Functional characterization of THO complex members THOC5 and THOC7

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Erklärung zur Dissertation:

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel “Functional characterization of THO complex members THOC5 and THOC7” selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe. Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, 2010 Anuja Guria

ABSTRACT

The TREX (Transcription/export) complex is evolutionarily conserved from yeast to humans and is required for coupled transcription elongation and nuclear export of mRNA. In eukaryotes the TREX complex is composed of UAP56, ALY and the THO–sub complex members. Although it is known that THO is not essential for the export of bulk poly (A)+ RNA, it has been observed that export of a subset of mRNA was affected by the depletion of THO complex members in *Drosophila*. However it is not known which genes depend on THO complex in mammalian system. Furthermore it is unknown whether THO complex plays any additional role other than export of mRNA to the cytoplasm.

This thesis consists of two parts:-

Firstly, THOC5 dependent genes were identified using conditional THOC5 knockout system followed by microarray analysis. Secondly, to identify novel binding partners of THOC7 which is a known interacting partner of THOC5 in cytoplasm by using tandem affinity purification followed by mass spectrometry.

1) Identification of THOC5 dependent genes

A mouse embryonic fibroblast cell line (MEF) THOC5/FMIP *flox/flox* was established by flanking exons IV and V region of FMIP with lox P sites, making it possible to inactivate THOC5/FMIP in a conditional manner. Upon infection of MEF THOC5/FMIP with adenovirus carrying Cre recombinase (Ade-GFP-Cre) THOC5/FMIP is downregulated more than 95% within 4 days at protein level. Microarray analysis of MEF with conditional knockdown of THOC5/FMIP revealed that only 72 functionally known genes were downregulated more than 3 fold. Strikingly half of the downregulated genes are known to be involved in differentiation and development. These data show that THOC5/FMIP plays a role

in exporting only a subset of genes, however it plays an important role in mouse development and differentiation.

2) Potential involvement of the cytoplasmic THOC7/THOC5 complex in translation process.

In this study Tandem affinity purification followed by mass spectrometry was performed to look for proteins interacting with THOC7 in order to determine its possible function.THOC7 gene was cloned in a commercially available TAP construct from stratagene and were transiently transfected into HEK293 cells. The THOC7 interacting proteins were Tap purified and identified by mass spectrometry. About 94 proteins were identified among them (40%) were cytoplasmic proteins involved in protein synthesis and translation. These results suggests a potential role of THOC7 in protein synthesis and translation.

Key words :- THO complex, Transcription export complex (TREX), THOC5, THOC7, mRNA export.

ZUSAMMENFASSUNG

Der TREX (Transkription/Export) Komplex ist evolutionär von der Hefe bis zum Mensch konserviert und ist notwendig für die gekoppelte Transkriptions elongation und der nuklearer Export von mRNA. In Eukaryoten besteht der TREX komplex aus den mitgliedern UAP56, ALY und dem THO-Subkomplex. In Drosophila ist THO nicht erforderlich für den Poly (A)+ RNA Export, da nur ein Teil vom mRNA Export durch Abbau der Mitglieder des THO Komplexes beeinflusst wird. Es ist jedoch nicht bekannt, welche Gene im Säugetiersystem abhängig vom THO Komplex sind. Ferner ist es unbekannt, ob der THO Komplex außer im mRNA Export zum Zytoplasma eine zusätzliche Rolle spielt.

Diese Arbeit besteht aus zwei Teilen:

Zunächst wurden THOC5-abhängige Gene unter Verwendung vom THOC5 Knock-out System mit einer anschließenden Microarray - Analyse identifiziert. Zweitens wurden neue Bindungspartner von THOC7, ein bekannter interagierender Partner von THOC5 im Zytoplasma mittels Tandem-Affinitäts-Aufreinigung (TAP) und mit anschließender Massenspektrometrie identifiziert.

1) Identifizierung von THOC5-abhängigen Genen:

Es wurde eine Maus *embryonale Fibroblasten* Zelllinie (MEF) THOC5/FMIP *flox/flox*

durch Flankieren der Exons IV und V Region von FMIP mit lox P Stellen etabliert. Diese ermöglicht die THOC5/FMIP-Inaktivierung in einer konditionalen Weise. Nach Infizierung der MEF THOC5/FMIP mit konditionalem Adenovirus, was eine Cre Rekombinase (Ade-GFP-Cre) trägt, ist THOC5/FMIP auf Proteinebene mehr als 95% innerhalb 4 Tagen herunterreguliert.

