAATATGAAG
Tho1	S.c. .	ccagggagcagcctcgATGGCAGATTATTCTTCCCTTACTGTTGTTC	gcaaagcaccggcctcgttaTCTTCTGTAACCAGAGCGGTTGCC

Iws1	S.c.	ccagggagcagcctcgATGAGTACAGCCGATCAAGAACAACCC	gcaaagcaccggcctcgttaTTTATGCTTCTTGTTTAATCTTGAAGTCAACCTCTTG
Mex67 (68-323)	C.t.	ccaggggcccgactcgatgGGTCGTGCAACCAGCCGTATGGCAC	cagaccgccaccgactgcttaCGGCAGCTGCGGCAGCGGTG
Mex67 (1-1971)	C.t.	ccaggggcccgactcgatgATGGCACCGCCTACAGGTCCGCG	cagaccgccaccgactgcttaAACCTGCGGCTGACTAATAAATGCTTCCGGAG
Yra1	C.t.	ccaggggcccgactcgatgATGAGCGGTAAACTGGATCAGACCCTGGATGAAATTC	cagaccgccaccgactgcttaCAGAACATCTTCTTCCATCGGTGCATCACCAC
Sub2	C.t.	ccaggggcccgactcgatgATGAGCGCAGAAGAAGATCTGATTGATTATTCCGATGATGAG	agaccgccaccgactgcttaGCTTGCCATATAGGTGCTTGCGTCAACACCTTC
Thoc7 (Sumo-tag)	H.s.	accaggaacaaaccggcggccgctcgatgATGGGAGCCGTGACTGACGACGAAGTTATAC	gcaaagcaccggcctcgttaTGGCTTAGGATCTGTTTCCATGCTTGCTTCCTG
Thoc7	H.s.	ccagggagcagcctcgatgATGGGAGCCGTGACTGACGACGAAGTTATAC	gcaaagcaccggcctcgttaTGGCTTAGGATCTGTTTCCATGCTTGCTTCCTG

The nucleotide sequence of the gene of interest was amplified from the genome of *Saccharomyces cerevisia*using a polymerase chain reaction . In the case of *Chaetomium thermophilum* the genes had to be synthesized as the genome was not available. The PCR reaction contained 2 µL genomic DNA, 17.5 pmol forward and revers LIC-primers (used Primers are displayed in table: 2) and 17.5 µL Phusion-flash master mix from finnzymes. The initial denaturing temperature was 98°C for 1 min, followed by 30 cycles of denaturing at 98°C for 10s, annealing at 55°C for 30s and extension at 72°C for 15s/kb. For completion of strand extension 72°C were maintained for 5min. For further processing, the sample was purified using agarose gel electrophoresis in a 1% agarose gel complemented with SYBRSafe. The PCR was specific in almost all cases, yielding the correct size products. Inserts were extracted from the gel using the Qiagen gel-extraction kit according to the manufacturer's guide. 600ng of the insert were processed for LIC-recombination by treatment with 0.4µL T4 DNA polymerase (LIC qualified) supplemented with 2µL T4-buffer, 25 µM dATP, 5 mM DTT in a 20 µL reaction mix. After 30min of incubation at room temperature the polymerase was inactivated at 75°C for 20 min. For integration of the insert into the vector 2 µL of processed insert was mixed with 1 µL of processed vector and incubated 10 min at room temperature. 1 µL 25mM EDTA was mixed and incubated for 10 more min. 2 µL of the reaction mix was transformed into XL1 Blue electro competent cells using electroporation. The cells were allowed to recover for 45 min at 37°C in 200µL SOC-media. The transformed cells were plated on LB-plates containing the corresponding antibiotic and grown over night at 37°C. The following day, colonies were picked and affirmed via colony PCR. The PCR was carried out as mentioned before, but instead of genomic DNA the reaction mix was inoculated with one picked colony. The same colony was

used to inoculate 4 mL of LB complemented with 4 µL antibiotic. The sizes of the amplified products were analysed by analytical agarose gel, and clones which showed the expected insert size were further processed for plasmid purification. Therefore, the 4mL cultures were grown over night and harvested the next morning by centrifugation at 3,220xg for 15 min in a tabletop centrifuge. The pellet is furthermore treated with a Miniprep kit from Qiagen following the manufacturer's protocol. The obtained plasmids were confirmed by sequencing [MPI core facility].

3.2. Protein Expression

For expression, the plasmids were transformed into BL21 gold expression by electroporation. After recovery in 200µL SOC-media at 37°C for 45 min the cells were plated on LB-plates containing the according antibiotic, as resistance marker for the plasmid, and Chloramphenicol, as resistance marker for the expression strain. The plates were incubated overnight at 37°C.
Expression tests were performed to check for soluble expression of the proteins with different tags. Therefore, 30 mL of LB-media complemented with the respective antibiotic and Chloramphenicol were inoculated with the BL21 gold cells from the expression plates. The cells were grown in a shaker at 37°C up to an OD_{600} of 0.4. Then, the temperature was reduced to 18°C and induced with 0.2 mM IPTG. After having grown over night, the OD_{600} is measured and the amount of cells corresponding to OD_{600}, when resuspended in 1 mL was pelleted at 3,220xg for 15 min,. The cells were lysed by sonication for 1 min with a pulsation of 0.4s on / 0.6s off at an amplitude of 25%. A sample of 5-10 µL total lysate was taken for SDS-PAGE. The sample is pelleted for 30 min at 15,700xg. The supernatant was added to 50µL washed His- or GSH-beads, and rotated for 1h at 4°C. The beads were subsequently washed three times with lysis buffer to remove unbound protein. Bound protein was eluted by addition of SDS-sample buffer. The elution and the lysate samples were incubated for 3 min at 95°C before loading onto a SDS-PAGE.

Proteins, shown to be soluble in the test-expression were then used for large-scale expression. Therefore, a preculture of 10 mL volume per 1L of planed culture was prepared. Therefore LB or TB-media was complemented with antibiotics and BL21 cells expressing the plasmid of interest. The preculture was incubated at 37°C overnight. For the large-scale expression, 330mL of LB or TB, for expression in 2L Erlenmeyer flasks, or 1L of media for expression in Tunair-flasks, were supplemented with antibiotics and inoculated with the preculture, and shaken at 220 rpm at 37°C. At an OD_{600} of 0.2 the temperature was reduced to 18°C and after 1 h the cultures were inoculated with 0.2 mM IPTG. After expression over night the cultures were harvested at 8,983xg for 10 min. The pellet was frozen and stored at -20°C.

3.3. Protein Purification

3.3.1. Lysis of cultures

Sonication was used to disrupt the cells. Therefore, the pellet was thawed in three times its volume of lysis buffer, supplemented with 1 mM PMSF, 1 $^{\mu g}/_{mL}$ DNase. In case of expected nucleotide contamination 50 mM Urea were supplemented to the lysate. For lysis of *E.coli* cultures, he sonotrode VS70T was used with a pulsation of 0.5 s on, 0.5 s off and an amplitude of 40% for 5-15 min, depending on the volume of lysate.

In case of insect-cell cultures a pulsation of 5 s on and 20 s off was chosen. During the whole process the sample was kept on ice, to prevent heating of the sample and denaturing of the protein. Afterwards, the cell debris were removed by pelleting at 75,600xg for 45 min.
In case of co-lysis both pellets were thawed in the same lysis buffer and pooled before sonication.

3.3.2. GST-Bead-purification

In case of beads packed in a column, same procedure was followed and the column was driven by a peristaltic pump.
In case of on-column/beads cleavage, 1 mg of TEV-protease were added onto the column per 50 mg of protein, or 10 µg of precission3C per 1mg of protein. The samples were incubated at 4°C overnight, and the protein was then collected in the flow through or supernatant.

3.3.3. Protease-cleavage

For TEV-cleavage, 1 mg of protease was added per 50 mg of protein, during dialysis over-night in $T_{20}N_{200}Gly_5\beta_2$. For precission3C-cleavage 10 µg of protease were supplemented per 1 mg of protein.

3.3.4. Ni-column

Ni-affinity purification was mainly used for TEV-cleaved samples, to remove the GST-tag and the TEV-protease. Therefore the dialysed sample was loaded onto a equilibrated Ni-column via a superloop. Therefore, the superloop was washed with buffer containing 20 mM Imidazole while collecting the flow-through. Afterwards, the bound protein was eluted with a buffer containing 0.5 M Imidazole. The desired protein could be detected in the flow through, whereas the protease, GST and uncleaved GST-tagged protein were detected in the elution, as shown by SDS-PAGE analysis.
In the case of Ni-affinity purification as a first step of protein-purification, the column was first washed in buffer with 20 mM Imidazole to remove loosely bound protein, followed by a high salt chaperone wash with $T_{20}N_{1000}Mg_5KCl_{50}ATP_2$, or $T_{20}Mg_5KCl_{50}ATP_2$ for complex purification. The protein of interest was eluted by a shallow gradient of high Imidazole buffer (T_{50} $N_{50}I_{500}$). Fractions containing protein, as detected by SDS-PAGE, were pooled and further processed.

3.3.5. Gravity columns

For purification with gravity columns the filtered lysate was incubated with the Ni-beads for 1h or over-night at 4°C. The samples were then transferred onto the gravity columns and washed with lysis buffer and chaperone buffer. Elution occurred by incubating with 2 column volumes elution buffer and then collection of the elution.

3.3.6. Heparin-column

Heparin affinity was used to remove bound nucleotides from the protein of interest after a primary affinity step, i.e. Ni-affinity. As binding is dependent on the salt concentration, elution in the previous step occurred via low-salt buffer. The eluate was loaded onto a Heparin column equilibrated in low-salt buffer, and eluted in a shallow gradient, or in a step wise manner with high-salt buffer ($T_{20}N_{1000}$).

3.3.7. Preparative Gel filtration

Gel filtration was used as the last step of purification. Therefore, the purified protein was concentrated to a volume of less than 300 µL (for semi-analytical gel filtration) or

less than 2.5 mL (for preparative gel filtration), loaded onto a loop and injected into the column. Dependent on the size of the protein, either S200 or S75 columns were used for semi-analytical gel filtration. For high yields of protein, and hence preparative purification, the high load 200 or 75 was used.

3.4. Protein Analysis

3.4.1. SDS Polyacrylamide Gel Electrophoresis

For analysis of protein size and content, the sample was separated via SDS-PAGE (Laemmli, 1970) using a 15% resolving gel (370 mM Tris pH8.8, 15% Acrylamide, 0.1% SDS, 0.1% APS, 0.01% TEMED) and a 5% stacking gel (125 mM Tris pH 6.8, 5% Acrylamide, 0.1 % SDS, 0.2% APS, 0.15% TEMED). The samples were prepared with 2x sample buffer and boiling at 95°C for 3 min. The gels were run at a voltage of 70V during passing of stacking gel. When the sample reached the resolving gel, the voltage was increased to 150V. Samples with high Glycerol content were run at a constant current of 6mA per gel. Gels were washed with water and stained with Coomassie-stain by heating briefly in a microwave.

3.4.2. Mass spectrometric analysis

Mass spectrometric analysis was performed by the MPI core facility with a micrOTOF. Therefore, the sample was diluted to a protein concentration of 1mg/mL per protein, in a volume of 20 µL.

3.4.3. Thermoflour-measurements

Thermoflour assays were performed, to test for better buffer conditions, enhancing protein stability. Tested conditions are shown in table 3.

Table 3: conditions tested in Thermoflour assays

	NaCl			Imidazol	Glycerol	
Mes pH 6.5	50 mM	200 mM	500 mM	10 mM	10%	$N_{200}I_{10}Gly_{10}$
Hepes pH 7.5	50 mM	200 mM	500 mM	10 mM	10%	$N_{200}I_{10}Gly_{10}$
Tris pH 8.0	50 mM	200 mM	500 mM	10 mM	10%	$N_{200}I_{10}Gly_{10}$

As environmentally sensitive fluorescent dye SYPRO® orange was used. The melting Temperatur (T_m) was calculated as the mid-point of a Boltzmann-blot. The best condition was chosen by the highest T_m and the biggest height.

3.5. Protein Interaction Studies

3.5.1. GST-Pull Down

For the GST-Pull Down pre-blocked beads were used, that have been carefully washed with buffer containing 20 mM Hepes (pH 7.5), 150 mM NaCl and 0.1% NP-40, and blocked with 400 mmol NaCl, 50 µg glycogen, 50 µg tRNA and 500 µg BSA for 2 h at 4°C. To remove unbound blocking substances, the beads were carefully washed again and stored at 4°C.

To analyse stable protein-protein interaction, a complex was formed with 2-4 µg of the GST-tagged bait, and 2-4 µg of un-tagged prey. In case of THO- and TREX-complexes 6 µg were used. The proteins were diluted together in 35 µL of $T_{20}N_{150}Gly_{10}$, and, after taking a 5 µL SDS-input-sample, 30 µL of buffer were added, consisting of 20 mM Hepes (pH 7.5), 250 mM NaCl, 2 mM Mg-Acetate, 2 mM Imidazole, 10% Glycerol, 0.1%

NP-40, 1 mM DTT. For complex-formation, the samples were incubated for 10 min at 4°C. 12 µL of 50% pre-blocked bead-slurry were added to the samples, and further diluted with 200 µL of 1xbuffer, containing 40 mM Hepes (pH 7.5), 70 mM NaCl, 4 mM Mg-Acetate, 4 mM Imidazole 12.5% Glycerol, 0.2% NP-40 and 2 mM DTT. After 1 h rotation at 4°C, the beads were washed 3 times with 1xbuffer, to remove unbound and unspecifically bound proteins. To elute, 20 µL of buffer containing, 30 mM Tris (pH 8.8), 150 mM NaCl, 1mM Mg-Acetate, 2 mM Imidazole, 20 mM reduced Glutathione, 0.1% NP-40, 14% Glycerol, 1 mM DTT. The sample was shaken 5 min at 1400 rpm at 30°C, and afterwards concentrated in a speed-vac for 15 min. The samples were then supplemented with SDS-sample buffer and analysed by SDS-PAGE.
To exclude unspecific interactions with the beads or GST, untagged proteins with GST and beads and with the beads alone were treated as controls.

3.5.2. ITC-measurements

ITC-measurements were used to assess the binding affinity of two proteins. Prior to the measurement, the proteins were heated to 25°C and degased. The first protein was filled into the reaction chamber in a concentration of around 17 µM in a volume of 2 mL. 500µL of the second protein were loaded into the injection syringe at a concentration of 200µM. Injection occurred in 10 µL steps, at an interval of 300 s.

3.5.3. Gel filtration

For formation of a complex of potentially interacting proteins, stoichiometric amounts of samples were incubated together for 15 min. The samples were then injected onto a pre-equilibrated S200 or S6 column. Eluted fractions were analysed by SDS-PAGE.

4. Result

4.1. Reconstitution of the Yra1-Sub2 and Yra1-Mex67 complex from *Chaetomium thermophilum*

The first project aimed for the complex reconstitution of the adaptor protein Yra1 with the TREX-complex subunit Sub2, as well as the export factor Mex67 from Chaetomium thermophilum.
Therefore, full-length ctYra1 and ctSub2 were successfully cloned as GST- as well as His-tagged constructs. Two construct were designed for ctMex67, a full-length and a truncated construct containing only the domains responsible for RNA-binding as well as Yra1-binding, namely the RRM- and the LRR-domain. The predicted unstructured N-terminus was furthermore excluded from this construct. The truncated construct of ctMex67 amino acid 68-323 is referred to as ctMex67tr in the following text.

4.1.1. Purification of GST-ctYra1

As first attempt, GST-ctYra1 was purified from a culture grown in TB media. The first step of purification was GSH-affinity. The high absorption ratio for 260nm/280nm of 1.8 indicated high nucleotide contamination. Gel filtration of the sample was hoped to remove these impurities, but it did not help much. The protein was severely degraded. Respective gels and chromatograms are shown in Fig.9. A list of buffers is shown in table 4.

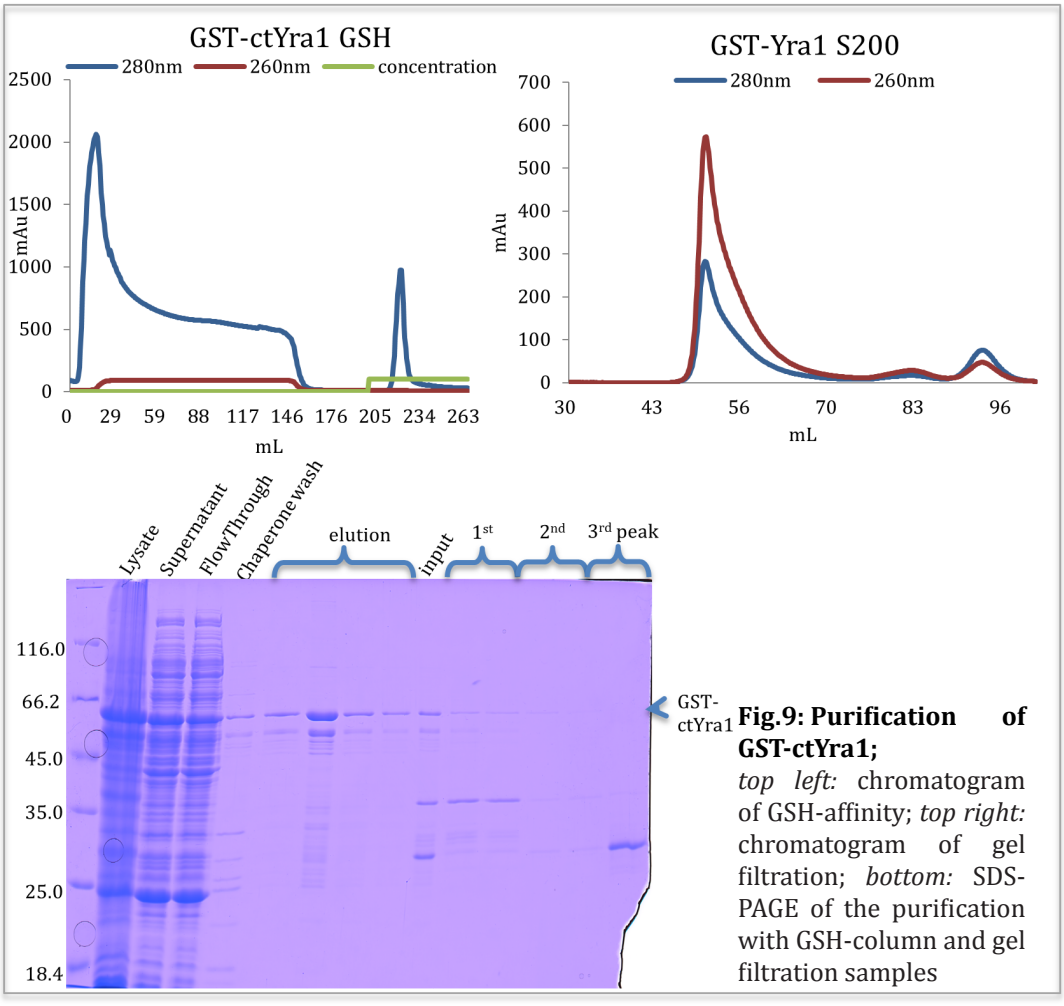


Fig.9: Purification of GST-ctYra1; *top left:* chromatogram of GSH-affinity; *top right:* chromatogram of gel filtration; *bottom:* SDS-PAGE of the purification with GSH-column and gel filtration samples

Table 4: List of buffers used for the purification of GST-ctYra1

Lysis	GSH-elution	GF S200
$T_{20}N_{200}Gly_5DTT_1$	$T_{20}N_{50}Glu_{20}$	$T_{20}N_{200}Gly_5DTT_1$

4.1.2. Co-expression of GST-ctYra1 with His-ctSub2 and with His- ctMex67tr

To circumvent protein degradation co-expression with putative interaction partners was tested. Therefore, GST-ctYra1 was co-expressed with His-ctSub2 as well as with His-ctMex67tr. Co-expression with His-Sub2 showed supressed expression of GST-ctYra1. However, His-ctSub2 could be purified by Ni-column. The tag was cleaved overnight by 3C protease in dialysis and furthermore the sample was purified by size exclusion. Corresponding chromatograms and gels are displayed in Fig.10 . Buffers used in the purification protocol are listed in table 5.

Table 5: List of buffers used for the purification of his-ctSub2

Lysis	Ni-elution	Dialysis	GF HL75
$T_{20}N_{200}Gly_{10}$	$T_{20}N_{100}I_{500}$	$T_{20}N_{200}Gly_5\beta_1$	$T_{20}N_{200}Gly_5DTT_1$

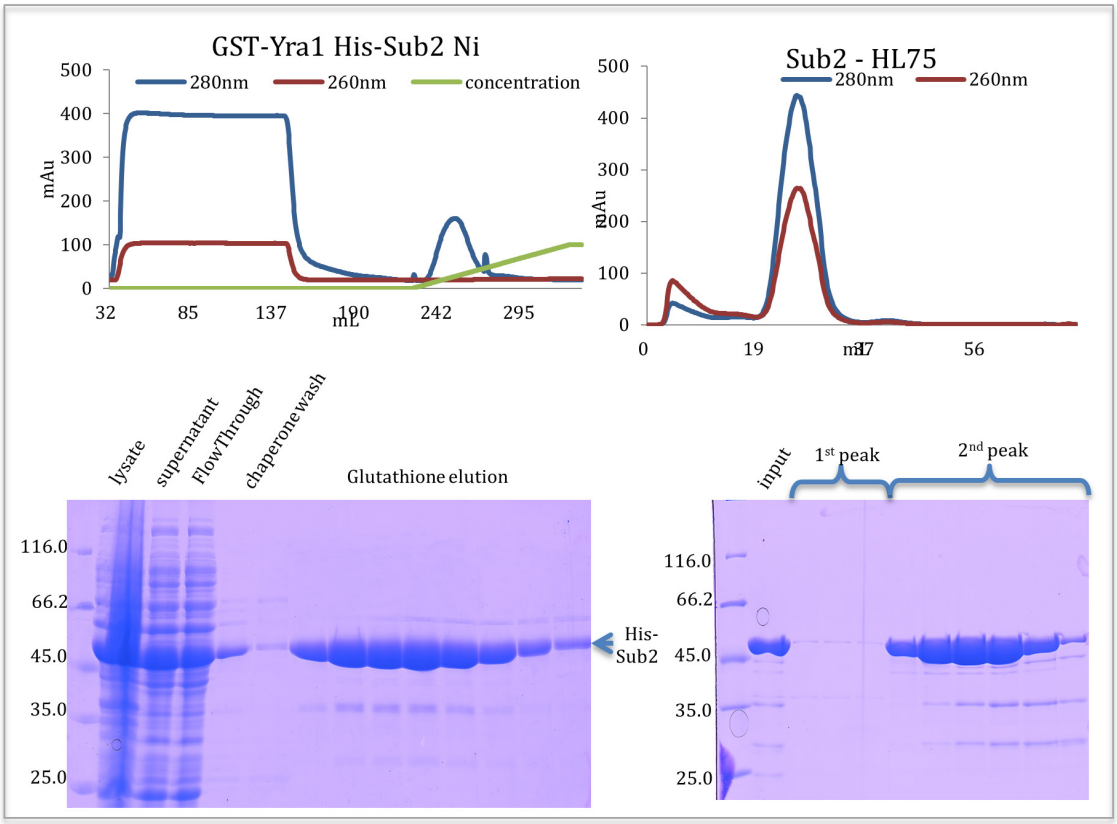


Fig.10: Coexpression of GST-ctYra1+His-ctSub2;
top left: chromatogram of GSH-affinity; *top right:* chromatogram of gel filtration; *bottom left:* SDS-PAGE of the purification with GSH-column; *bottom right:* SDS-PAGE of gel filtration samples

Co-expression of GST-ctYra1 with His-ctMex67tr showed expression of both proteins, however a complex could not be purified. The 260nm/280nm ratio of 2.1 indicates high nucleotide contaminations that might inhibit an interaction of Yra1 and Mex67. To remove these impurities the eluate of the GSH-column, was loaded onto a Heparin-column, and eluted by a gradient of increasing salt concentration. Since the flowthrough from the GSH column contained His-ctMex67, it was loaded onto a Ni-column to allow purification of Mex67. The Ni-column was washed and the eluted protein was gel filtrated. (Fig.11)
All buffers used in the purification are listed in table 6.

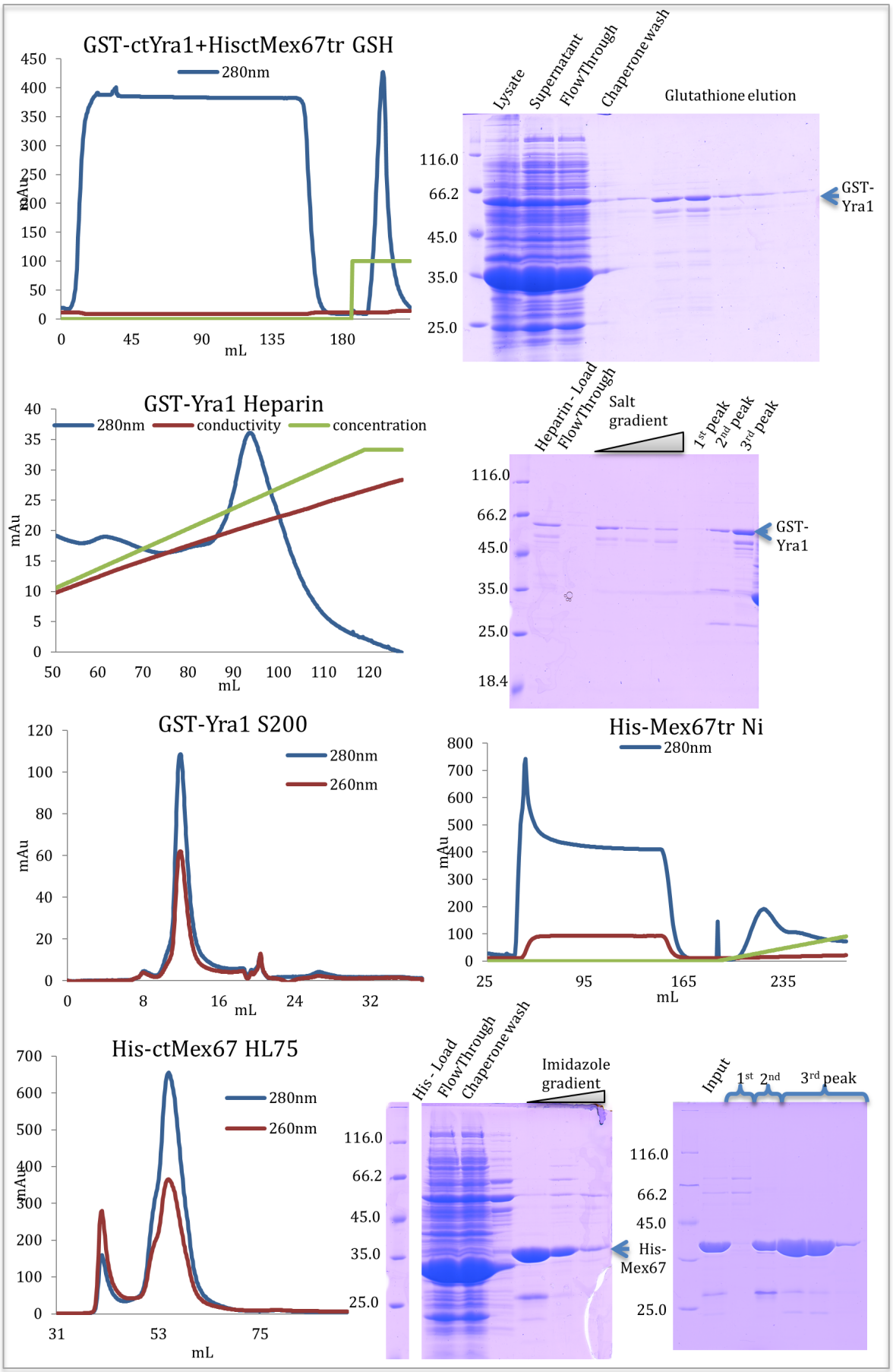


Fig.11: Coexpression of GST-ctYra1+His-ctMex67;
top: chromatogram of GSH-affinity and Ni-affinity; *2nd row:* SDS-PAGE of the purification with GSH-column (left) and the Ni-affinity (right); *3rd row:* gel filtration of GST-Yra1 S2000 (left) and His-Mex67 HL75 (right); *bottom:* SDS-PAGE of GST-Yra1 Heparin-purification and gel filtration (Left) and of His-Mex67 gel filtration (right)

Table 6: List of buffers used for the purification of GST-ctYra1 and His-ctMex67tr

Lysis	GSH-elution	Heparin - low salt	Heparin - high salt	GF S200
$T_{20}N_{100}DTT_1$	$T_{20}N_{50}Glu_{20}$	$T_{20}N_{100}DTT_1$	$T_{20}N_{1000}$	$T_{20}N_{200}Gly_5DTT_1$
Ni-column - low I	**Ni-column - high I**	**GF HL75**		
$T_{20}N_{100}$	$T_{20}I_{500}$	$T_{20}N_{150}Gly_5DTT_1$		

4.1.3. Interaction studies

Interactions between the purified proteins were tested by pull-down experiments using GSH-beads. GST-ctYra1 was tested for binding to ctSub2 and His-ctMex67tr, as well as both proteins separately in the presence and absence of AMP-PNP and RNA. Resulting SDS-PAGE analyses is shown in Fig.12. Only an interaction of GST-ctYra1 to His-ctMex67tr could be seen, regardless of the presence of ATP or RNA, however, no interaction with ctSub2 could be detected under the conditions tested.

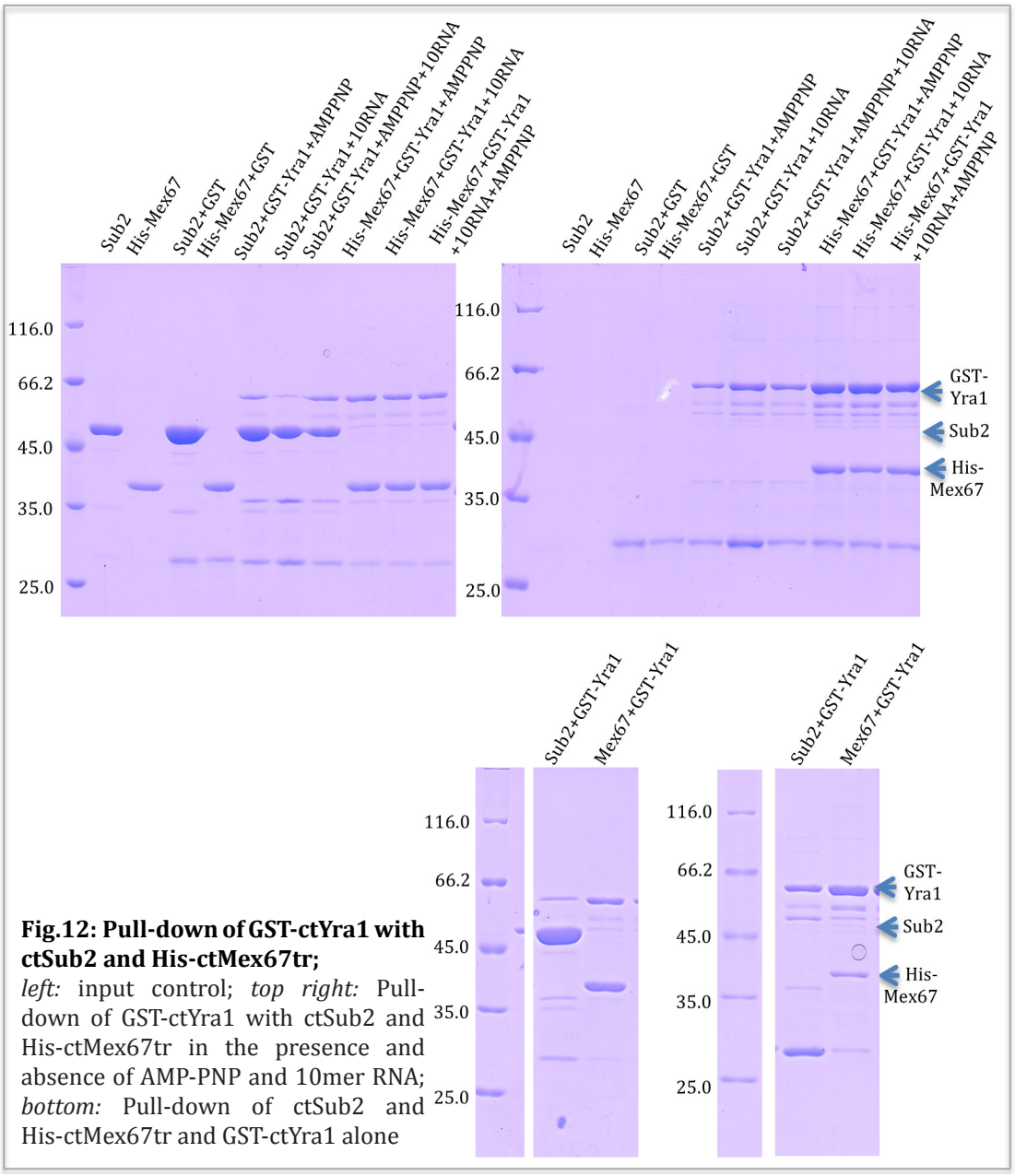


Fig.12: Pull-down of GST-ctYra1 with ctSub2 and His-ctMex67tr;
left: input control; *top right:* Pull-down of GST-ctYra1 with ctSub2 and His-ctMex67tr in the presence and absence of AMP-PNP and 10mer RNA; *bottom:* Pull-down of ctSub2 and His-ctMex67tr and GST-ctYra1 alone

To furthermore analyse the binding behaviour, analytical gel filtration was performed with GST-ctYra1 with ctSub2, as well as GST-ctYra1 with His-ctMex67. Therefore, GST-ctYra1 was incubated with its putative interaction partner in a 3:1 ratio and injected into an Superdex 200 column in $T_{20}N_{100}Gly_5$. The corresponding chromatograms and SDS-PAGES are displayed in Fig.13.
His-ctMex67tr and GST-ctYra1 showed elution in two peaks, indicating either no complex formation under the condition tested or dissociation of the complex during gel filtration. In case of ctSub2 and GST-ctYra1 only inconclusive results were obtained as degradation products prevent successful interpretation of the data.

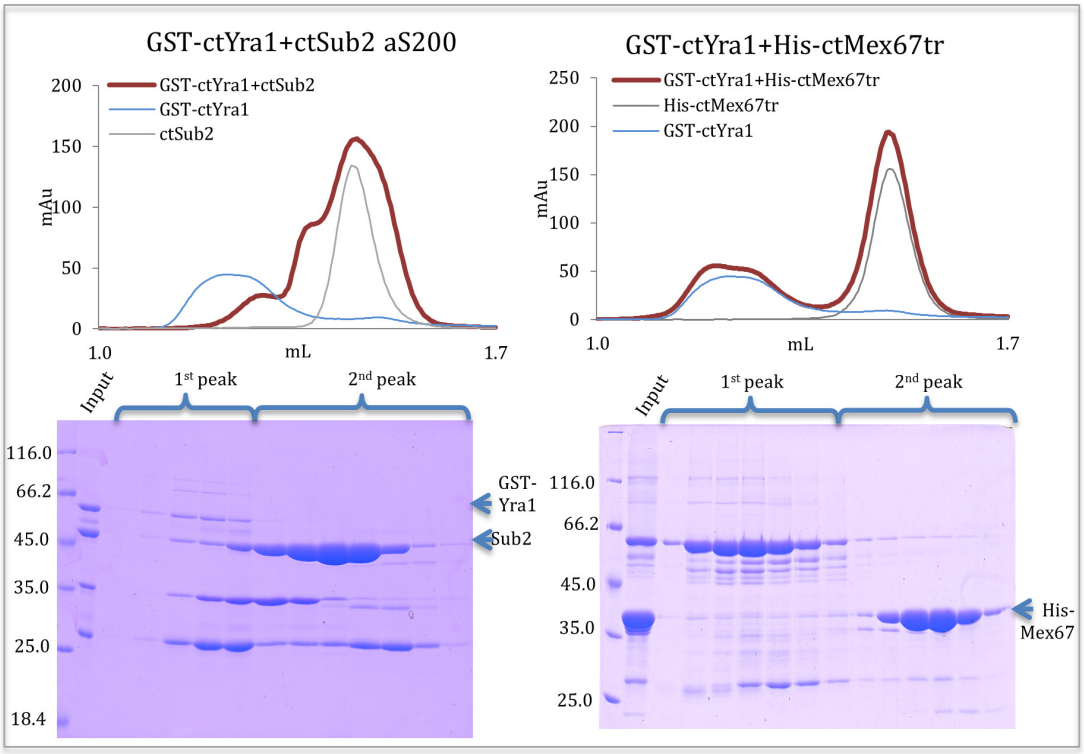


Fig.13: analytical gel filtration;
top left: chromatogram of analytical gel filtration of GST-ctYra1 and ctSub2; *top right:* chromatogram of analytical gel filtration of GST-ctYra1 and His-ctMex67tr; *bottom left:* SDS-PAGE of GST-ctYra1+ ctSub2; *bottom left:* SDS-PAGE of GST-ctYra1+His-ctMex67tr

4.1.4. Tag-cleavage of GST-ctYra1

As the GST-tag could potentially interfere with complex formation, removal of the GST-tag by precision3C cleavage was tested. Protease sensitivity of ctYra1, lead to the attempt to cleave in the presence of a binding partner, as complex formation was hoped to stabilize ctYra1. Therefore, purified GST-ctYra1, as described above, was loaded onto a GSH-column and supplemented with ctSub2 in a 1:1 ratio, as well as precission3C protease. The sample was incubated overnight and the protein collected in the flow through. However, ctYra1 was completely degraded (Fig.14).

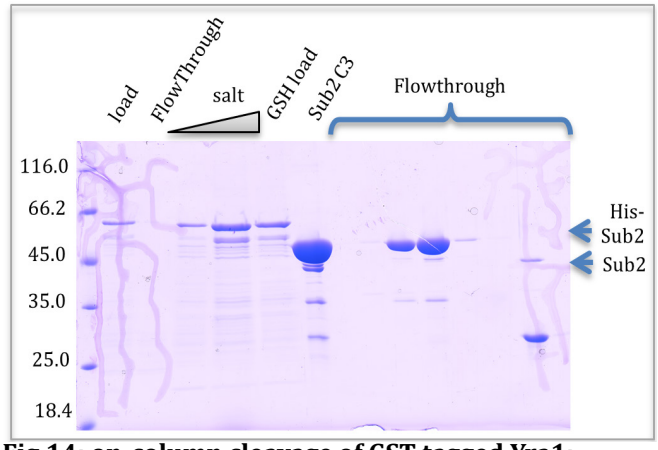


Fig.14: on-column cleavage of GST-tagged Yra1;
first four lanes: Heparin-column elution, *last eight lanes:* GSH-column supplemented with Sub2 and C3

4.1.5. Purification of His-ctYra1

6xHis-tag was hoped to impede complex formation less than GST-tag. Therefore, His-ctYra1 was expressed and purified by Ni-affinity. High nucleotide content as indicated by the 260nm/280nm ratio of 1.4 requested a Heparin purification step. The 260nm/280nm ratio of 0.8 indicated a decrease in nucleotide contamination (Fig.15). However, degradation products could be seen in SDS-PAGE analysis. A list of buffers is shown in table 7.

Table 7: List of buffers used for the purification of His-ctYra1

Lysis	Ni-elution	Heparin - low salt	Heparin - high salt
$T_{20}N_{200}Gly_5DTT_1$	$T_{20}N_{100}I_{500}$	$T_{20}N_{100}$	$T_{20}N_{1000}$

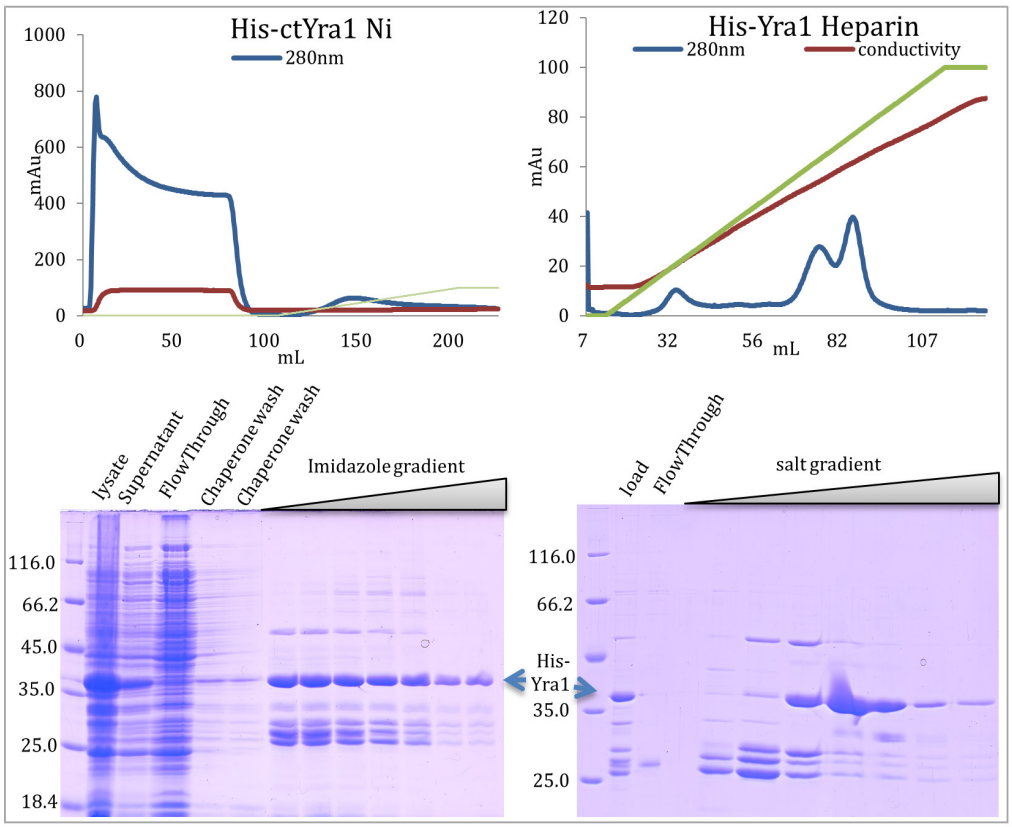


Fig.15: Purification of His-ctYra1;
top left: chromatogram of Ni-column; *top right:* chromatogram of Heparin column; *bottom left:* SDS-PAGE of Ni-column; bottom left: SDS-PAGE of Heparin column

4.1.6. Interaction studies

Pull-down experiments were performed with His-ctYra1 and ctSub2 using Ni-beads. However, SDS-PAGE analysis was inconclusive, as unspecific interactions between ctSub2 and the Ni-beads could be observed. Hence, a pull-down effect of His-ctYra1 on ctSub2 cannot be verified. (Fig.16)

Analytical gel filtration of His-ctYra1 with ctSub2 as well as with His-ctMex67 was performed in $T_{20}N_{100}Gly_5$. The proteins were mixed in a ratio of 3:1, with an

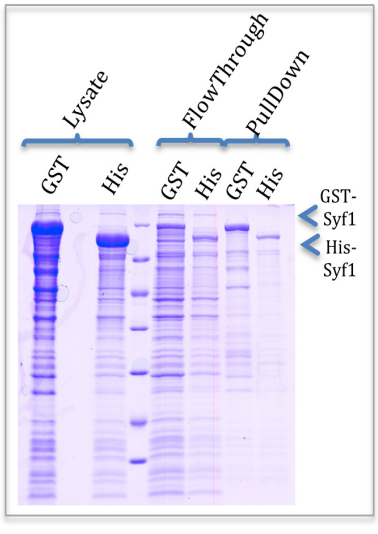


Fig.16: Pull-down of His-Yra1 with Sub2;
left: Input-gel; *right:* Pull-down, *first lane:* pull-down, *second lane:* control

excess of His-ctYra1, taking the degradation products into account. Chromatograms and SDS-PAGE analysis are shown in Fig.17. Broad peaks in the elution profile complicate interpretation of the obtained data. Hence, complex formation could not be verified, as overlapping of the peaks would result in the same elution profile as complex formation.