Microarray Analysen von MEF mit konditionalem Knockdown von THOC5/FMIP zeigten, dass nur 72 funktionell bekannte Gene mehr als 3-Fach herunterreguliert wurden. Auffallig ist, dass die Hälfte von herunterregulierten Genen ander Differenzierung und Entwicklung beteiligt sind. Diese Daten zeigen, dass THOC5/FMIP eine Rolle beim Export von einer bestimmten Gruppe von Genen spielt. es Jedoch, eine wichtige Rolle in der Maus bei der Entwicklung und Differenzierung spielt.

2) Mögliche Beteiligung des zytoplasmatischen THOC7/THOC5 Komplexes im Translationsprozess:

In dieser Arbeit wurde eine Tandem-Affinitäts-Aufreinigung mit anschließender Massenspektrometrie durchgeführt, um die THOC7-interagierende Proteine zu suchen und ihre eventuelle Funktion festzustellen. Das THOC7 Gen ist in einem kommerziell erhältlichen TAP Konstrukt von Stratagene kloniert und transient in HEK293 Zellen transfiziert. Die THOC7-interagierenden Proteine sind mittels TAP aufgereinigt und durch Massenspektrometrie identifiziert worden. Etwa 94 Proteine sind identifiziert worden, darunter (40%) waren zytoplasmatische Proteine, die an der Proteinsynthese und Translation beteiligt sind. Diese Ergebnisse deuten auf eine neue potenzielle Funktion von THOC7 in der Proteinsynthese und Translation hin.

Schlagwörter :- THO komplex, Transkription export komplex (TREX), THOC5, THOC7, mRNA export.

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1 INTRODUCTION

The flow of genetic information occurs from DNA to RNA to proteins. In eukaryotes, mRNA is transcribed from DNA as primary transcripts and has to be processed in the nucleus before they are exported to the cytoplasm for translation. The processing steps include capping at the 5'end, splicing of introns, and polyadenylation at the 3'end. Further, the integrity of mRNA is also checked by quality control mechanisms such as nonsense mediated decay (NMD), which eliminates improper mRNAs with premature termination codons. The processed mRNA has to be exported across the nuclear membrane into the cytoplasm for translation. Nuclear export of mRNA is a complex procedure and involves elaborate nuclear transport pathways.

1.1 Export of eukaryotic RNA

In eukaryotes the process of gene expression occurs in different compartments of the cell. Several complex mechanisms exist to transport macromolecules across compartment boundaries. The trafficking of macromolecules between the nucleus and cytoplasm occurs via nuclear pore complex (NPC). Each NPC is an assembly of several different proteins that form a pore across the nuclear membrane. Transport through NPCs is an active process and is assisted by a family of conserved nuclear transport receptors known as karyopherins or importins. Karyopherins that are involved in the import of the cargo are called importins and those involved in the export of cargo are called exportins. These cargo carriers recognize certain short peptide signal on the cargo that could be either a nuclear localization signal (NLS) or a nuclear export signal (NES). Two key features of karyopherins, their ability to bind nucleoporins and to form complexes with Ran-GTPase, are essential for nuclear export.

Karyopherins are also involved in the export of RNA and recognize specific nucleotide motifs in RNA cargos. Different RNA species within the cell (mRNA, snRNA, rRNA, tRNA) use

separate pathways to ease their movement from the nucleus to the cytoplasm. Export of tRNA, microRNA (miRNA), and ribosomal (r)RNA follows this normal pattern that involves exportins and the RanGTPase (Rodriguez, M. S., 2004). The export of small nuclear (sn)RNA is carried out by CRM1 (also known as exportin1), which recognizes the proteins that contain Leu rich- type NES (Fornerod, M., 1997). CRM1 does not directly interact with the snRNA cargo, but requires the cap-binding complex (CBC) and a NES-containing adaptor protein called PHAX to be targeted to the 5′ cap of the snRNA (Hamm, J.1990, Ohno, M., 2000). Phosphorylation of PHAX in the nucleus is required for recruitment of CRM1 and RanGTP to the CBC-bound snRNA complex (Figure 1.1).