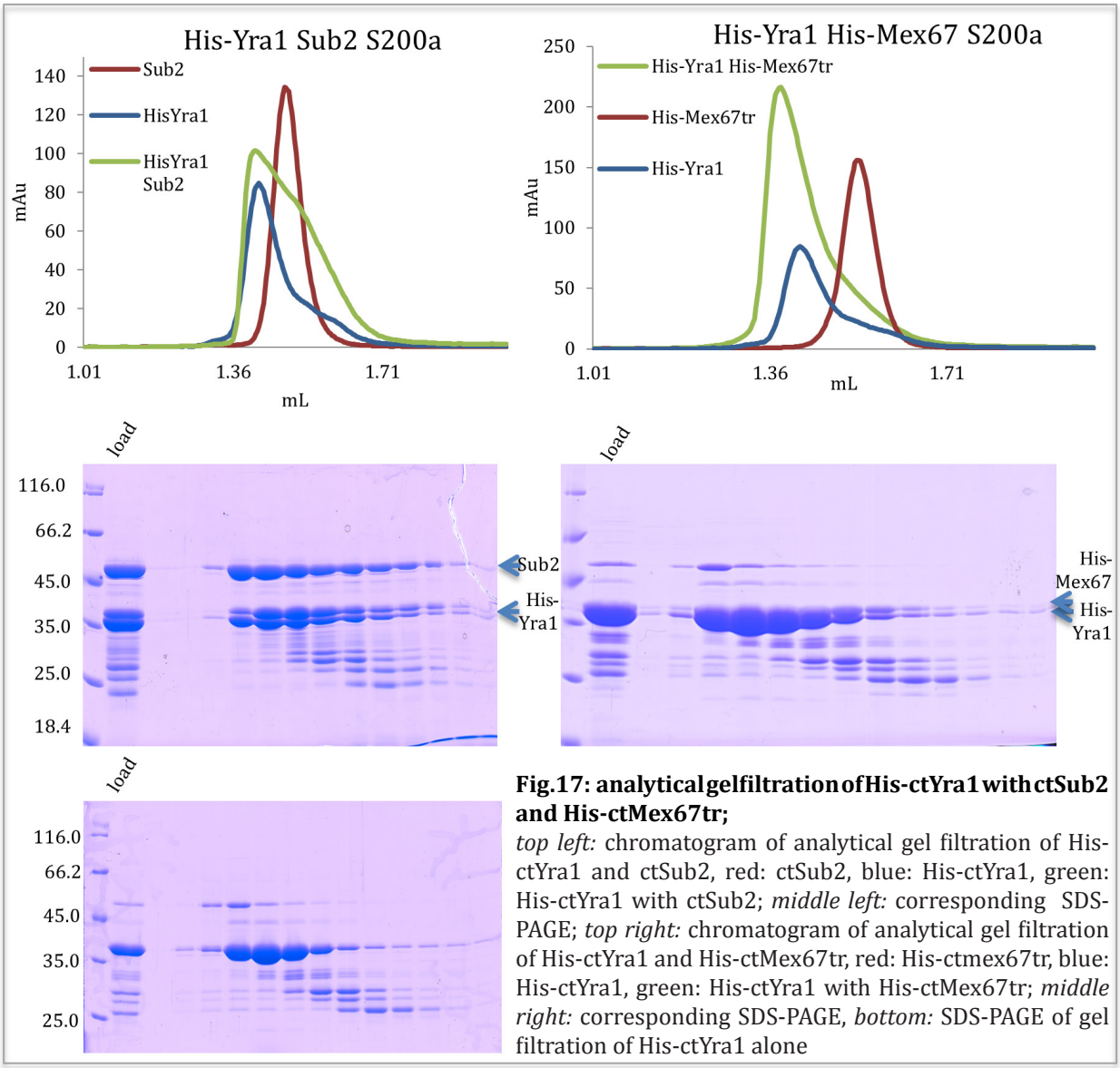


Fig.17: analytical gelfiltration of His-ctYra1 with ctSub2 and His-ctMex67tr;
top left: chromatogram of analytical gel filtration of His-ctYra1 and ctSub2, red: ctSub2, blue: His-ctYra1, green: His-ctYra1 with ctSub2; *middle left:* corresponding SDS-PAGE; *top right:* chromatogram of analytical gel filtration of His-ctYra1 and His-ctMex67tr, red: His-ctmex67tr, blue: His-ctYra1, green: His-ctYra1 with His-ctMex67tr; *middle right:* corresponding SDS-PAGE, *bottom:* SDS-PAGE of gel filtration of His-ctYra1 alone

Positive Pull-down assays and inconclusive gel filtration, lead to the idea of a complex, not stable enough to be detected by gel filtration. This would be the case for a complex with high dissociation constant breaking apart during gel filtration. Therefore, ITC measurements were performed, to test for this hypothesis. 17 µM His-ctYra1 were used in the measuring cell and 200 µM His-ctMex67tr was loaded into the needle. However, no dissociation constant could be obtained from ITC-measurements. (Fig.18). ITC-measurements were performed with the help of Claire Basquin.

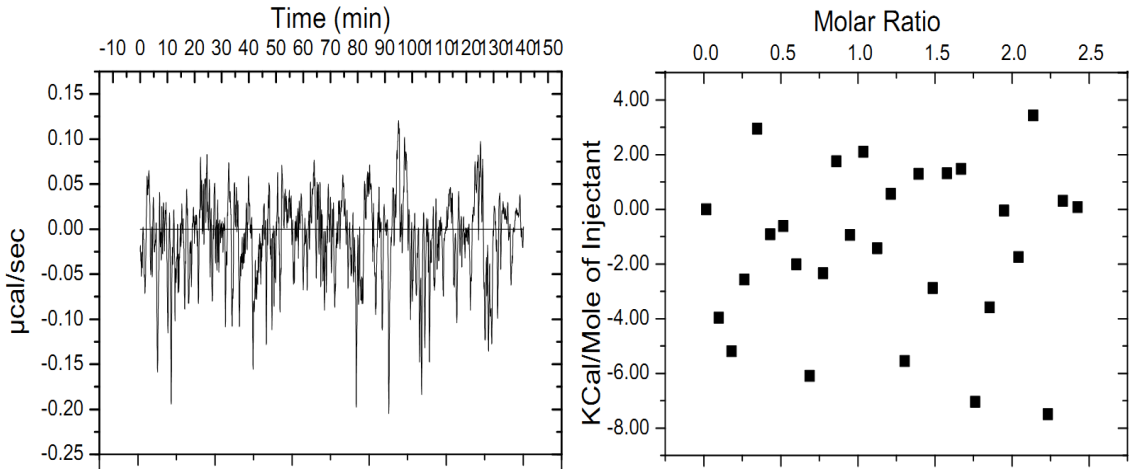


Fig.18: Results of the ITC-measurements

4.1.7. Purification of His-ctMex67fl

To ensure, that complex stability of ctYra1-ctMex67 is not affected by the truncation of ct-Mex67tr, full-length His-ctMex67 was expressed and purified. The protein was extracted from the lysate by Ni-affinity. Due to high nucleotide content with a 260nm/280nm ratio of 1.32, the eluted protein was applied onto a Heparin column. Nucleotides were effectively removed, as the 260nm/280nm ratio was 0.49 (Fig.19). Buffers used for the purification are listed in table 8.

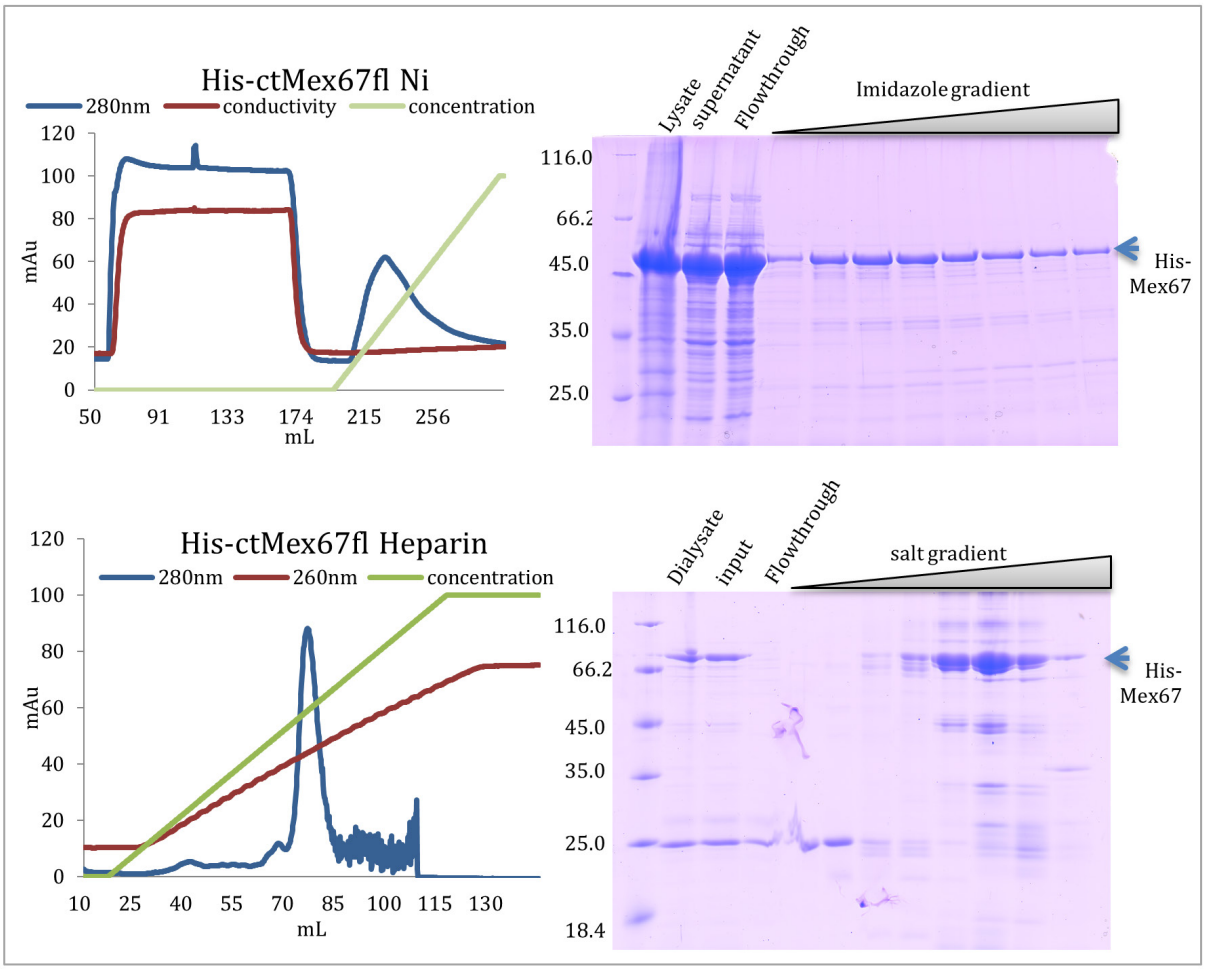


Fig.19: Purification of His-ctMex67fl;
top left: chromatogram of Ni-affinity column; *top right:* corresponding SDS-PAGE *bottom left:* Chromatogram of Heparin column; *bottom right:* SDS-PAGE of Heparin column

Table 8: List of buffers used for the purification of His-ctMex67fl

Lysis	Ni-elution	Heparin - low salt	Heparin - high salt
$T_{20}N_{200}Gly_5$	$T_{20}N_{100}I_{500}$	$T_{20}N_{100}$	$T_{20}N_{1000}$

4.1.8. Interaction studies

To check for complex formation analytical gel filtration was performed with threefold excess of His-ctYra1. $H_{20}N_{100}MgAc_2Gly_{10}DTT_1I_2$ was used as gel filtration buffer, to exclude the buffer differences between pull down and gel filtration. Due to bigger size differences of His-ctYra1 and His-ctMex67fl in comparison to the truncated protein, better resolution and hence better separation was expected on the gel filtration column, in case of no complex formation. The Chromatogram and the corresponding SDS-PAGE are shown in Fig.20.

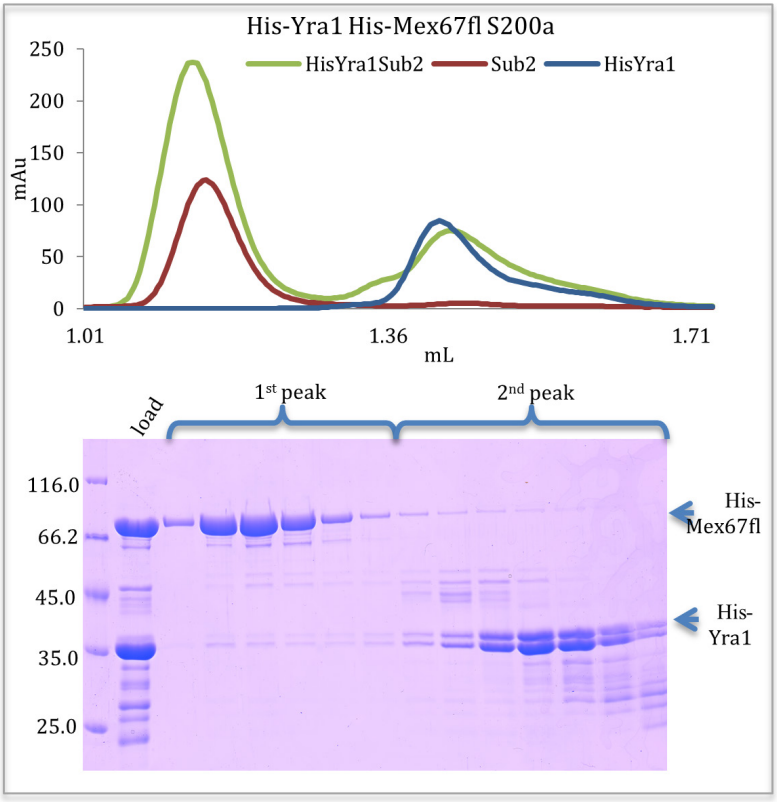


Fig.20: analytical gel filtration of His-ctYra1 with His-ctMex67fl; *top:* chromatogram of analytical gel filtration of His-ctYra1 and His-ctMex6fl in $H_{20}N_{100}MgAc_2Gly_{10}DTT_1I_2$; *bottom:* corresponding SDS-PAGE

Separation of the two proteins could be clearly detected, and only a faint band of His-ctYra1 could be detected in the first peak. This indicates a complex dissociating during the stringend conditions of gel filtration with only a small amount of proteins eluted as a complex.

4.2. Putative TREX-complex interacting proteins in yeast

As the adaptor proteins from *Chaetomium thermophilum* did not display enhanced stability, no advantage in using the proteins from the thermophilic organisms were obtained. As a next step, interaction partners of the yeast TREX partners were investigated in.

4.2.1. Sto1

Purification

Sto1 was shown to be soluble in the expression test in BL21 gold cells as a N-terminal GST-tagged construct. (Fig.21)
Cells expressing GST-Sto1 were grown in LB media. For purification of GST-tagged Sto1, the GSH-beads protocol was followed. The protein was pooled, flash frozen and stored at -80°C.
To obtain Sto1 without tag, the GSH eluate was dialyzed overnight along with TEV protease. The dialysate was then passed through His-column so that the tag and some impurities could be removed (Fig.22). The flow through was concentrated and injected into a gel filtration column (Fig.23). Buffers used for the purification are displayed in table: 9.

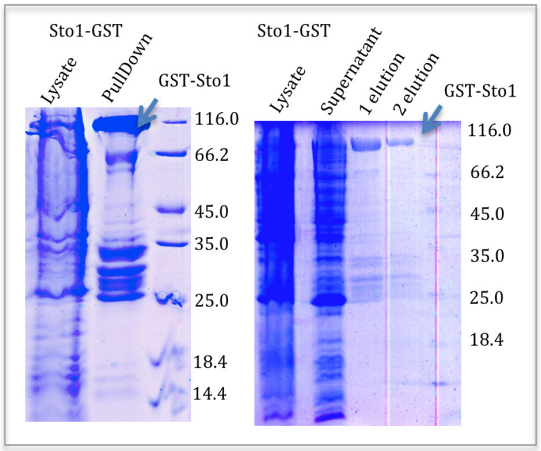


Fig.21: GST-Sto1 test expression and purification;
left: GST-Sto1 test-expression in BL21 gold cells;
right: GST-Sto1 purification with GSH-beads

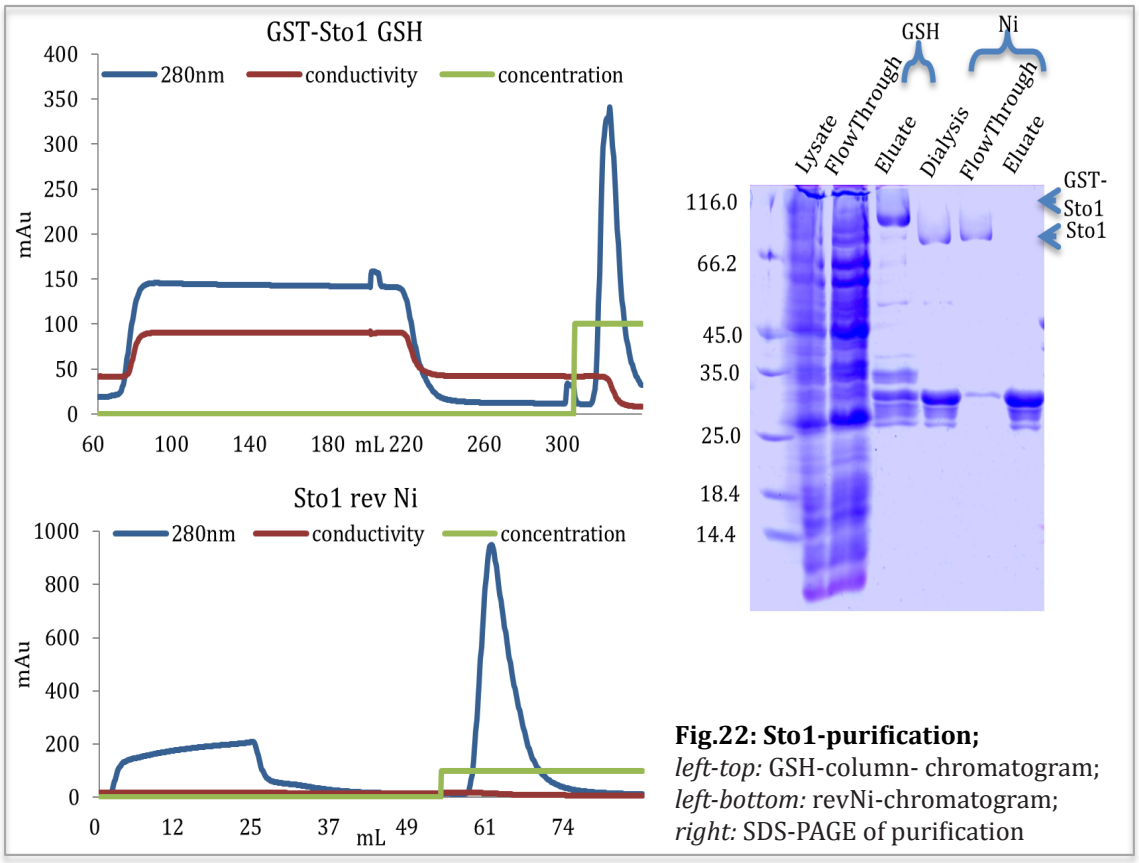


Fig.22: Sto1-purification;
left-top: GSH-column- chromatogram;
left-bottom: revNi-chromatogram;
right: SDS-PAGE of purification

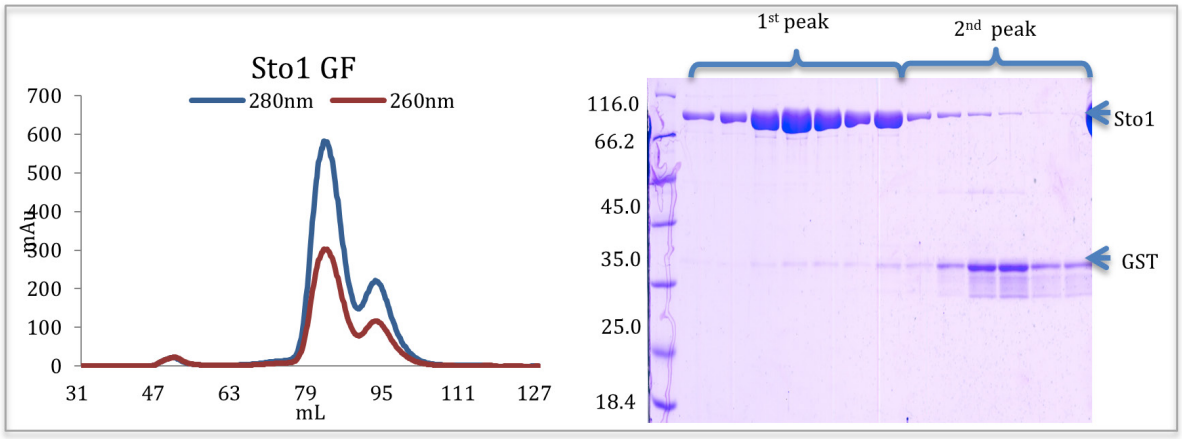


Fig.23: Gel filtration of Sto1;
left: chromatogram; *right:* SDS-PAGE of gel filtration

Table 9: List of buffers used for the purification of Sto1

Lysis	GSH-elution	Dialysis	revNi – low I	revNi - high I	Gel filtration
$T_{20}N_{500}DTT_1$	$T_{20}N_{50}Glu_{20}$	$T_{20}N_{200}Gly_5\beta_2$	$T_{20}N_{200}Gly_2I_{20}\beta_1$	$T_{20}I_{500}$	$T_{20}N_{150}Gly_5DTT_1$

Interaction Studies

Purified Sto1 was used for gel filtration with a Sub2 construct from amino acid 50 to 446, available in the lab. $T_{20}N_{150}Gly_5DTT_1$ was used as running buffer on an S200-column. Samples were mixed in a molar ration of 1:1.

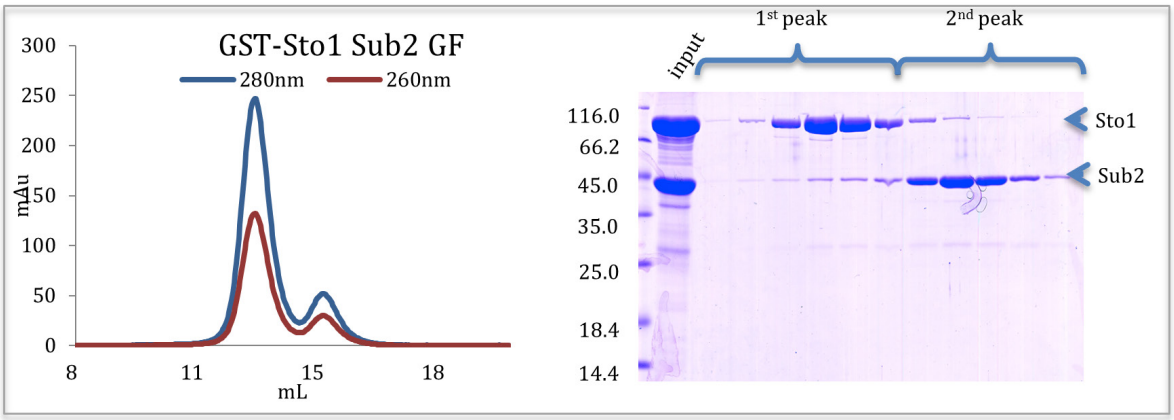


Fig.24: Gel filtration of Sto1 with Sub2 construct;
left: chromatogram; *right:* SDS-PAGE

The proteins clearly eluted in 2 peaks, as confirmed by SDS-PAGE (Fig.24). This indicates no stable complex formation in the used buffer system.

In order to explore the binding of Sto1 with different THO complex constructs pull down assays were performed with GST-tagged Sto1. However, no binding of Sto1 to either the THO/TREX complex, nor Sub2 could be shown under the conditions tested. Furthermore, addition of AMP-PNP, an ATP-analogue, did not change the binding behaviour (Fig.25).

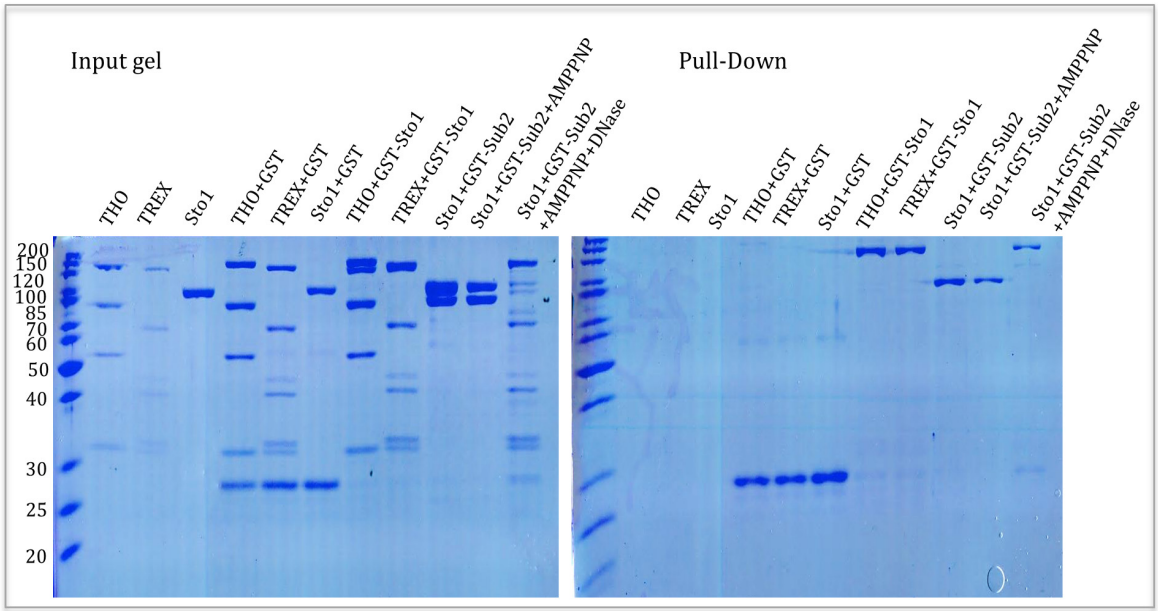


Fig.25: Pull Down of THO/TREX complex with GST-Sto1& Sto1 with GST-Sub2;
left: input gel, right: Pull Down samles; with negative controls (first 6 lanes), interaction of Sto1 with THO and TREX complex (7th and 8th lane), interaction of Sto1 with full length Sub2 without AMP-PNP (9th lane) with AMP-PNP (10th lane) and with AMP-PNP and DNase (11th lane)

4.2.2. Syf1

Purification

Syf1 could be soluble expressed in BL21 gold cells for the His-tagged, as well as for the GST-tagged construct. The Ni-pull down is shown in Fig.26.
GST-Syf1 was successfully expressed in LB media, and purified by Ni-affinity followed by overnight dialysis with TEV cleavage of the GST-tag. As a last step size exclusion was carried out to remove cleaved GST. (Fig.27).
GST-Syf1 was purified from a culture grown in LB-media by GSH-column with subsequent overnight dialysis and yielded 1 mg of protein (Fig.28).
Buffers used for the purifications are displayed in table 10.

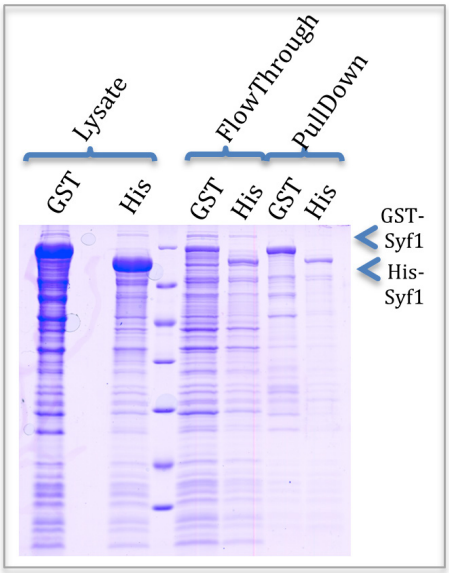


Fig.26: Test expression of His-Syf1 and GST-Syf1

Table 10: List of buffers used for the purification of Syf1 and GST-Syf1

	Lysis	GSH-elution	Ni-elution	Dialysis	Gel filtration
Syf1	$T_{20}N_{500}DTT_1$		$T_{20}I_{500}$	$T_{20}N_{200}Gly_5\beta_2$	$T_{20}N_{200}Gly_5DTT_1$
GST-Syf1	$T_{20}N_{500}Gly_5DTT_1$	$T_{20}N_{50}Glu_{20}Gly_5$		$T_{20}N_{200}Gly_5\beta_2$	

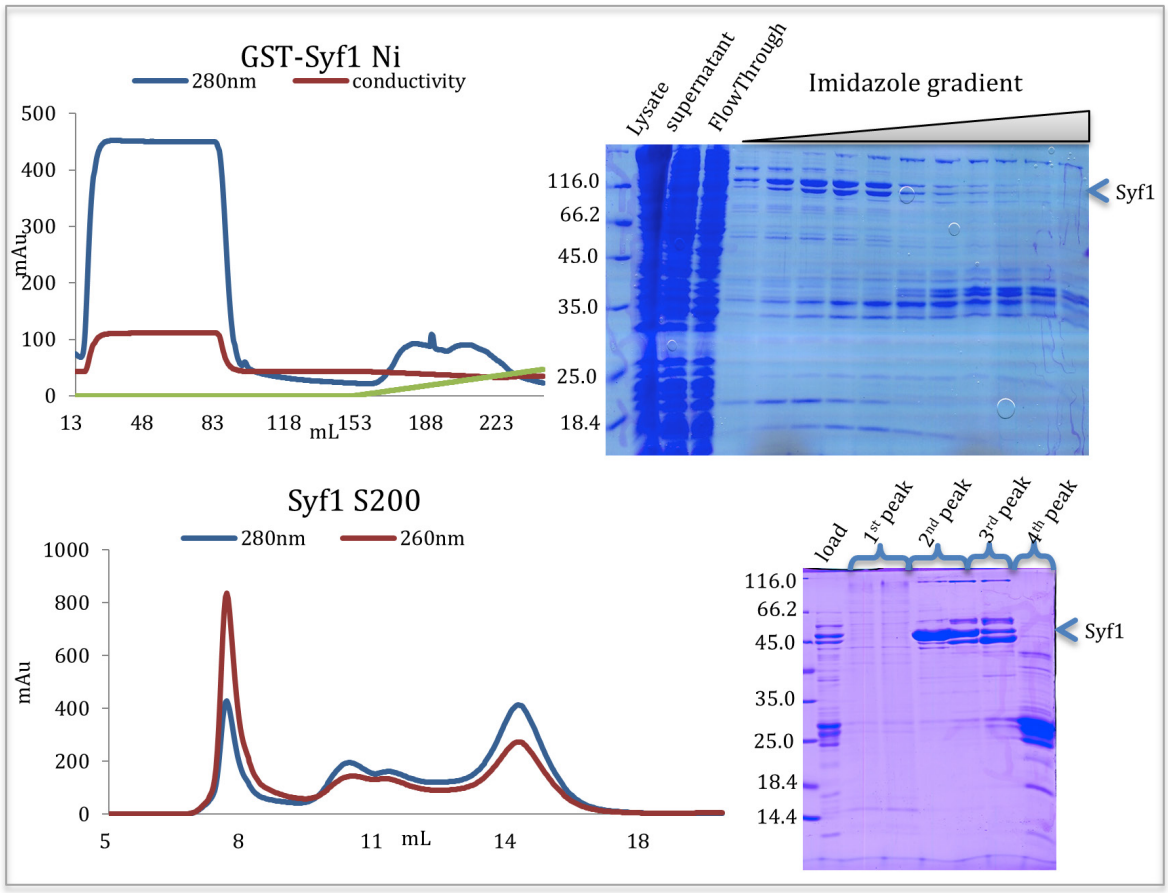


Fig.27: Purification of Syf1;
top left: chromatogram of Ni-affinity purification; *top right:* corresponding SDS-PAGE of samples taken from Ni-affinity purification; *bottom left:* chromatogram of gel filtration; *bottom right:* corresponding SDS-PAGE from gel filtration fractions

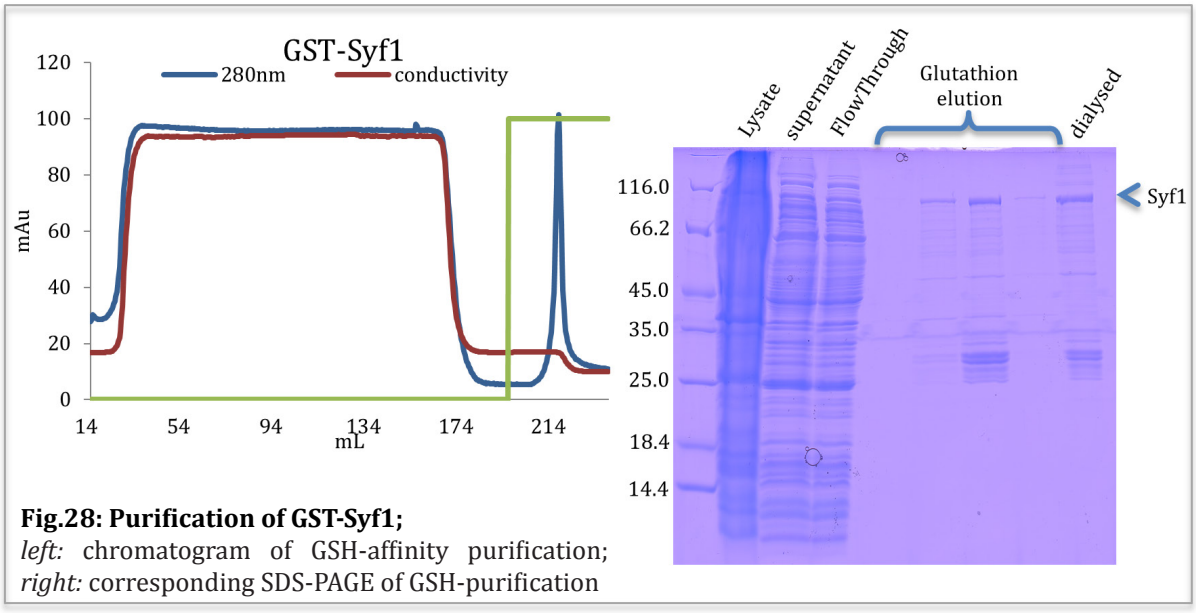


Fig.28: Purification of GST-Syf1;
left: chromatogram of GSH-affinity purification; *right:* corresponding SDS-PAGE of GSH-purification

Interaction studies

To study binding of Syf1 to the THO complex, gel filtration of both proteins in a ratio of 1:2 was performed after 1.5h incubation at 4°C, to allow complex formation. A S6 column equilibrated with $T_{20}N_{125}Gly_5DTT_1$ was used. The chromatogram showing an overlay of Syf1 gel filtration alone (green), TREX complex gel filtration alone (red) and of expected complex formation (blue), as well as the corresponding SDS-PAGE are shown in Fig.29.

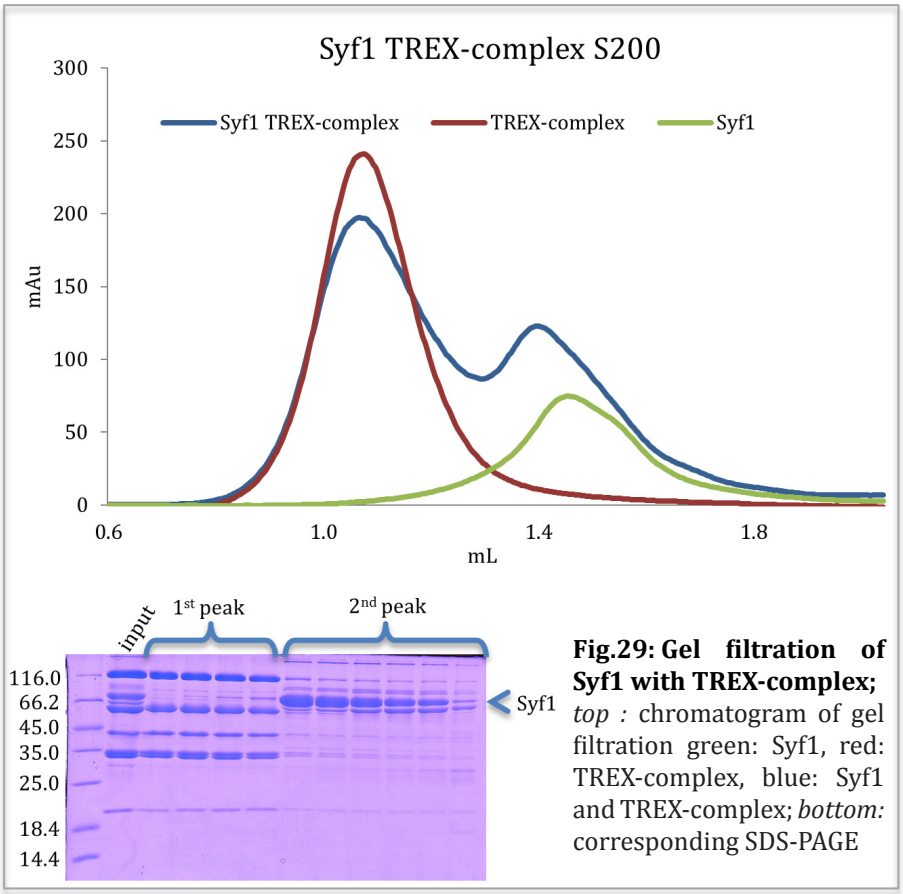


Fig.29: Gel filtration of Syf1 with TREX-complex; *top :* chromatogram of gel filtration green: Syf1, red: TREX-complex, blue: Syf1 and TREX-complex; *bottom:* corresponding SDS-PAGE

Syf1 and the TREX-complex clearly elute in two separate peaks, indicating that there is no complex formation under the conditions tested.

To further test binding of Syf1 to the TREX complex, a pull down assay was carried out. GST-Syf1 was incubated with the THO complex, as well as with the TREX complex. However, GST-Syf1 failed to pull down either THO or TREX-components. The SDS-page is shown in Fig.30.

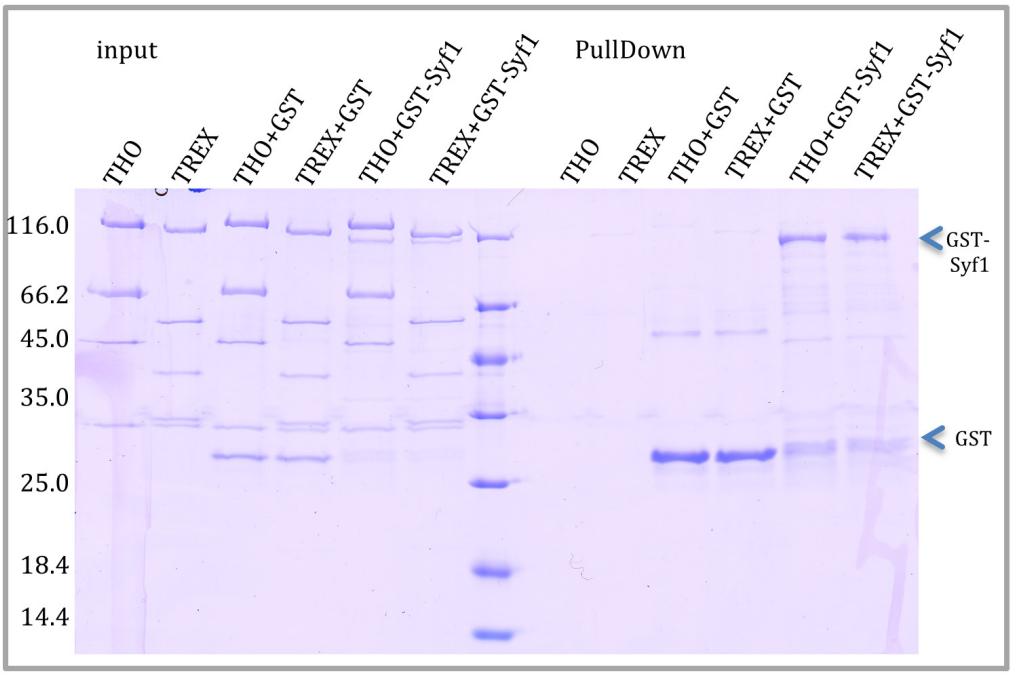


Fig.30: Pull Down of THO- and TREX- complex with GST-Syf1;
Input gel on the left half of the gel, Pull down samples on the right half; with controls *(first 4 lanes)*, interaction of THO with GST-Syf1 *(5th lane)* and TREX with GST-Syf1*(6th lane)*

4.2.3. Tho1

Purification

His-tag, as well as Z-tag and GST-tag construct could be expressed in BL21 gold cells and is observed to be soluble in the test expression (Fig. 31).

Tho1 and GST-Tho1 were expressed in LB-media. After purification using GSH-column, 60% of the amount of protein was cleaved by TEV-protease in dialysis. The other part was directly injected into gel filtration. Protease and GST were removed from the sample by Ni-affinity, and the flow through was concentrated and further purified by gel filtration (Fig.32). Buffers used in the purification are displayed in table 11.