However, general mRNA export is different as it uses a transport receptor that is not related to karyopherins and does not directly depend on the RanGTP–RanGDP gradient (Reed, R.2002, Conti, E. 2001). Export of mRNA is mediated by a group of evolutionarily conserved proteins called nuclear mRNA export factors. Among these, the key mediator of mRNA export is an heterodimer of Tap and a small cofactor p15 (Figure 1.1). Atleast six such export factors in humans, four in *Drosophila* and two in *Caenorhabditis elegans* have been identified. Yeast has only a single ortholog of Tap-p15 termed as Mex67p-Mtr2p (JA Erkmann 2004). These proteins are distinct from the prototypical importin-exportin family of proteins as they lack the characteristic Ran-binding domain found in all karyopherins. Hence the directionality of mRNA transport would have to be mediated by other mechanisms.

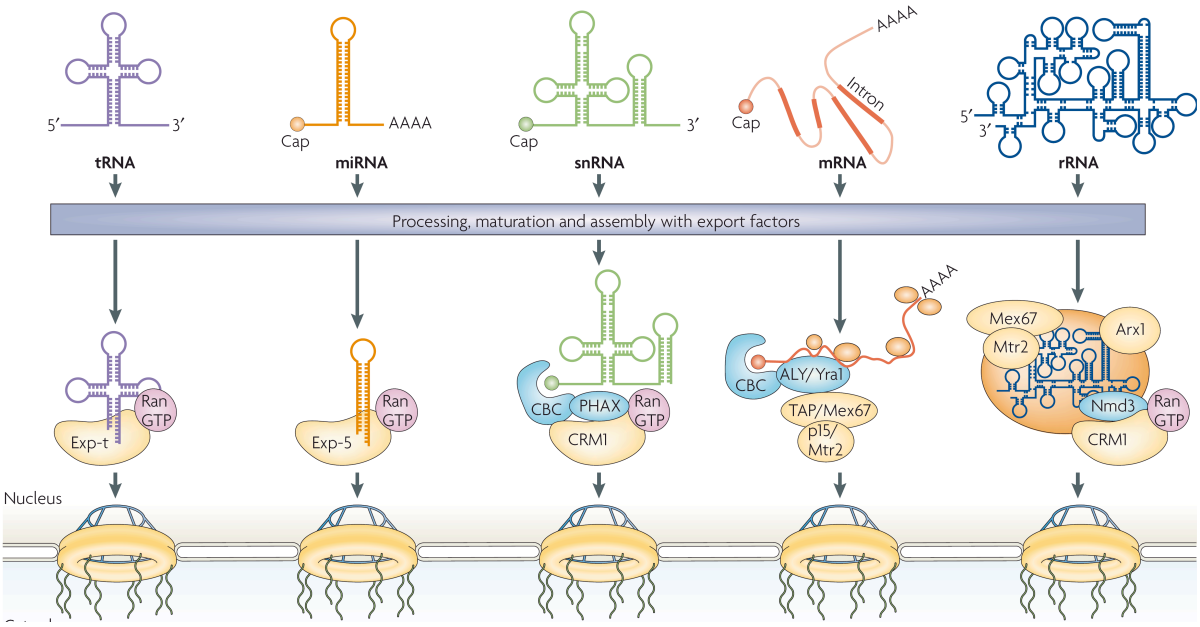


Figure 1.1 The different RNA export pathways. The major RNA export routes are shown, snRNA, mRNA, In both case, the primary RNA transcript is shown, as well as the transport-competent RNA after it has undergone processing, maturation and assembly with export factors (adapted from Alwin Köhler 2007).

1.2 THO/TREX complex in mRNA export

In order to increase the fidelity of gene expression, transcription, splicing and mRNA export is often coupled to each other. There is enough evidence suggesting that highly conserved mRNA export machinery, the transcription export (TREX) complex is involved in this process. TREX is a multisubunit complex composed mainly of THO complex, UAP56 and RNA export factor 1 (REF1) also known as ALY. Studies in yeast associate TREX complex with transcription elongation and co-transcriptional recruitment of the mRNA export machinery (Fischer et al 2002, Jimeno et al 2003, Huertas and Aguilera 2003). In mammalian cells TREX complex has been shown to colocalize with the splicing factors. Moreover, recruitment of TREX complex to the mRNA during splicing reaction suggest that TREX

complex also plays a role in coupling mRNA export to splicing. The role of TREX complex in mRNA export has been studied in different species including yeast, *Drosophila* and humans (Table 1.1). Recent reports have also identified the existance of a yeast homolog of THO-complex in plants (Furumizu.C et al 2010).