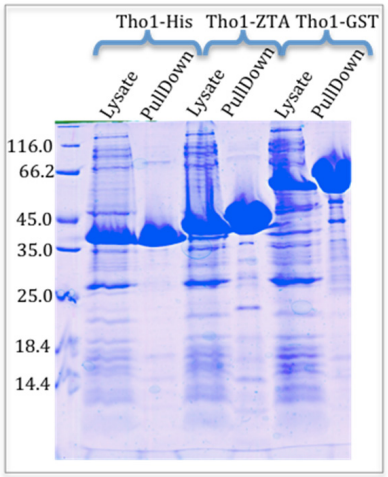


Fig.31: Testexpression of Tho1;
constructs with His-tag, Z-tag and GST-tag were used

Table 11: List of buffers used for the purification of Tho1 and GST-Tho1

	Lysis	GSH-elution	Dialysis	revNi – low I	revNi – high I	Gel filtration
Tho1	$T_{20}N_{500}Gly_5DTT_1$	$T_{20}N_{50}Glu_{20}$	$T_{20}N_{200}Gly_5\beta_2$	$T_{20}N_{200}Gly_5I_{20}\beta_1$	$T_{20}N_{100}I_{500}$	
GST-Tho1	$T_{20}N_{500}Gly_5DTT_1$	$T_{20}N_{50}Glu_{20}$				$T_{20}N_{200}Gly_5DTT_1$

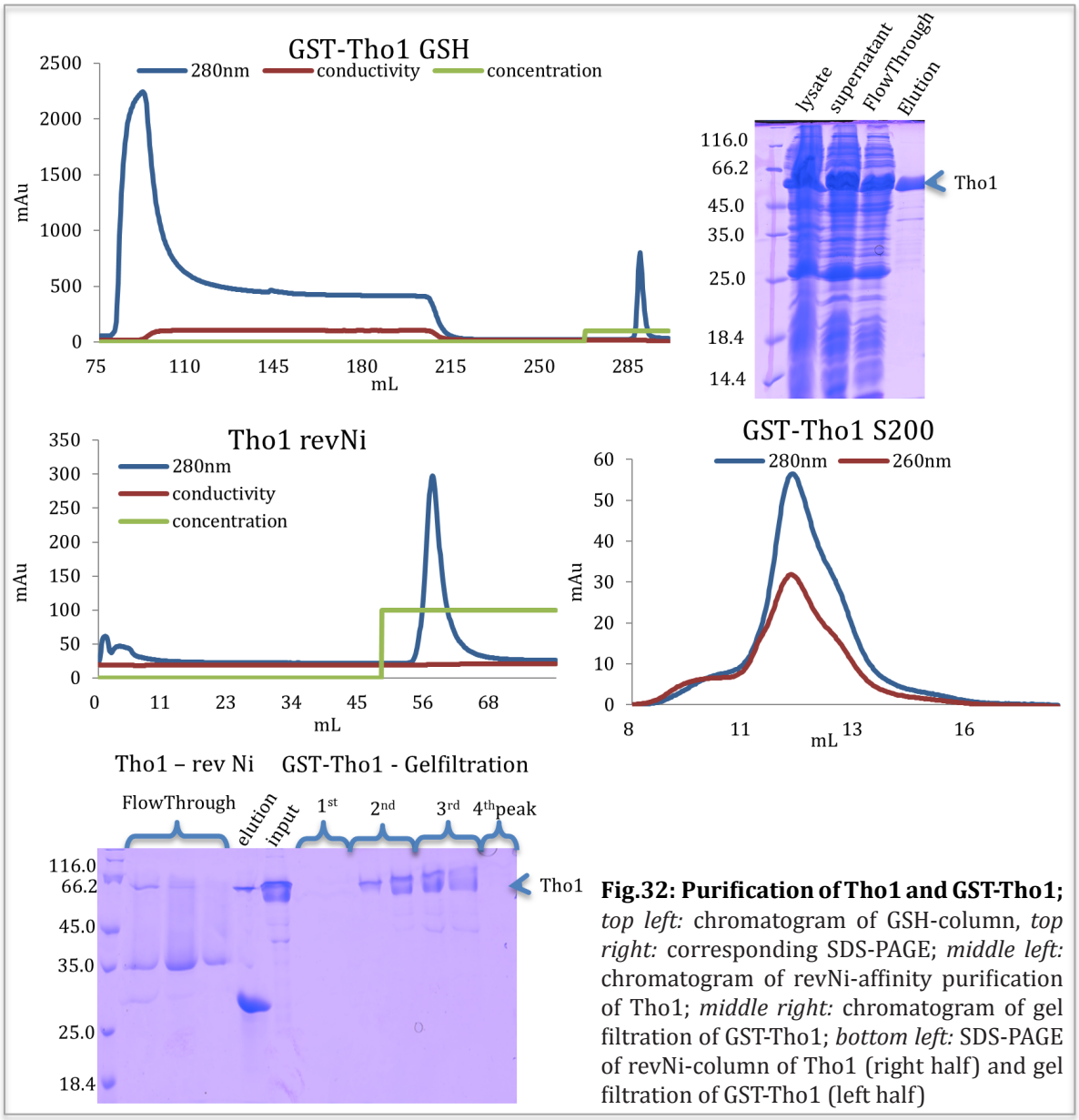


Fig.32: Purification of Tho1 and GST-Tho1; *top left:* chromatogram of GSH-column, *top right:* corresponding SDS-PAGE; *middle left:* chromatogram of revNi-affinity purification of Tho1; *middle right:* chromatogram of gel filtration of GST-Tho1; *bottom left:* SDS-PAGE of revNi-column of Tho1 (right half) and gel filtration of GST-Tho1 (left half)

Interaction studies

Pull down assays were performed to study the interaction of Tho1 with the THO-complex. Therefore, either GST-Tho1 was used to study the interaction with Sub2 or GST-Yra1, to test for binding to Sub2 and Tho1. Interaction of Sub2 with Yra1 was furthermore used as positive control.

The pull down showed no interaction of Tho1 neither with Sub2 alone, nor in complex with Yra1 in the used buffer system. (Fig.33) However, interaction of Yra1 with Sub2 indicates, that the pull down experiment was fine.

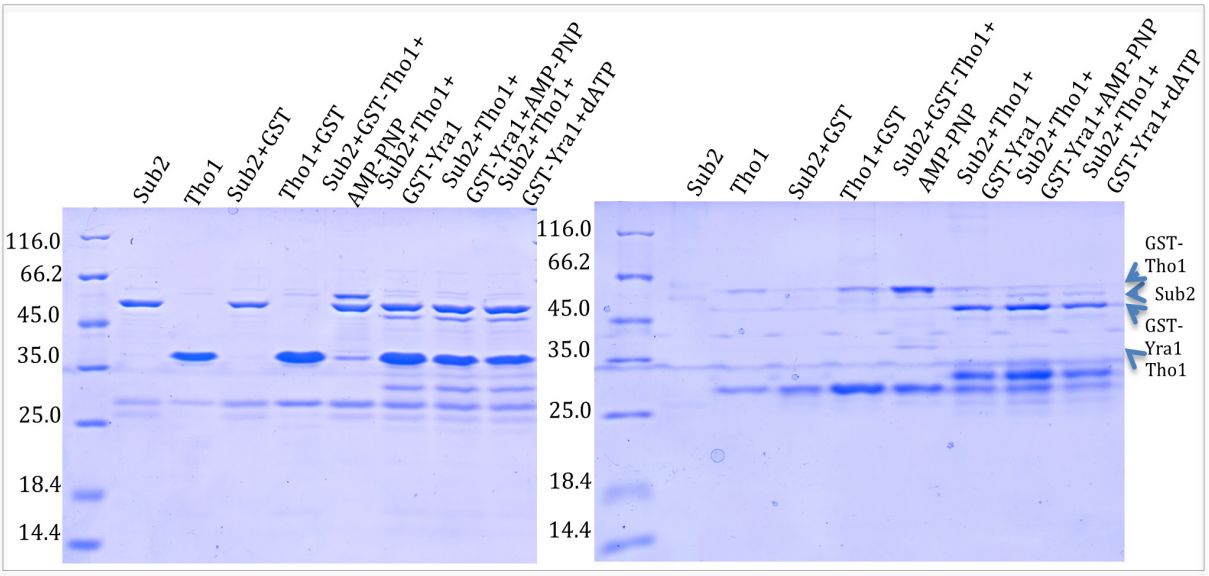


Fig.33: Pull down assays with Sub2 and GST-Tho1 and Sub2, Tho1 and GST-Yra1;
left: input gel; *right:* pull down samples analysed by SDS-PAGE, with controls (first 4 lanes), and interaction studies of Sub2 with GST-Tho1 and AMP-PNP (5th lane), and Sub2 and Tho1 with GST-Yra1 (6th lane) with AMP-PNP (7th lane) and dATP (8th lane)

4.2.4. Iws1

Purification

Expression test of Iws1 shows soluble expression in BL21 gold cells as His- and GST-tagged construct (Fig.34).

Iws1 was expressed in LB media and purified by GSH-column, followed by TEV-cleavage and Ni-affinity purification (Fig.35). Buffers used in the purification are shown in table 12.

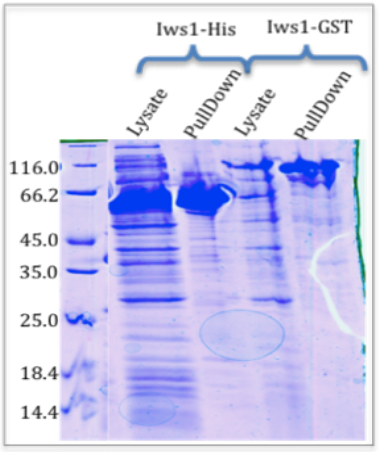


Fig.34: Testexpression of Iws1;
with His-tag, and GST-tag

Table 12: List of buffers used for the purification of Iws1

Lysis	GSH-elution	Dialysis	revNi - low I	revNi - high I
$T_{20}N_{500}Gly_5DTT_1$	$T_{20}N_{50}Glu_{20}$	$T_{20}N_{150}Gly_5\beta_2$	$T_{20}N_{250}Gly_{10}I_{20}$	$T_{20}I_{500}$

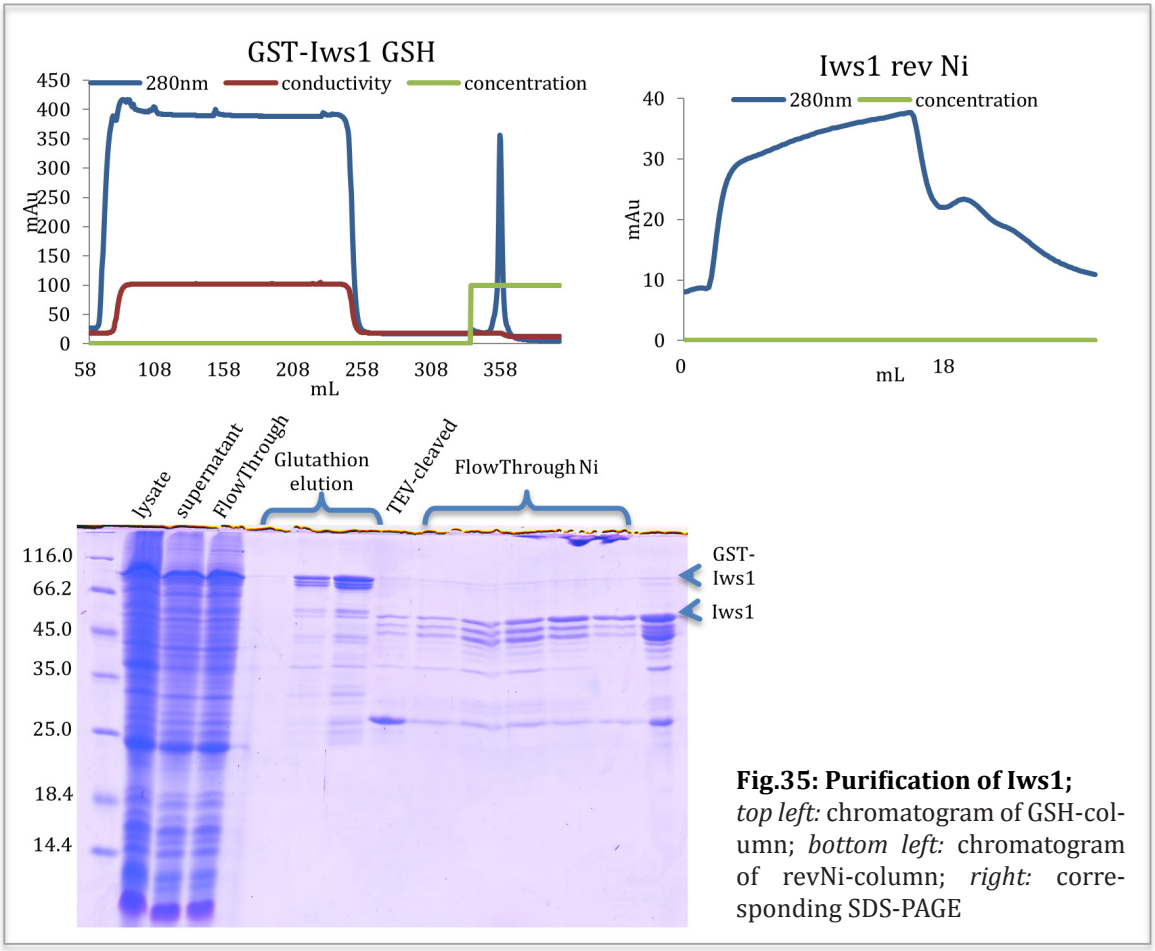


Fig.35: Purification of Iws1; *top left:* chromatogram of GSH-column; *bottom left:* chromatogram of revNi-column; *right:* corresponding SDS-PAGE

Interaction studies

Using pull down assay, the binding of Iws1 to Yra1 was explored. Iws1 was tested for interaction with 2 different Yra1 constructs (named YraI and Yra II), differing in the linker between the protein and the GST-tag. Also binding in the presence and absence of AMP-PNP was tested.

However, GST-Yra1 failed to pull down Tho1, independent of the presence of AMP-PNP (Fig.4.16).

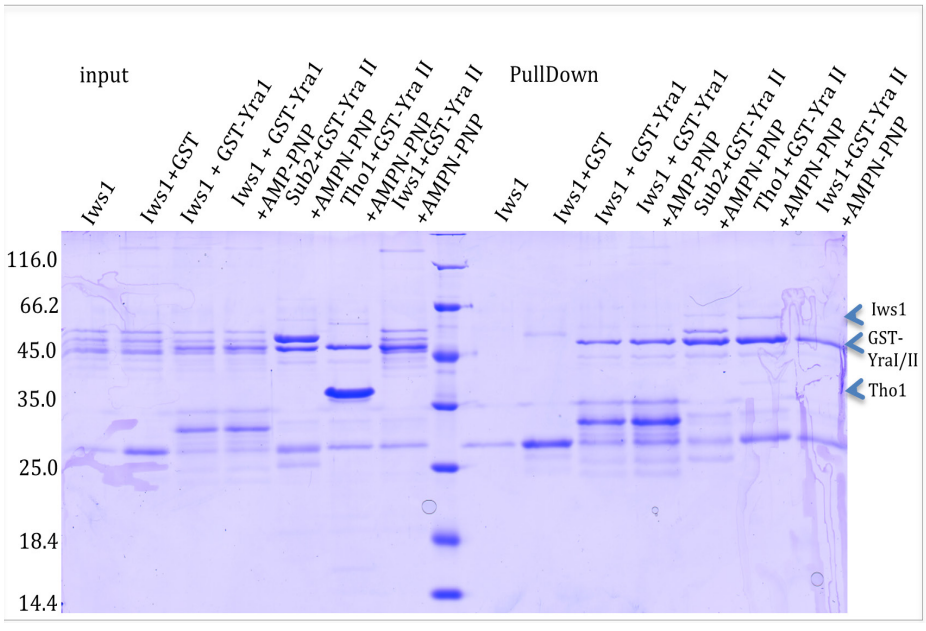


Fig.36: Purification of Iws1; The input gel is displayed in the left half of the gel, the right half shows the pull down samples; with controls (first 2 lanes) interaction of Iws1 with GST-Yra1 with and without AMP-PNP (3rd to 7thlane)

4.3. Reconstitution of the subcomplex of Thoc5 and Thoc7

The aim of the third project was to express and purify Thoc5 and Thoc7of the human TREX complex, and to reconstitute the binary complex of these two proteins.

4.3.1. Purification of GST-Thoc7

A culture grown in TB media was lysed and purified by GSH-affinity. The obtained protein was concentrated and further purified by gel filtration. (Fig.37) The chromatogram showed GST-Thoc7 eluted as an aggregate in the void volume. Buffers used for the purification are shown in table 13.

Table 13: List of buffers used for the purification of GST-Thoc7

lysis	GSH-elution	GF S200
$T_{20}N_{200}Gly_5DTT_1$	$T_{20}N_{50}Glu_{20}$	$T_{20}N_{200}Gly_5$

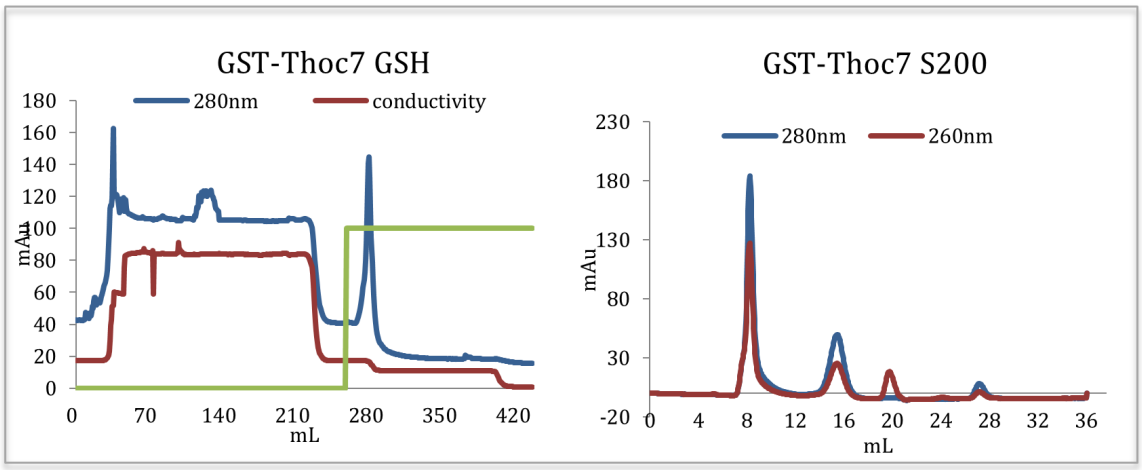


Fig.37: Purification of GST-Thoc7
left: chromatogram of GSH-affinity; *right:* chromatogram of gel filtration

4.3.2. Thermoflour-assays

In order to improve the protein stability a Thermoflour assay was carried out by claire Basquin. However, it revealed no significant input to the stability, probably due to the fact that the protein was aggregated before.

4.3.3. Purification of His-Thoc5

Thoc5 was purified using Ni-affinity resin. The eluted protein was reasonably pure, but showed degradation products. Hence, size exclusion was carried out to further purify the protein (Fig.:38). Used buffers are listed in table 14.

Table 14: List of buffers used for the purification of His-Thoc5

Lysis	Ni-elution	GF S200
$T_{20}N_{200}Gly_5DTT_1$	$T_{20}N_{100}I_{500}$	$T_{20}N_{100}Gly_5$

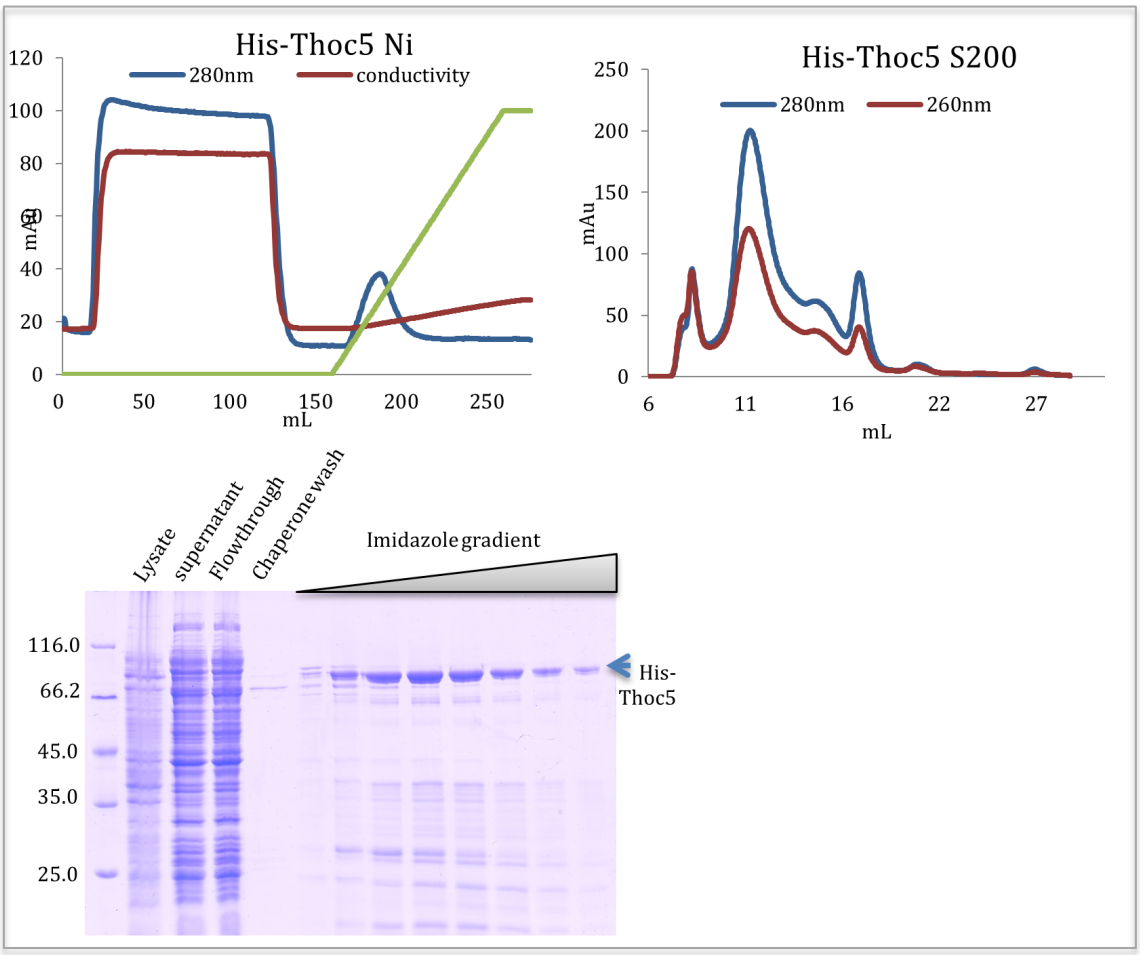


Fig.38: Purification of His-Thoc5
top left: chromatogram of Ni-affinity; *right:* chromatogram of gel filtration; *bottom:* SDS-PAGE of ni-affinity column

4.3.4. Complex assembly

Complex assembly of GST-Thoc7 with His-Thoc5 was expected to increase the solubility of GST-Thoc7. Therefore, GST-Thoc7 was incubated with His-Thoc5 in a ratio of 1:1, and injected into gel filtration. However, GST-Thoc7 eluted as an aggregation peak, and Thoc5 could not help to make it soluble (Fig.39).