1.2.1 Yeast

In yeast, TREX complex contains the mRNA export factors Sub2 and Yra1 and the THO complex components Tho2, Hpr1, Mft1, and Thp2 (Chavez et al 2000). Mutants of these genes showed a transcription-dependent hyper-recombination phenotype and impairment of transcription, giving the first hint that TREX complex has a role in gene expression. As the transcription is in progress the adaptor protein Yra1 (Aly/REF1 in humans) interacts with Sub2p (UAP56 in humans) of transcription elongation factor, and further helps in recruiting the members of THO complex to nascent mRNA (Zenklusen et al. 2002; Abruzzi et al. 2004; Jimeno et al. 2006). Recently it has been proposed that THO/Sub2p may also be involved in mRNA 3'-end processing. This was supported by the observations that mutants of THO/Sub2p showed defective 3'-end processing (Saguez et al. 2008), and that THO together with Sub2p participates in an mRNP remodeling event that follows mRNA 3'-end processing (Rougemaille et al. 2008).

1.2.2 *Drosophila*

As in yeast, the TREX complex in *Drosophila* is composed of UAP56, Aly/THOC4 and members of THO complex THOC1 (HPR1 in yeast), THOC2 and TEX1. Apart from this, the THO complex also consists of three other proteins THOC5, THOC6 and THOC7, the orthologs of which have not been identified in yeast (Rehwinkel et al., 2004). It has been reported that UAP56 is essential for bulk mRNA export whereas the role of Aly in this process is dispensable. Gene knockdown experiments performed with cultured fruit fly cells shows that <20 % of genes expression depend on THO complex, whereas expression of

nearly 75% genes are affected upon UAP56 depletion (Gatfield et al, 2001; Herold et al, 2001; Gatfield and Izaurralde, 2002; Farny et al, 2008). Although THO complex functions in mRNA exportin *Drosophila*, majority of mRNAs are transcribed and exported independent of THO complex (Rehwinkel et al 2004).

1.2.3 Humans

The THO complex in humans consist of hHpr1, hThoc2, fSAPs (functional spliceosome-associated proteins) fSAP79, fSAP35, fSAP24, now known as THOC5, THOC6, THOC7 and hTex1 (THOC3 in *Drosophila*; Masuda et al 2005, Dufu et al 2010) (Table 1.1). The THO complex with UAP56 and Aly forms the TREX complex. Unlike in yeast, the human TREX complex is associated with splicing machinery and is not a transcription coupled mechanism. The recruitment of THO to RNA therefore requires nuclear cap binding complex (CBC) and several splicing factors (Masuda et al., 2005; Cheng et al., 2006). The TREX component ALY assists the recruitment of THO complex to 5' end of the mRNA by interacting with the CBC component CBP80. This interaction ensures the transport of mRNA in a 5' to 3' direction through the nuclear pore.

Table 1.1: THO complex members in different species

Saccharomyces cerevisiea		*Drosophila melanogaster*		*Homosapiens*	
	Protein length		**Protein length**		**Protein length**
THO2	**1597**	**THO2**	**1641**	**THOC2**	**1478**
HPR1	**752**	**HPR1**	**701**	**THOC1**	**657**
-	**-**	**THOC5**	**616**	**THOC5**	**683**
-	**-**	**THOC6**	**350**	**THOC6**	**317**
-	**-**	**THOC7**	**287**	**THOC7**	**204**
TEX1	**422**	**TEX1**	**320**	**THOC3**	**350**
MFT1	**392**	**MFT1**		**-**	
THP2	**261**	**THP2**		**-**	

1.3 THO complex and its members

Most of the information about the THO complex has been obtained from studies in yeast system and it has been proposed that the THO complex has a role in at least three cellular processes: transcription, mRNA export and genomic stability (Rougemaille et al 2008).

1.3.1 THO complex in transcription

Association of THO complex in transcription was proposed based on the observation that expression of the bacterial LacZ gene in yeast cells was defective in THO mutants. However, in these mutants other genes were efficiently expressed under LacZ promoter and this led to the conclusion that transcription activation was not defective in THO mutants rather it was faulty transcription elongation that was specific to long or G+C rich genes such as LacZ (Chavez et al., 2001). Other studies have proposed that this defective transcription could also be due to decreased processivity of RNAPII in THO mutants. However, conclusive biochemical evidance for this proposed mechanism is lacking. In another study Huerats and

Aguilera observed defects in transcription elongation as a consequence of formation of R-loops, a structure formed when the nascent mRNA folds back and hybridizes with DNA. This could be attributed to the failure in functional mRNP assembly, the role of which is to prevent the mRNA from hybridizing to the transcribed DNA. Interestingly, depletion of THO complex in human cell extracts did not show any defects in transcription but severe defects in gene expression, altogether suggesting a different role of THO complex in humans.