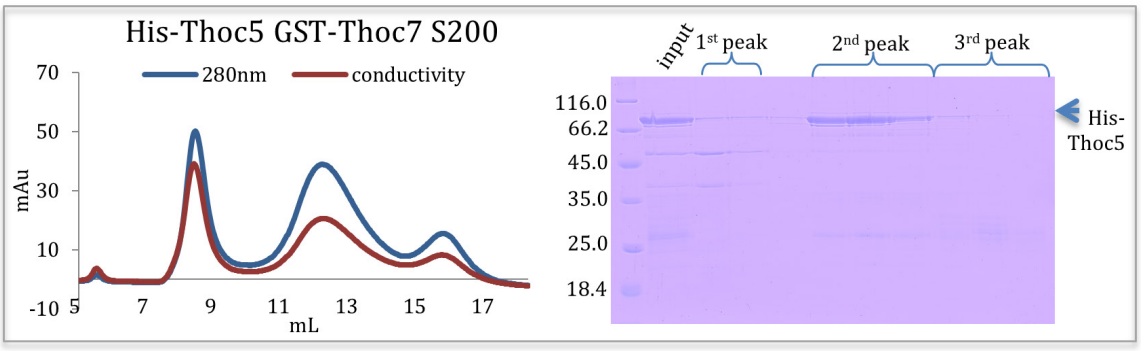


Fig.39: Complex formation of His-Thoc5 and GST-Thoc7
left: chromatogram of gel filtration in S200; *right:* corresponding SDS-PAGE

4.3.5. Co-expression of His-Thoc5 and GST-Thoc7

Co-expression with a binding partner was tried to improve the solubility of GST-Thoc7. His-Thoc5 and GST-Thoc7 were co-transformed into BL21 cells and grown in TB-media. The cells were resuspended and lysed. The lysate was applied onto Ni- column. Subsequent gel filtration showed His-Thoc5 with degradations. GST-Thoc7 could only be detected in the lysate before centrifugation, showing aggregated protein in the pellet (Fig.40). Buffers used in the purification are displayed in table 15.

Table 15: List of buffers used for the purification co-expressed His-Thoc5 and GST-Thoc7

Lysis	Ni-elution	GF S200
$T_{20}N_{100}$	$T_{20}N_{100}I_{500}$	$T_{20}N_{100}Gly_5$

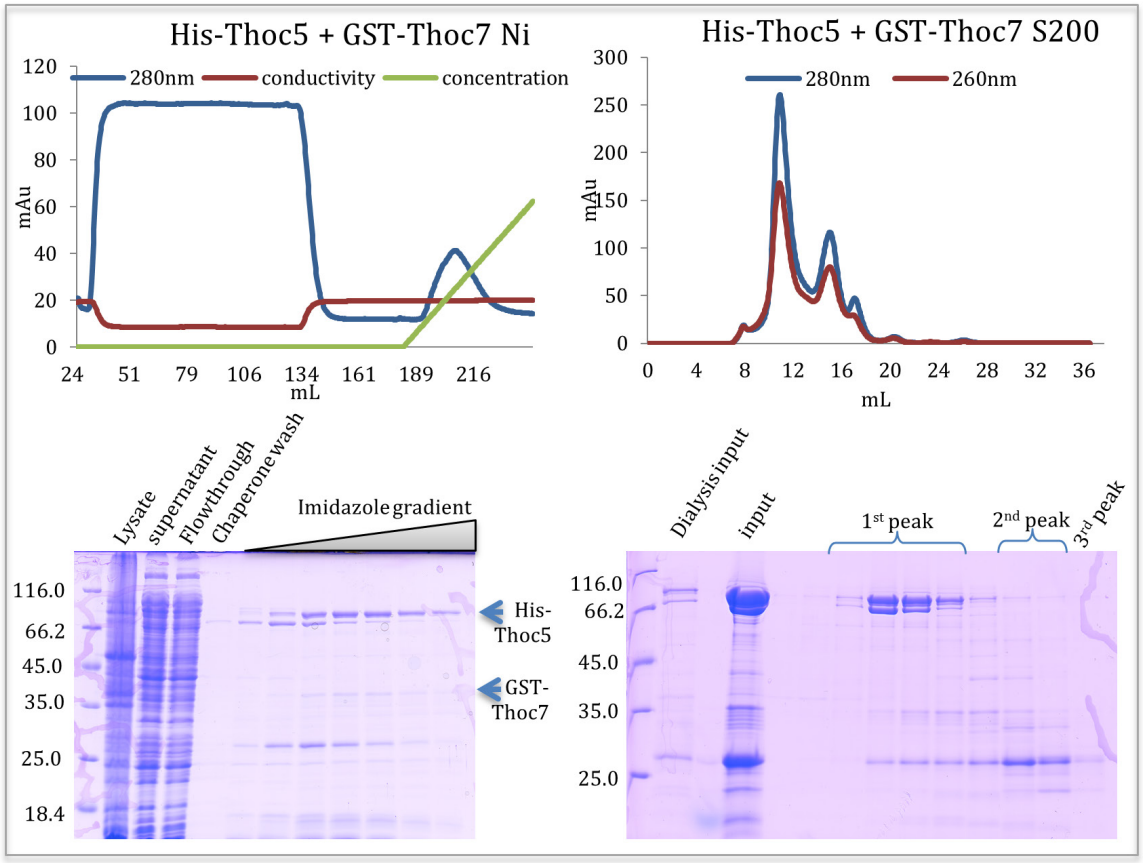


Fig.40: Coexpression of His-Thoc5 and GST-Thoc7

top left: chromatogram of Ni-column; *top right:* chromatogram of gel filtration in S200; *bottom left:* SDS-PAGE of Ni-affinity; *bottom right:* SDS-PAGE of gel filtration

4.3.6. Co-lysis of His-Thoc5 and GST-Thoc7

As further approach to obtain a stable complex, co-lysis of two independently grown pellets of His-Thoc5 and GST-Thoc7 was tested. The cells were separately resuspended and merged before lysing. The lysate was loaded onto a Ni-affinity column and washed with salt-free chaperone buffer. SDS-Page analysis showed only a faint elution of His-Thoc5 and most of the protein being aggregated (Fig.41). Buffers used for the purfication are listed in table 16.

Table 16: List of buffers used for the purification co-expressed His-Thoc5 and GST-Thoc7

Lysis	Ni-elution
$T_{20}N_{100}$	$T_{20}N_{100}I_{500}$

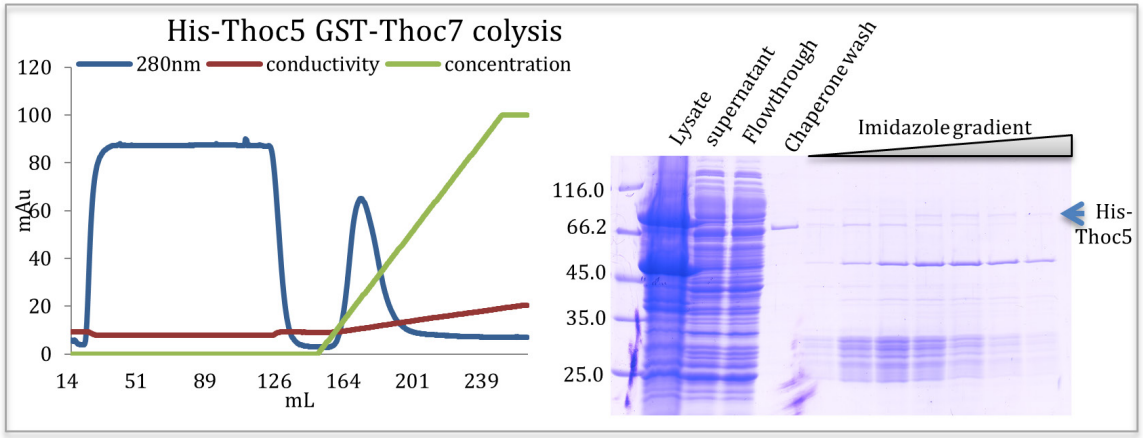
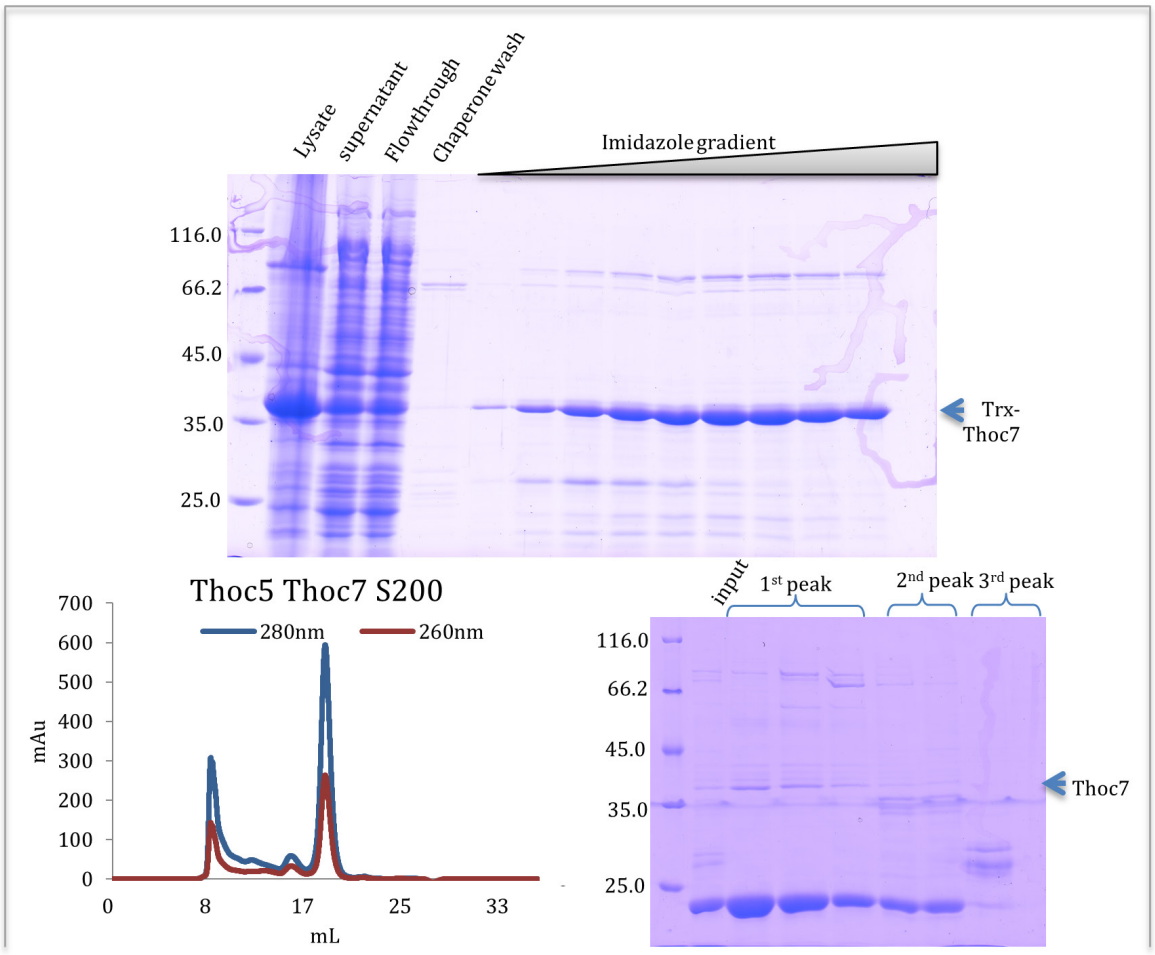


Fig.41: Co-lysis of His-Thoc5 and GST-Thoc7
left: chromatogram of Ni-column; *right:* SDS-PAGE of Ni-affinity

4.3.7. Co-expression of Trx-Thoc7 and His-Thoc5

To enhance the solubility, different tags were tried. First Trx-tagged Thoc7 was cloned and co-expressed with His-Thoc5 in BL21 gold cells. For purification the cells were lysed and applied onto a Ni-column. SDS-PAGE analysis revealed high expression of Trx-Thoc7 and purification of the soluble protein. His-Thoc5 was expressed poorly, with several degradation products. Half of the sample was cleaved with TEV-protease to remove the tag in overnight dialysis. Cut and uncut sample were injected into gel filtration. SDS-PAGE showed only a faint double-band of His-Thoc5 and degradation products, and Trx-Thoc7 showed unspecific interaction with the column material, as protein was detected in every fraction (Fig.42). Table 17 lists the buffers used for the purification.



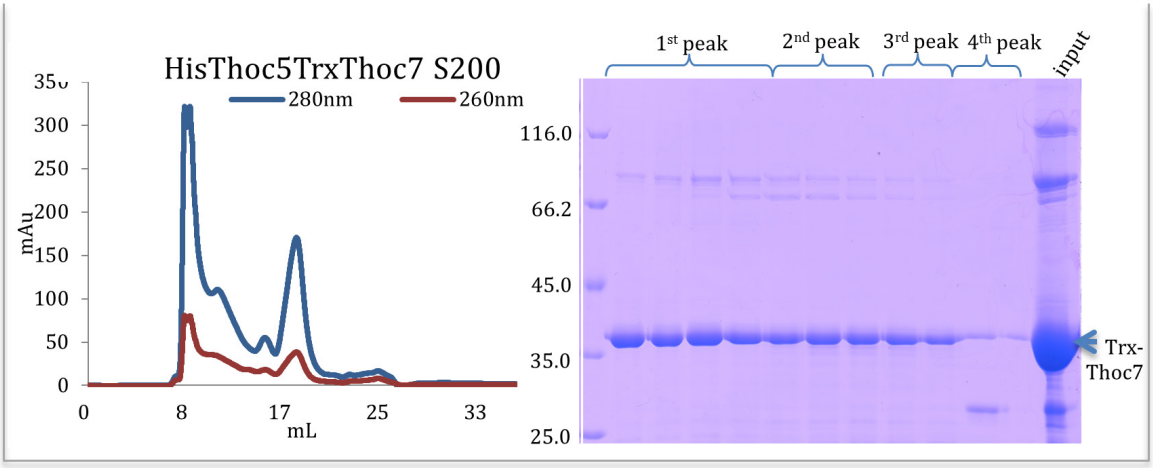


Fig.42: Purification of Trx-Thoc7

top: SDS-PAGE of Ni-affinity purification; *middle left:* Chromatogram of gel filtration of Trx-cleaved Thoc7 with His-Thoc5; *middle right:* Chromatogramm of gel filtration of Trx-Thoc7 with His-Thoc5; *bottom left:* SDS-PAGE of Thoc7-His-Thoc5 gel filtration; *bottom right:* SDS-PAGE of Trx-Thoc7 – His-Thoc5 gel filtration

Table 17: List of buffers used for the purification co-expressed His-Thoc5 and GST-Thoc7

Lysis	Ni-elution	Dialysis
$T_{20}N_{200}$	$T_{20}N_{100}I_{500}$	$T_{20}N_{100}Gly_5DTT_1$

4.3.8. Purification of Sumo-Thoc7

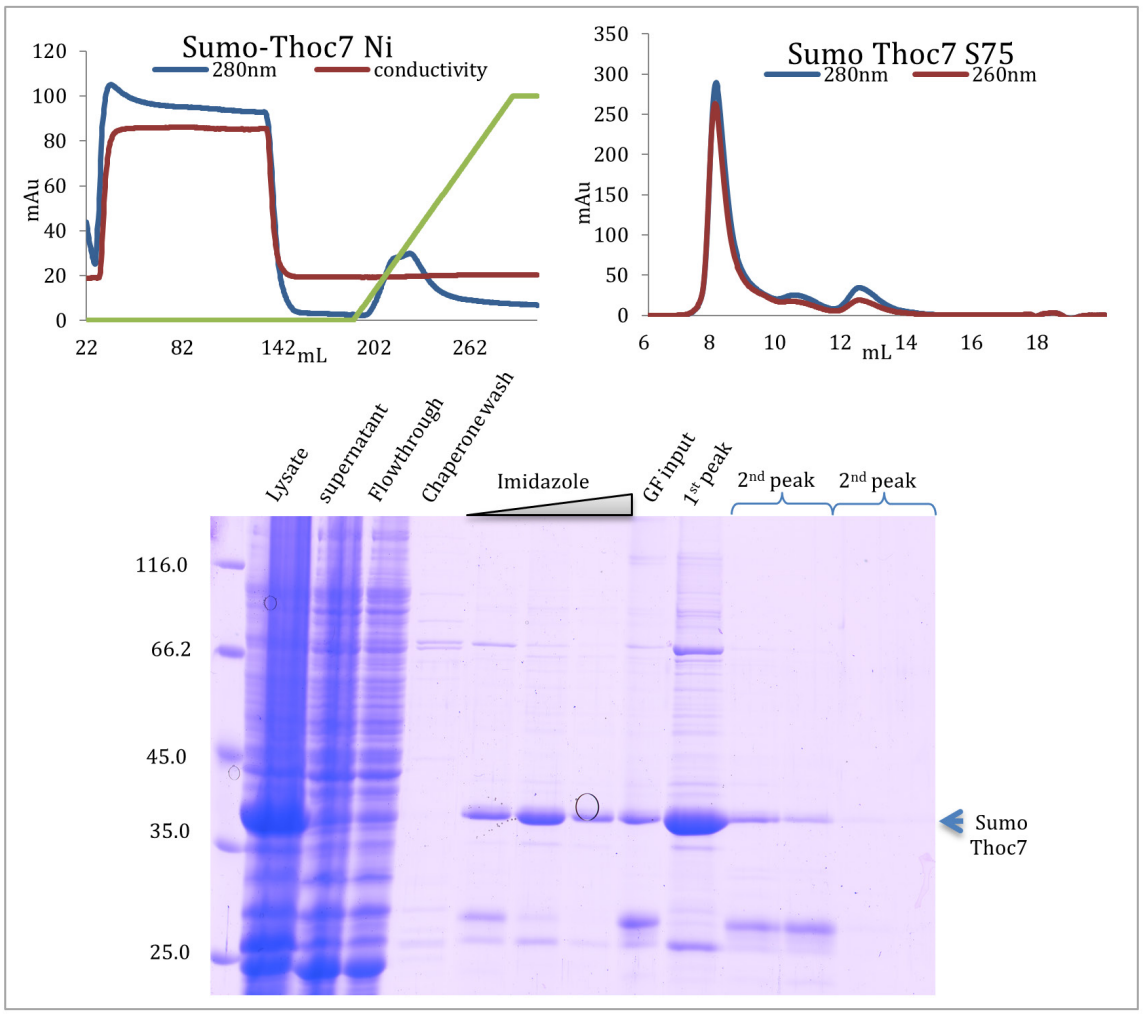


Fig.43: Purification of Sumo-Thoc7

top left: Chromatogram of Ni-column; *top right:* Chromatogram of gel filtration of Sumo-Thoc7; *bottom:* corresponding SDS-PAGE

Sumo fusions have been shown to improve the solubility. Therefore, Sumo-Thoc7 was cloned and expressed in BL21 gold cells, and purified using a Ni-affinity column. Subsequent gel filtration with SDS gel analysis showed a small peak of non-aggregated sample eluting after the aggregation peak (Fig.43), which was quite promising. Buffers used for the purification are listed in table 18.

Table 18: List of buffers used for the purification of Sumo-Thoc7

Lysis	Ni-elution	Dialysis
$T_{20}N_{200}$	$T_{20}N_{100}I_{500}$	$T_{20}N_{100}Gly_5DTT_1$

4.3.9. Complex assembly

To test for complex formation with His-Thoc5, gel filtration of Sumo-Thoc7 with His-Thoc5 was performed. A molar ratio of 3:1 for Sumo-Thoc7:His-Thoc5 was chosen to compensate for aggregated Sumo-Thoc7. SDS-PAGE analysis of the elution profile showed an elution of both His-Thoc5 and Sumo-Thoc7 over all peaks. Furthermore, a non-stoichiometric complex elution indicates a co-elution by incomplete peak separation, rather than a stable complex formation. (Fig.44) The double band of His-Thoc5 indicates degradation products of the protein.