1.3.2 THO complex and mRNA export

As mentioned above THO complex interacts with both RNA and DNA and at the same time forms complex with the export factors Sub2p and Yra1p. Hence it was proposed that THO complex forms an interface between transcription and mRNA metabolism (Jimeno et al., 2002; Strasser et al., 2002).

In agreement with a role in mRNA export, deletion of an individual THO component in yeast results in rapid accumulation of poly(A)+ transcripts within the nucleus (Schneiter et al., 1999; Libri et al., 2002; Strasser et al., 2002; Zenklusen et al., 2002). However THO complex is not required for the export of all mRNAs, rather it is needed for specific heat shock mRNAs. In case of *Drosophila* it has been shown that less than 20% of its transcriptome is regulated by THO (Rehwinkel et al., 2004).

1.3.3 THOC1

A nuclear matrix protein hHpr1/p84/THOC1 was identified in yeast two-hybrid screening using the amino terminal domain of the retinoblastoma tumor suppressor protein as bait (Durfee T et al 1994). THOC1 is a component of the TREX (Transcription/Export) complex that physically couples the elongating RNA polymerase II with factors important for messenger ribonucleoprotein particle (mRNP) formation, RNA processing, and mRNA export (Abruzzi KC et al 2004, Rondon AG et al 2003, Strasser K et al 2002). THOC1 is expressed in most tissues throughout the cell cycle (Durfee T, et al 1994), except in the G0 phase

(Gasparri F et al 2004). THOC1 overexpression can trigger p53-independent apoptosis that is inhibited by binding of hHpr1/p84/THOC1 to the retinoblastoma tumor suppressor protein (Doostzadeh-Cizeron J, 1999, 2001).

Depletion of hHpr1/p84/THOC1 decreases growth rates in multiple cancer cell lines, such as Hela, 293T, HCT116, U2OS, and MDA-MB-231 (Guo S et al 2005). In humans, high levels of hHpr1/p84/THOC1 have been observed in breast cancer cells and are strongly associated with tumor size and aggressiveness, implying potential significance of this protein in tumor transformation, progression, and metastasis (Guo S et al 2005). THOC1 requirement was compared in the proliferation and survival of isogenic normal and oncogene transformed cells (Li Y et al 2007). It was found that neoplastic cells rapidly lose viability via apoptotic cell death following depletion of pTHOC1. In contrast, viability of normal cells is largely unaffected by pTHOC1 loss, suggesting that THOC1 may provide a novel molecular target for cancer therapy (Li Y et al 2007).

1.3.4 THOC7

THOC7 was identified as a binding partner of a putative transcriptional repressor, Ngg1 interacting factor like 1 (NIF3L1) by yeast two hybrid screening (Tascou, S et al 2003). THOC7 is a member of THO/TREX complex and it interacts with other THO complex member THOC5. THOC7 is a protein of molecular weight 24kDa and at its C-terminal region it contains a leucine zipper (LZ) (figure 1.2).

Figure 1.2 Scheme showing potential functional domains in THOC7, LZ:leucine zipper

Though THOC7 lack a nuclear localization signals (NLS) motif, still it was detected in the nucleus and cytoplasm. Interaction of THOC7 with THOC5 is therefore required for its nuclear translocation. FMIP/THOC5 binding domain of THOC7 is located within the centre portion of THOC7. THOC7 binds to THOC5 at its N-terminal domain (1-199) the exact binding region of which has been mapped using Myc tagged THOC5 mutants (El. bounkari et al 2009). Mouse and human THOC7 show 90% homology at the nucleotide level and 97% homology at the amino acid level.

1.3.5 THOC5/FMIP

THOC5/Fms interacting protein (FMIP), a member of the THO complex, was originally identified as a substrate for the macrophage colony stimulating factor (M-CSF) receptor tyrosine kinase, Fms (Tamura et al., 1999). FMIP is a nuclear cytoplasmic shuttling protein with a NLS (nuclear localization signal), two LxxLL motifs, a PEST domain and a putative leucine zipper (Figure 1.3). The N-terminal domain of FMIP (1-199) binds to THOC7 directly and the C-terminal domain (559-683) is required for its interaction with other member of THO complex THOC1. Mouse and human FMIP share 89.6% and 95.6% identity at the nucleotide and the amino acids levels respectively.

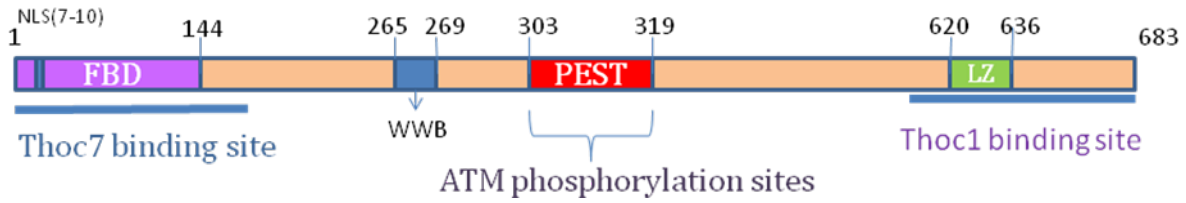


Figure 1.3 Scheme showing functional domains of FMIP/THOC5 (adapted from T.Tamura et al 1999). NLS: nuclear localization signal, FBD: Fms binding domain, LZ: leucine zipper, PEST: PEST domain, WWB: binding sites for proteins with WW motif, numbers represent amino acid position.

FMIP/THOC5 is phosphorylated not only by several tyrosine kinases such as Fms, (Tamura et al 1999) Bcr-Abl, c-kit, and Tel- PDGF (Pierce et al., 2008), but also by protein kinase C

(Mancini et al., 2004), downstream kinase from insulin stimulus (Gridley et al., 2005) or ATM kinase (Matsuoka et al., 2007), suggesting that extracellular stimulation regulates the function of THOC5/FMIP.

Depletion of THOC5/FMIP by siRNA or ectopic expression causes abnormal hematopoiesis and abnormal muscle differentiation in myeloid progenitor or mesenchymal progenitor cell lines, indicating that the THO complex is essential for differentiation process in mammals (Carney et al 2009, Tamura et al 1999, Mancini et al 2007). Recently it was shown that THOC5 binds directly to the M-domain of Tap, which has an Ntf2-like fold. Knockdown of THOC5 using siRNA showed nuclear accumulation of HSP70 mRNA but bulk poly (A) $^{+}$ RNA was not affected. THOC5 together with ALY functions in the nuclear export of heat shock mRNAs (J. Katahira et al 2009) (Figure 1.4).