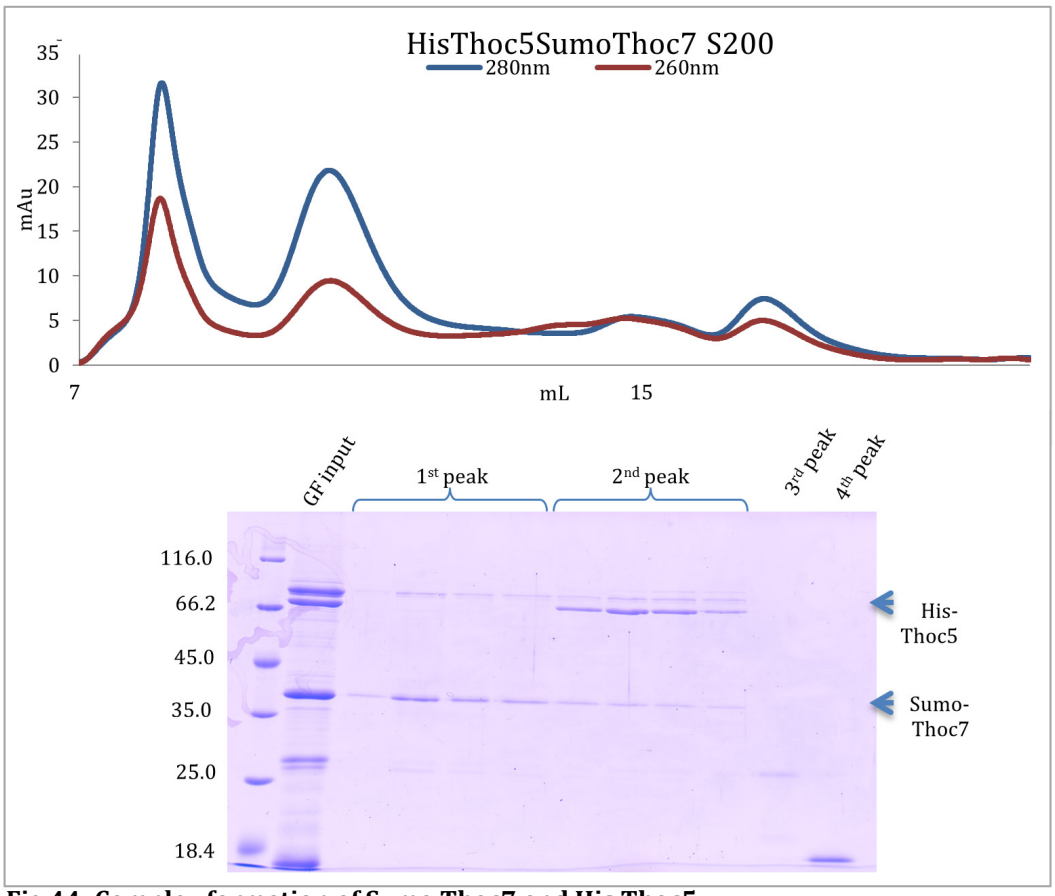


Fig.44: Complex formation of Sumo-Thoc7 and His-Thoc5
top: Chromatogram of gel filtration; *bottom:* corresponding SDS-PAGE

4.3.10. Thermoflour-assays

Thermoflour assays of the non-aggregated fraction of Sumo-Thoc7 was hoped to indicate buffer conditions more suitable for protein stability, yielding less aggregated sample. The sample was used at a concentratioin of 2 mg/mL. Highest melting temperature and biggest height of the curve was obtained for a Tris-based buffer at pH 8, supplemented with 10 mM Imidazole. The meassurements were perforemed by Clarie Basquin.

4.3.11. Purification of Sumo-Thoc7 in $T_{20}N_{50}I_{10}$

Purification in the buffer conditions obtained from Thermoflour was tested. The protein was lysed in $T_{20}N_{50}I_{10}$ and after sonication and centrifugation incubated with the Ni-beads for 1 h. The beads were then applied onto gravity columns and extensively washed with lysis-buffer and high-salt chaperone buffer, and eluted with $T_{20}N_{100}I_{500}$. The obtained protein was subjected to gel filtration. SDS-PAGE analysis showed no improvement in yield, as most of the protein remains aggregated. (Fig.45)

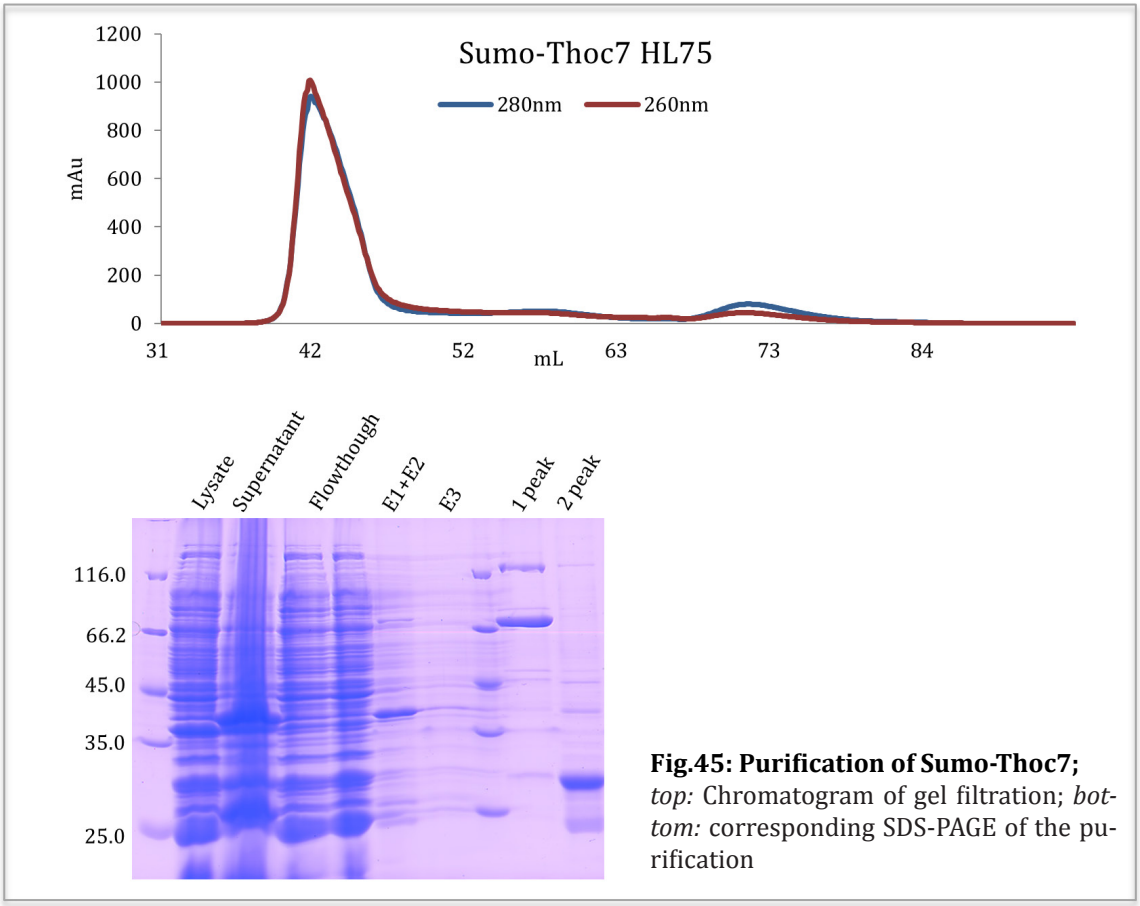


Fig.45: Purification of Sumo-Thoc7; *top:* Chromatogram of gel filtration; *bottom:* corresponding SDS-PAGE of the purification

5. Discussion and Outlook

5.1. Reconstitution of the Yra1-Sub2 and Yra1-Mex67 complex from *Chaetomium thermophilum*

Based on the well-known behaviour of Yra1, Sub2 and Mex67 from Saccharomyces cerevisiae, ctYra1 was expected to build a stable complex with ctSub2 and a transient complex with ctMex67. However, pull down assays and gel filtration of revealed no binding of ctYra1 to ctSub2 and a binding of ctYra1 to ctMex67 was only detected in pull down assays, not in gel filtration. One possible explanation for this behaviour is a dilution effect in gel filtration. Transient complexes possess high dissociation constants, leading to a disassembly in highly diluted samples, such as in gel filtration experiments. To measure the K_D of this transient complex ITC measurements were performed, however, no K_D value was measurable. These inconclusive results might be due to several reasons: First, ctYra1 might have been degraded during ITC measurements. As the measurement occurred at 25 °C for a longer time, His-ctYra1 might not have been stable enough. A loss of the entire or a partial binding domain might decrease the amount of interacting proteins, and hence a non-detectable binding. Increasing the concentration of His-ctYra1 might be required for successful measurements. Second, pull-down assays were performed with GST-ctYra1, whereas for ITC measurements His-ctYra1 was used. Partial unfolding of His-ctYra1 during purification might be one explanation for a lack of binding of ctYra1 to ctMex67 in the ITC measurement. Unstructured ctYra1 might lack the binding epitope for ctMex67. To test for this hypothesis in further experiments, untagged ctMex67 should be used in pull down assays with His-ctYra1.

```
CtYra1      MSGKLDQTLDEILSSQRRDRNRRRSRRSSGAKATTNTAPAGGVQKSSKAARNATKPTPAK 60
ScYra1      MSANLDKSLDEIIGSNKAGSNRARVGGTRGN----------GPRRVGKQVGSQRRSLPNR 50
            **.:**::****:.*:: . ** *   : *           * :: .* . .  :. * :

CtYra1      TGGLTGESKIVVSNLPKDVSEAQIKGILGGRLDPCDTMSWSLGTGRRRLSTLGLEEYFQQ 120
ScYra1      RGPIRKNTRAPPNAVAR----------VAKLLDTTREVKVNVEGLPRDIKQDAVREFFAS 100
             * :  :::   . :.:          :.  **.   :. .:    * :.  .:.*:* .

CtYra1      SVGQVKKVELSYGPGGVSRGIAHVTFHHADGASKAFASLNGLLIDNKPVKVEVVVANADL 180
ScYra1      QVGGVQRVLLSYNERGQSTGMANITFKNGELARRAVERFNGSPIDGGRSRLRLNLIVDPN 160
            .** *::* ***.  * * *:*::**::.: * :*.  :**  **.   ::.: :

CtYra1      IPQPKPLAARIAQPKAQPKSAATVKNTNGNKEGGAGKKKTRRGRSNRPVKKTLEELDSEM 240
ScYra1      QRPVKSLADRIKAMPQKGGNAPRPVKRGPNRKAAMAKSQNKPKR-EKPAKKSLEDLDKEM 219
                *.** **     :  .*.   : . *::.. .*.:.:  * ::*.**:**:**.**

CtYra1      ADYFASANTNDTNANNANGTSSATAAGGDAPMEEDVL 277
ScYra1      ADYFEKK------------------------------ 226
            **** .

score: 29.0
```

Fig.46: Sequence alignment of Chaetomium thermophilum and Saccharomyces cerevisiae Yra1

by http://www.ebi.ac.uk/Tools/services/web_clustalw2

The unexpected behaviour of ctYra1 and ctSub2 could have different reasons: Although unlikely, it cannot be excluded, that mRNA export functions differently in Chaetomium thermophilum than in Saccharomyces cerevisiae. High homologies of export and adaptor factors (Figs.46+47+48) throughout the eukaryotic kingdom indicates an evolutionary conserved pathway of mRNA export. More likely is that the

applied in-vitro system does not represent native conditions. GST- and His-tag might prevent a binding of ctSub2 to tagged ctYra1. However, untagged ctYra1 showed to be not stable enough for purification and interaction studies. Furthermore, truncations of ctYra1 might have increased affinity to ctSub2, but might be lacking the affinity tag for pull-down analysis. This might lead to a false-negative pull-down assay. Pull-down assays with tagged Sub2 an untagged Yra1 might be suitable for testing this hypothesis. In addition, RRM-containing proteins are known for instability and un-predictable behaviour, explaining the inconclusive gel filtration analysis.

```
CtMex67      MAPPTGPRRGGSAQKTALRPTRGGGISKHRPAPKTDIDGDIAMGGPPADTSKRGSGSSGR 60
ScMex67      -------------------------------------------------MSGFHNVGN 9
                                                                 *  . *.

CtMex67      GARAGRGGRATSRMAQNIRNYATELAVGSNGAKTQPNKTVIKILGLKNSKAASNPDGGLR 120
ScMex67      INMMAQQQMQQNRIKISVRNWQNATMN------------------------------- 36
                 .:     .*:  .:**: .

CtMex67      SLLDFLERKSKEKITLGRGIIDGDYVWLKVN-KDDAQHLLRLNGFTYAGATLTIEETNEP 179
ScMex67      DLINFISRNAR--VAVYDAHVEGPLVIGYVNSKAEAESLMKWNGVRFAGSNLKFELLDN- 93
             .*::*:.*:::  :::  . ::*  *   ** * :*: *:: **. :**:.*.:*  ::

CtMex67      MPALSSQSKLSQAAQETKQKLTSCLARRYNAEQKLLDLSALGTDETLSSLGSFNNQSLAE 239
ScMex67      -------NGASAGTSDTISFLRGVLLKRYDPQTKLLNLGALHSDPELIQKGVFSSISTQS 146
                    .  * .:.:* . * . * :**:.: ***:*.** :*  * . * *.. *  .

CtMex67      KSFKALMHLVSNEYKDPEQKNEAIQAVSLARNDILDVGQVYSLAVTLPRLRRLDLSGNNL 299
ScMex67      KMFPAMMKLAS------TEKSLIVESVNLADNQLKDISAISTLAQTFPNLKNLCLANNQI 200
             * * *:*:*.*       :*.  :::*.** *:: *:. : :** *:*.*:.* *:.*::

CtMex67      ENLSKISKWQQEFRFLEELHLTGNPVTTLPNYATEIKKWFPSLQILDGQQIRTPQEAAES 359
ScMex67      FRFRSLEVWKNKFKDLRELLMTNNPITTDKLYRTEMLRLFPKLVVLDNVIVRDEQKLQTV 260
              .: .:. *:::*: *.** :*.**:**   * **: : **.* :**.  :*  *:

CtMex67      LKSSFPTPLPQLPSNVRDGENNVASTFLQAFFQLWDHDRLTLIPQFYDS-------ETTF 412
ScMex67      YS--LPMKIQQFFFEN-DALGQSSTDFATNFLNLWDNNREQLLNLYSPQSQFSVSVDSTI 317
              .  :*  : *:  :  *. .: :: *   *::***::*  *:  :  .       ::*:

CtMex67      SVVFATDSPQDPASSSCSKFSRNLNILSPRHPSTLQRLFVGSNLIADLWKVLPATRHPSL 472
ScMex67      PPSTVTDSDQTPAFGYYMSSSRNISKVSSEK-SIQQRLSIGQESINSIFKTLPKTKHHLQ 376
             .   .*** * ** .   . ***:. :*..: *  *** :*.: * .::*.** *:*

CtMex67      DQTSQWLIDCHTFPHLA----------DPTGMAPYAMGLMINVNG-------QCEEADIS 515
ScMex67      EQPNEYSMETISYPQINGFVITLHGFFEETGKPELESNKKTGKNNYQKNRRYNHGYNSTS 436
             :*..:: ::  ::*::           : ** .    .   . *.       :    . *

CtMex67      QNLYGTRTFSRCFILGPSKPGAPHPYRVLSD-QLTLHTWK------PQPAPQVG------ 562
ScMex67      NNKLSKKSFDRTWVIVPMNNSVIIASDLLTVRAYSTGAWKTASIAIAQPPQQQASVLPQV 496
             :*  ..::*.* ::: * : ..  .  :*:    :  :**      .**. * .

CtMex67      -------TVPPPAVAAPMPGAVPIPTATPAVAPVPPVMGDEPDDAT-----KAQLIAEVS 610
ScMex67      ASMNPNITTPPQPQPSVVPGGMSIPGAPQGAMVMAPTLQLPPDVQSRLNPVQLELLNKLH 556
                    *.** . .: :**.:.** *. ..  :.*.:   **  :     : :*: ::

CtMex67      RRTGMNVEYSQMCLTGAANWNLELALQSFEQQKANVPPEAFISQPQV 657
ScMex67      LETKLNAEYTFMLAE-QSNWNYEVAIKGFQSSMNGIPREAFVQF--- 599
              .* :*.**: *     :*** *:*::.*:..  .:* ***:.

score 23.0
```

Fig.47: Sequence alignment of Chaetomium thermophilum and Saccharomyces cerevisiae Mex67

by http://www.ebi.ac.uk/Tools/services/web_clustalw2

Further experiments would require an optimization of ctYra1 purification. Only homogenous, stable protein allows precise interpretation of the experiments. One possibility to enhance ctYra1 purity might be by inserting longer His-tags on C- and N-terminus. This might increase the affinity of His-ctYra1 to the Ni-column, leading to a

higher yield by reducing protein loss in the flow-through and to a purer sample with less protease contamination. Furthermore, other interaction partners of ctYra1, like Nab2, might stabilize the protein and the binding to ctMex67 of ctSub2.

```
CtSub2      MS--AEEDLIDYSDDE----------LNNESTGPSANGKKADAAASTQNVDKKGSYVGIH 48
ScSub2      MSHEGEEDLLEYSDNEQEIQIDASKAAEAGETGAATSATEGDNNNNTAAGDKKGSYVGIH 60
            **  .****::***:*           :  .**.::...:.*   .*   **********

CtSub2      STGFRDFLLKPELLRAIADCGFEHPSEVQQTCIPQAMLGGDIICQAKSGLGKTAVFVLTT 108
ScSub2      STGFKDFLLKPELSRAIIDCGFEHPSEVQQHTIPQSIHGTDVLCQAKSGLGKTAVFVLST 120
            ****:******** *** ************  ***:: * *::****************:*

CtSub2      LQQVEPVPGECSVLVMCHTRELAFQIRNEYNRFSKYMPEIKTGVFYGGTPIQKDAEILKN 168
ScSub2      LQQLDPVPGEVAVVVICNARELAYQIRNEYLRFSKYMPDVKTAVFYGGTPISKDAELLKN 180
            ***::***** :*:*:*::****:****** *******::**.********.****:***

CtSub2      KDTHPHIIVGTPGRLNALVREKYLRLGSVRMFVLDECDKMLDQIDMRRDVQEIFRATPQQ 228
ScSub2      KDTAPHIVVATPGRLKALVREKYIDLSHVKNFVIDECDKVLEELDMRRDVQEIFRATPRD 240
            *** ***:*.*****:*******: *. *: **:*****:*:::**************::

CtSub2      KQVMMFSATLSDEIKPICRKFMQNPTEHYVDEDTKLTLHGLQQYYIALEEREKNRKLNEL 288
ScSub2      KQVMMFSATLSQEIRPICRRFLQNPLEIFVDDEAKLTLHGLQQYYIKLEEREKNRKLAQL 300
            ***********:**:****:*:*** * :**:::************ ********** :*

CtSub2      LDELQFNQVIIFVKSTLRATELDKLLRECNFPSIAVHSGISQEERIRRYKEFKEFNKRIC 348
ScSub2      LDDLEFNQVIIFVKSTTRANELTKLLNASNFPAITVHGHMKQEERIARYKAFKDFEKRIC 360
            **:*:*********** **.** ***. .***:*:**. :.***** *** **:*:****

CtSub2      VATDVFGRGIDIERINLAINYDMPADADSYLHRVGRAGRFGTKGLAISFVSSDQDREVLK 408
ScSub2      VSTDVFGRGIDIERINLAINYDLTNEADQYLHRVGRAGRFGTKGLAISFVSSKEDEEVLA 420
            *:*******************:. :**.**********************.:*.***

CtSub2      AIEKRFEVALPEFPKEGVDASTYMAS 434
ScSub2      KIQERFDVKIAEFPEEGIDPSTYLNN 446
             *::**:* :.***:**:*.***: .

Score 71.0
```

Fig.48: Sequence alignment of Chaetomium thermophilum and Saccharomyces cerevisiae Sub2

by http://www.ebi.ac.uk/Tools/services/web_clustalw2

5.2. Putative TREX-complex interacting proteins in yeast

5.2.1. Sto1

An interaction between subunits of the CBC and components of the TREX complex was reported in human to direct the export of intronless mRNA from the nucleus. However, gel filtration of yeast Sto1 with one component of the TREX complex, Sub2, as well as pull down assays with the THO and TREX complex showed no binding of any component to Sto1, neither in presence nor absence of an ATP analog.
One explanation of the different behavior in yeast from the reported binding in human could be insufficient homology between the yeast and human protein. This can be seen in a sequence alignment of Sto1 with different subunits of the human CBC (Fig.49) With a scoring of 5 and 12 only low homology can be seen between the human and the yeast proteins.

However, also only the whole CBC might display the binding interface between the human subunit and the TREX-subunits. Hence, only a complex of Sto1 and CBC2, forming the entire yeast CBC would show an interaction with the TREX complex. This would represent the low sequence homology between yeast and human and indicate a functional homology. However, a whole complex as interaction partner would be not suited to facilitate crystallization.