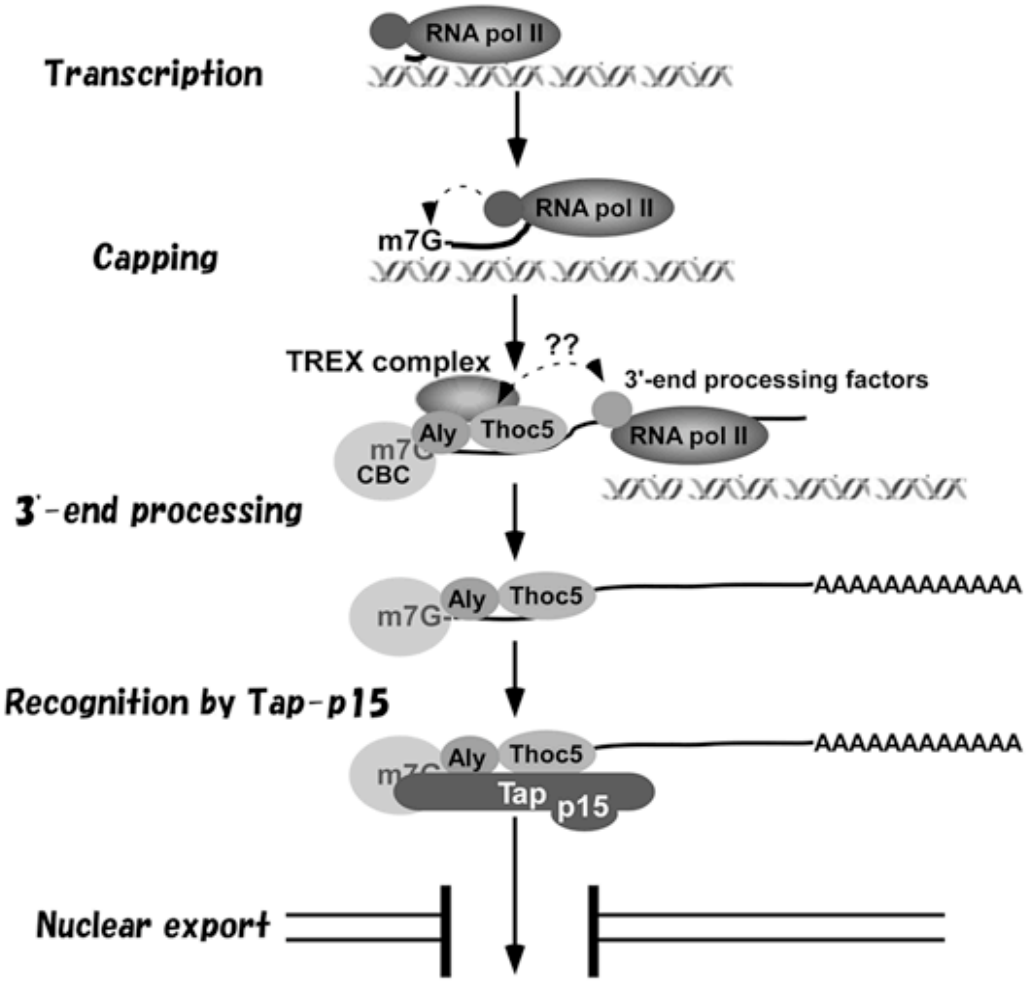


Figure 1.4 Model for nuclear export of HSP70 in mammalian cells. The human TREX components THOC5 and Aly bind to the different domains of the export receptor Tap-p15 heterodimer and act as an adaptor and coadaptor for nuclear export of *HSP70* mRNA. Whether loading of the human TREX complex on the target mRNA depends on interactions with the nuclear cap binding complex (CBC) and/or the 3'-end processing factors is still unknown (indicated by question marks).(adapted from Katahira et al 2009).

1.3.6 Conditional knockout mouse THOC5

The major insights into the fucntion of a particular protein *in vivo* is obtained from specific gene knockout mouse models. Attempts to generate THOC5/FMIP knockout failed as the THOC5 deficiency was embryonic lethal. Most of the information about the THOC5/FMIP *in vivo*, was obtained from conditional knockout mice. A floxed THOC5/FMIP allele (THOC5/FMIP *flox)* was generated by recombination in embryonic stem (ES) cells (Niendorf

S et al 2007). The THOC5/FMIP gene spans 20 exons in a 33, 523 kb region on chromosome 11. A targeting strategy was adopted whereby flanking exons IV and V with loxP sites, THOC5/FMIP could be inactivated in a conditional manner. The deletion of exons IV/V of THOC5/FMIP causes a frame shift of product and the truncated protein obtained was 83 amino acids long which lacks THOC1 binding domain (El. Bounkari et al 2009).

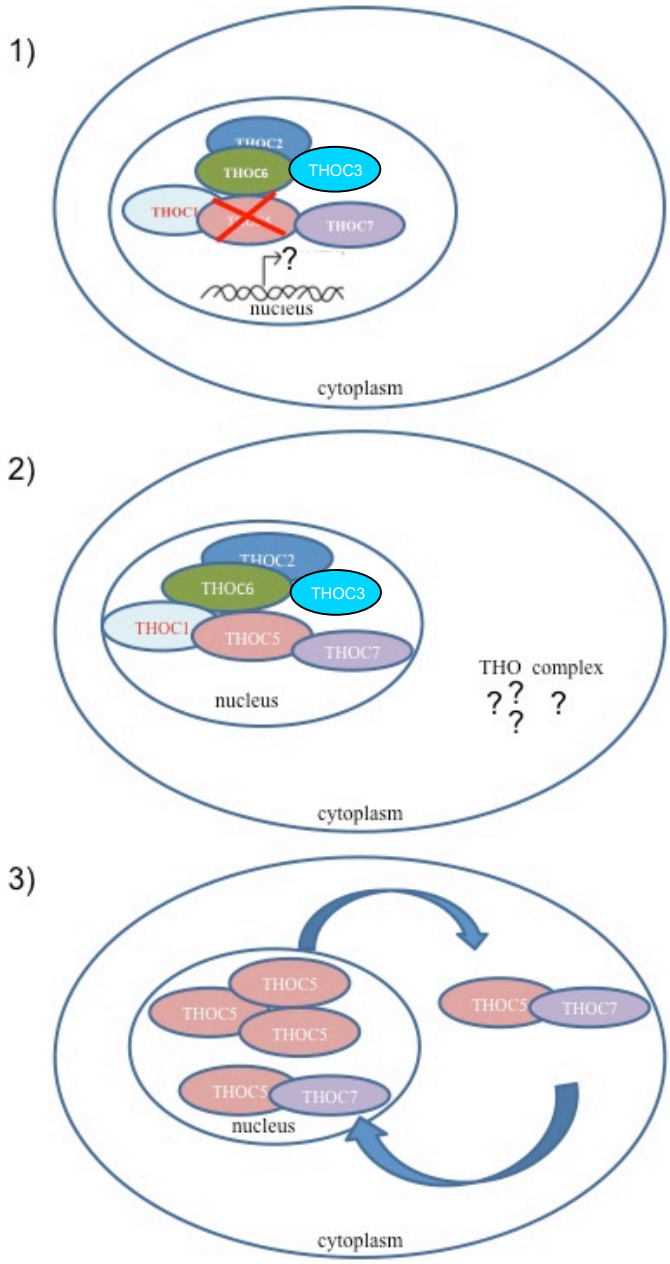
The interferon-inducible *cre*-recombinase based conditional THOC5 knockout mouse, died within the first 2 weeks. It was observed that conditional knockout mice had developed acute leukocytopenia (reduction in white blood cell numbers) and anemia (reduction in red blood cell numbers). The number of blood cells in peripheral blood was decreased; probably because bone marrow cells became apoptotic, and due to loss of committed myeloid progenitor cells and of cells with long-term reconstituting potential. Normal bone marrow cells were transferred to rescue the mice from death. After bone marrow transplantation 9 out of 14 THOC5/FMIP depleted mice survived over 2 months. These data suggests that THOC5 plays an important role during early embryogenesis and hematopoietic differentiation (Mancini et al 2010).