The different binding behavior in yeast and human could also indicate different recruitment models. Export mechanism of splicing-independent mRNA in human could differ from the export of intronless mRNA in yeast, being more related to the export of spliced mRNA in human than to mRNA export in yeast.

```
Sto1            MFNRKRKGDFDDEDYHDYRPRMPKRQRIPPVVQLCKEMMPDIRTIG-ESVKAFEEDIQFL 59
NCBP1           -MSRRRHSDENDG------GQPHKRRKTSDANETEDHLESLICKVGEKSACSLESNLEGL 53
                 :.*:*:.* :*        :  **:: . . :  ..: . * .:* :*. ::*.::: *

Sto1            SDAIVNEFGHEEYFNDALLQTFRAVVLEQPQKLPAIALLTMVVNSRNEAAGKGVVNFFFS 119
NCBP1           AGVLEADL---PNYKSKILRLLCTVARLLPEKLTIYTTLVGLLNARNYNFGGEFVEAMIR 110
                :..:  ::     ::. :*: : :*.   *:**.  : *. ::*:**   *  .*: ::

Sto1            ELQKYCNQSVDPEYKPESSDTGPWNKIKLILRFLATLS--PMLLNDELISIFKDFLQLSI 177
NCBP1           QLK-------------ESLKANNYNEAVYLVRFLSDLVNCHVIAAPSMVAMFENFVSVTQ 157
                :*:             ** .:. :*:   ::***: *    ::   .::::*::*:.::

Sto1            DLNQSDTNKRNPLSEALYTNTLLNIPYLFFFNRNDDDLKQKVGSLLEFVEDNYKVKSTEI 237
NCBP1           EEDVPQVRRDWYVYAFLSS---LPWVGKELYEKKDAEMDRIFANTESYLKR---RQKTHV 211
                : : .:..:   :   * :   *      :::::* ::.: ...  .:::     :.*.:

Sto1            NLLREYNNGAPFDSAELVQLVLSNVKKSLMDDLKDLEQLFPDWIHLLTEQSGDQGFNDPL 297
NCBP1           PMLQVWTADKPHPQEEYLDCLWAQIQKLKKDRWQERHILRP-----------YLAFDSIL 260
                 :*: :. . *. . * :: : ::::*   *  :: . * *             .*:. *

Sto1            QLPSIEDLAEYMDLGHQRGSVDSMWHTPRYVFKVYVPNETHDFETVIPITTYAGQLFNDI 357
NCBP1           CEALQHNLPPFTPPPH---TEDSVYPMPRVIFRMFDYTDDPEGPVMPGSHSVERFVIEEN 317
                  .  .:*. :    *   : **::  ** :*:::  .:  :  .:    :    ::::

Sto1            IIDLVESLEFNRKEVARQVVSLDLFFKKGIFAEPGISIAQLSNVYEENPLATTFKIEDLA 417
NCBP1           LHCIIKSHWKERKTCAAQLVSY-------------------------PGKNKIPLNYHI 351
                :  :::*   :**  * *:**                          *  ..: ::

Sto1            IETIISLIFKLPDVSQPFAYFYTLLVEICQNSPKAIAPVFGRAFRFFYSHLKNMDFELRL 477
NCBP1           VEVIFAELFQLPAPPHIDVMYTTLLIELCKLQPGSLPQVLAQATEMLYMRLDTMNTTCVD 411
                :*.*:: :*:**  .:  . : ***:*:*: .* ::. *:.:* .::* :*..*:
```

```
Sto1            RYLDWFSIQMSNFNFSWKWNEWEDDSKNLSKSFYDPNINFIRNLIHKELRLTSNPIDVEE 537
NCBP1           RFINWFSHHLSNFQFRWSWEDWSD---CLSQDPESPKPKFVREVLEKCMRLS-------- 460
                *:::*** ::***:* *.*::*.*    **:.  .*: :*:*:::.* :**:

Sto1            SLPEEFKQYLDSSYIAHDALVAYYQSFYVDYTVEPINIKKNDFYFKHESSPLRDVVVELL 597
NCBP1           ----YHQRILDIVPPTFSALCPANPTCIYKYGDESSNSLPG----------HSVALCLAV 506
                     .:: **    :..** .   :   .*  *. *   .             .:   :

Sto1            DYIHKPNNTREVSELEQLLEKIKANHGSIIKNFDRFIIVLIVQALLESGSRSLSHANKYI 657
NCBP1           AFKSKATNDEIFSILKDVPNPNQDDDDDEGFSFNPLKIEVFVQTLLHLAAKSFSHSFSAL 566
                 :  *..* . .* *::: :  : :...   .*: : * ::**:**. .::*:**: . :

Sto1            SDLKDDFKYVLDKIELDQDQKEFIIIEAVIRFWNSNSQNGYLIVDAFKFAELVSSRSIIN 717
NCBP1           AKFHEVFKTLAE-----SDEGKLHVLRVMFEVWRNHPQMIAVLVDKMIRTQIVDCAAVAN 621
                :.::: ** : :     .*: :: ::..::..*..:.*   ::** :  :::*.. :: *

Sto1            FALNEELANNYG-LVDSTAIEAIFRTLSHEITLEIEHADDFEFVLEKLCIIINNTVSQLN 776
NCBP1           WIFSSELSRDFTRLFVWEILHSTIRKMNKHVLKIQKELEEAKEKLARQHKRRSDDDDRSS 681
                : :..**:.::  *.    :.: :*.:.:.:    :. :: :  * :     .:  .: .

Sto1            IQLD--EDIDVPQIFELTDGDNASDLAAYDLKWKYYTSIVFIKSL--------------- 819
NCBP1           DRKDGVLEEQIERLQEKVESAQSEQKNLFLVIFQRFIMILTEHLVRCETDGTSVLTPWYK 741
                 : *   : :: :: * .:. ::.:   : : :: :  *:  : :

Sto1            -----LRKYSLKYKSLSDKILGNLDTAVPHQSTKEQIKIWLGELNSI-- 861
NCBP1           NCIERLQQIFLQHHQIIQQYMVTLENLLFTAELDPHILAVFQQFCALQA 790
                     *::  *:::.: :: : .*:. :   . . :*   : :: ::
```

Fig.49: Sequence alignment of yeast Sto1 and human NCBP1,
by http://www.ebi.ac.uk/Tools/services/web_clustalw2

5.2.2. Syf1

Interference with loading of the TREX-complex in Syf1 deletion mutants and synthetic lethality assays showed interaction of a TREX-subunit with Syf1. However, no binding could be seen in pull down assays and gel filtration under all tested conditions. This indicates only indirect interaction of Syf1 with the TREX-complex via the Prp19 complex (Fig.50). Hence it is likely, that low occupancy of the TREX complex on mRNA in Syf1-deletion is due to lost binding of the Prp19 complex to its upstream effector, not due to a lost Prp19-TREX-complex interaction.

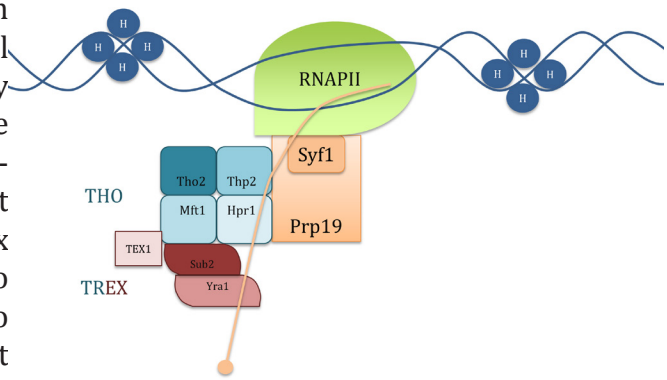


Fig.50: Recruitment model of the TREX complex dependent on Prp19 and Syf1

5.2.3. Tho1

Reported interaction of the human proteins Cip29, Uap56 and Aly lead to the idea of a possible trimeric complex between the yeast homologues Tho1, Sub2 and Yra1. However, pull-down of purified samples in presence and absence of an ATP analogue showed no interactions between the Yra1-Sub2 subcomplex and Tho1.
The sequence alignment of yeast Tho1 with human Cip29, as shown in Fig.51, shows only moderate homology with a scoring of 18.0. This might be the reason for the species specific differences in the binding behaviour. Involvement of Tho1 in the mRNA export pathway is based on its capability of suppressing Hpr1-deletion phenotype by over expression of Hpr1. Results from the pull-down assays conclude an interaction of Tho1 with the mRNA export pathway different than Cip29.

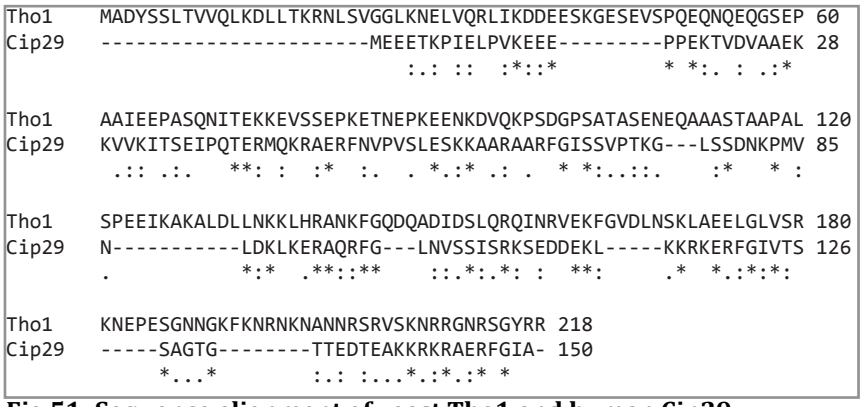
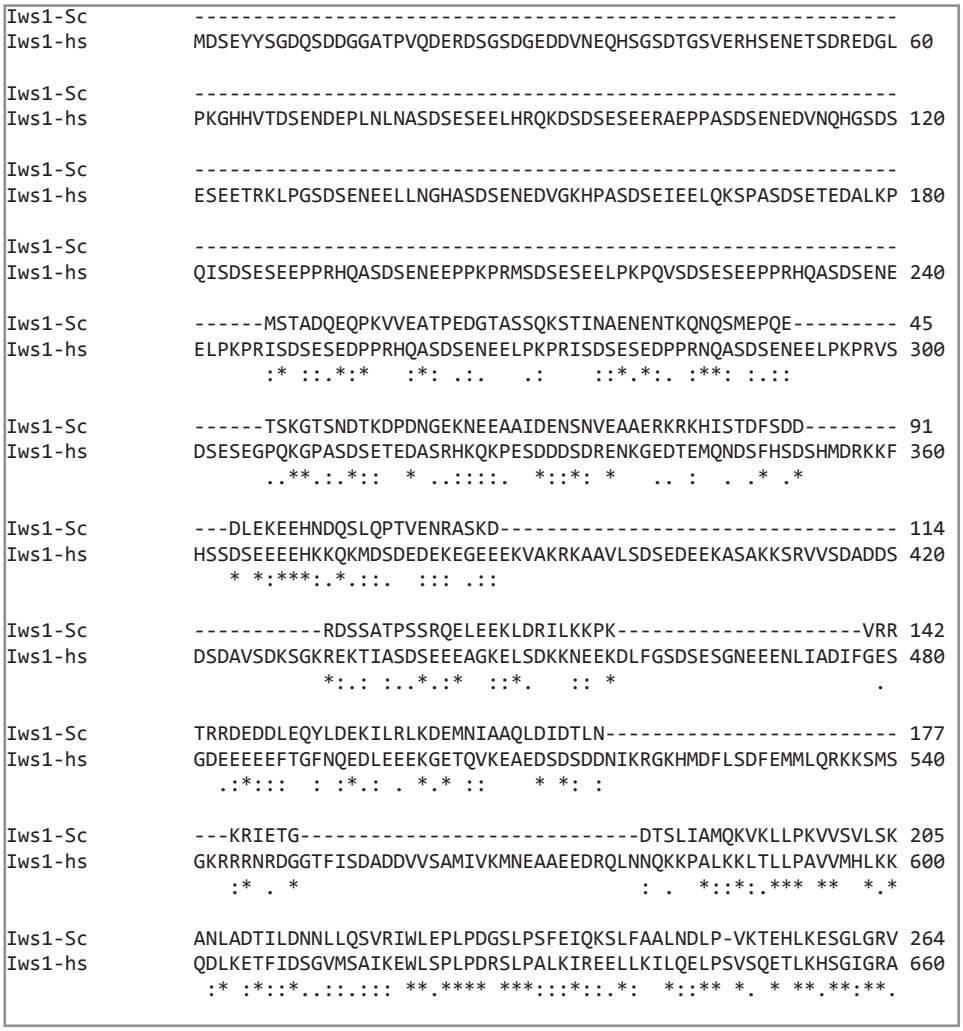


Fig.51: Sequence alignment of yeast Tho1 and human Cip29,

by http://www.ebi.ac.uk/Tools/services/web_clustalw2

5.2.4. Iws1

Human Iws1 was reported to function as adaptor between Spt6 and Aly/REF. This interaction couples transcription elongation via the histone-chaperone Spt6 with mRNA export. Sequence alignment of yeast Iws1 with human Iws1 shows an elongated N-terminus in the human homologue, not present in yeast (Fig.52). This N-terminus might be required for an interaction with Aly/REF. Functions fulfilled by the core of Iws1 might still be conserved, however the bridging of transcription elongation to mRNA export might either be performed by a different component in the cell or might indicate differences in the recruitment and quality control pathway.



```
Iws1-Sc         VIFYTKSKRVEAQLARLAEKLIAEWTRPIIGASDNYRDKRIMQLEFDSEKLRKKSVMDSA 324
Iws1-hs         VMYLYKHPKESRSNKDMAGKLINEWSRPIFGLTSNYKG-------MTREEREQRDLEQMP 713
                *::  *  : . .   :* *** **:***:* :.**:.       :  *: .::.: : .

Iws1-Sc         KNRKKKSKSGEDPTSRGSSVQTLYEQAAARRN-----RAAAPAQTTTDYKYAPVSNLSAV 379
Iws1-hs         QRRRMNSTGGQTPRRDLEKVLTGEEKALRPGDPGFCARARVPMPSNKDYVVRPKWNVEME 773
                :.*: :*..*: *    ..* *  *:*    :     ** .*  :..**   *  *:.

Iws1-Sc         PTNARAVGVGSTLNNSEMYKRLTSRLNKKHK--------------- 410
Iws1-hs         SSRFQATSKKGISRLDKQMRKFTDIRKKSRSAHAVKISIEGNKMPL 819
                .:. :*..  .  . .:  :::*.  :*.:.
```

Fig.52: Sequence alignment of yeast and human Iws1,
by http://www.ebi.ac.uk/Tools/services/web_clustalw2

5.3. Reconstitution of the subcomplex of Thoc5 and Thoc7

The third project aimed for the reconstitution of the binary complex of Thoc5 and Thoc7 from the human TREX-complex. Purification of the individual proteins showed the formation of soluble aggregates in case of Thoc7. Changes of the tag to enhance solubility showed only little improvements. Co-expression of both proteins led to a suppression of Thoc7 expression, and Co-lysis yielded both proteins aggregated in the pellet.

Further attempts to purify the complex could include mixing of the samples after lysis and before loading onto Ni-column. This would lead to dilution of Thoc7 and might help to decrease aggregation of Thoc7 by first decreasing Thoc7-Thoc7-interactions and second by stabilizing Thoc7 by binding to Thoc5. Resuspension of the Thoc7 culture in bigger volumes of lysis buffer might furthermore decrease the amount of soluble aggregate. Aggregation during expression could be decreased, by enhancing chaperone expression in the culture. Higher levels of chaperone might help proper folding of Thoc7 and might stabilize the monomers. Increased chaperone expression could be achieved by heat-shock response through growing the culture at 40-45°C for a short period before induction.

If attempts to improve binary complex formation remain unsuccessful, it might indicate that the binary complex is alone not stable enough and may require a further interaction partner. Therefore, other subunits of the TREX-complex should be tested for direct interaction with the binary complex.

6. References

Abruzzi, K.C., Lacadie, S., and Rosbash, M. (2004). Biochemical analysis of TREX complex recruitment to intronless and intron-containing yeast genes. Embo J 23, 2620-2631.
Chanarat, S., Seizl, M., and Strasser, K. (2011). The Prp19 complex is a novel transcription elongation factor required for TREX occupancy at transcribed genes. Genes Dev 25, 1147-1158.
Chavez, S., Beilharz, T., Rondon, A.G., Erdjument-Bromage, H., Tempst, P., Svejstrup, J.Q., Lithgow, T., and Aguilera, A. (2000). A protein complex containing Tho2, Hpr1, Mft1 and a novel protein, Thp2, connects transcription elongation with mitotic recombination in Saccharomyces cerevisiae. Embo J 19, 5824-5834.
Cook, A., Bono, F., Jinek, M., and Conti, E. (2007). Structural biology of nucleocytoplasmic transport. Annu Rev Biochem 76, 647-671.
Cook, A.G., and Conti, E. (2010). Nuclear export complexes in the frame. Curr Opin Struct Biol 20, 247-252.
Dufu, K., Livingstone, M.J., Seebacher, J., Gygi, S.P., Wilson, S.A., and Reed, R. (2010). ATP is required for interactions between UAP56 and two conserved mRNA export proteins, Aly and CIP29, to assemble the TREX complex. Genes Dev 24, 2043-2053.
Espinet, C., de la Torre, M.A., Aldea, M., and Herrero, E. (1995). An efficient method to isolate yeast genes causing overexpression-mediated growth arrest. Yeast 11, 25-32.
Haun, R.S., Serventi, I.M., and Moss, J. (1992). Rapid, reliable ligation-independent cloning of PCR products using modified plasmid vectors. Biotechniques 13, 515-518.
Hautbergue, G.M., Hung, M.L., Golovanov, A.P., Lian, L.Y., and Wilson, S.A. (2008). Mutually exclusive interactions drive handover of mRNA from export adaptors to TAP. Proc Natl Acad Sci U S A 105, 5154-5159.
Hsin, J.P., and Manley, J.L. (2012). The RNA polymerase II CTD coordinates transcription and RNA processing. Genes Dev 26, 2119-2137.
Katahira, J. (2012). mRNA export and the TREX complex. Biochim Biophys Acta 1819, 507-513.
Katahira, J., and Yoneda, Y. (2009). Roles of the TREX complex in nuclear export of mRNA. RNA Biol 6, 149-152.
Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.
MacKellar, A.L., and Greenleaf, A.L. (2011). Cotranscriptional association of mRNA export factor Yra1 with C-terminal domain of RNA polymerase II. J Biol Chem 286, 36385-36395.
Maris, C., Dominguez, C., and Allain, F.H. (2005). The RNA recognition motif, a plastic RNA-binding platform to regulate post-transcriptional gene expression. Febs J 272, 2118-2131.
Marzluff, W.F., Wagner, E.J., and Duronio, R.J. (2008). Metabolism and regulation of canonical histone mRNAs: life without a poly(A) tail. Nat Rev Genet 9, 843-854.
Masuda, S., Das, R., Cheng, H., Hurt, E., Dorman, N., and Reed, R. (2005). Recruitment of the human TREX complex to mRNA during splicing. Genes Dev 19, 1512-1517.
Nojima, T., Hirose, T., Kimura, H., and Hagiwara, M. (2007). The interaction between cap-binding complex and RNA export factor is required for intronless mRNA export. J Biol Chem 282, 15645-15651.
Pena, A., Gewartowski, K., Mroczek, S., Cuellar, J., Szykowska, A., Prokop, A.,

Czarnocki-Cieciura, M., Piwowarski, J., Tous, C., Aguilera, A., et al. (2012). Architecture and nucleic acids recognition mechanism of the THO complex, an mRNP assembly factor. Embo J 31, 1605-1616.
Piruat, J.I., and Aguilera, A. (1998). A novel yeast gene, THO2, is involved in RNA pol II transcription and provides new evidence for transcriptional elongation-associated recombination. Embo J 17, 4859-4872.
Portman, D.S., O'Connor, J.P., and Dreyfuss, G. (1997). YRA1, an essential Saccharomyces cerevisiae gene, encodes a novel nuclear protein with RNA annealing activity. Rna 3, 527-537.
Reed, R., and Cheng, H. (2005). TREX, SR proteins and export of mRNA. Curr Opin Cell Biol 17, 269-273.
Stewart, M. (2010). Nuclear export of mRNA. Trends Biochem Sci 35, 609-617.
Strasser, K., and Hurt, E. (2000). Yra1p, a conserved nuclear RNA-binding protein, interacts directly with Mex67p and is required for mRNA export. Embo J 19, 410-420.
Strasser, K., and Hurt, E. (2001). Splicing factor Sub2p is required for nuclear mRNA export through its interaction with Yra1p. Nature 413, 648-652.
Strasser, K., Masuda, S., Mason, P., Pfannstiel, J., Oppizzi, M., Rodriguez-Navarro, S., Rondon, A.G., Aguilera, A., Struhl, K., Reed, R., et al. (2002). TREX is a conserved complex coupling transcription with messenger RNA export. Nature 417, 304-308.
Valkov, E., Dean, J.C., Jani, D., Kuhlmann, S.I., and Stewart, M. (2012). Structural basis for the assembly and disassembly of mRNA nuclear export complexes. Biochim Biophys Acta 1819, 578-592.
Yoh, S.M., Cho, H., Pickle, L., Evans, R.M., and Jones, K.A. (2007). The Spt6 SH2 domain binds Ser2-P RNAPII to direct Iws1-dependent mRNA splicing and export. Genes Dev 21, 160-174.
Zenklusen, D., Vinciguerra, P., Wyss, J.C., and Stutz, F. (2002). Stable mRNP formation and export require cotranscriptional recruitment of the mRNA export factors Yra1p and Sub2p by Hpr1p. Mol Cell Biol 22, 8241-8253.
Zhang, M., and Green, M.R. (2001). Identification and characterization of yUAP/Sub2p, a yeast homolog of the essential human pre-mRNA splicing factor hUAP56. Genes Dev 15, 30-35.