1.4 Aim of the thesis:-

THO complex is involved in mRNA processing and export, yet the role of individual THO members in this process remains unclear. Furthermore, the genes that depends on THO complex for its expression remains elusive (except HSP70).

THO complex is originally isolated from the nuclear fraction. However certain members of THO complex like THOC1, THOC5 and THOC7 has been identified to be proteins shuttling between nucleus and cytoplasm. Hence it is not known if a counterpart of THO complex also occurs in cytoplasm. Therefore the major aim of this study was

1) To Identify THOC5 dependent mRNA by microarray using MEF cells from conditional THOC5 knockout mouse.
2) To examine if THOC7 or THOC5 has any additional functions other than mRNA export and which member of THO complex forms a complex with THOC7.



THO complex participates in mRNA export, however in mammalian system only the export of HSP70 has been identified to be dependent on THO complex. Therefore we wanted to identify other genes that depend on THO complex for mRNA export.

THO complex and its members have been isolated from the nuclear fraction (Masuda et al 2005). Certain members of THO complex have also been found in cytoplasm.Hence the presence of THO complex in cytoplasm is not yet known.

THOC5 forms a complex with THOC7 and imports to the nucleus (El.bounkari et al 2009). To understand the role THOC7 in cytoplasm.

Figure 1.5 Scheme representing the basic aim of the thesis.

1) Which genes are THO dependent?

2) Does THOC7 forms a complex with other THO complex members in cytoplasm?

3) Is THOC7 involved in some novel function in cytoplasm?

2 MATERIALS

All standard chemicals for making buffers and solutions were purchased from the suppliers listed below:-

Ambion	Austin, TX, USA
Amersham	Buckinghamshire, UK
ATCC	Manassas, VA, USA
Bayer	Leverkusen, Germany
BD Biosciences	San Jose, CA, USA
Beckman Coulter Inc	Fullerton, CA, USA
Biochrom	Berlin, Germany
Biometra	Gottingen, Germany
Biorad	Munich, Germany
Biozym GmbH	Hess.Oldendorf, Germany
Boehringer	Ingelheim, Germany
Clontech	Palo Alto, CA, USA
Dianova	Hamburg, Germany
DNA Star.Inc	Madison, WI, USA
DSMZ	Braunschweig, Germany
Eppendorf	Hamburg, Germany
Eurogentec	Seraing, Belgium
Falcon	Heidelberg, Germany
Fuji photo film	Kanagawa, Japan
GFL	Burgwedel, Germany
Gibco BRL	Carlsbad, CA, USA
Heraeus	Hanau, Germany

Hettich	Tuttlingen, Germany
Invitek	Berlin, Germany
Invitrogen	Carlsbad, CA, USA
Kodak	Rochester, NY, USA
Kuhn & Bayer	Nidderau, Germany
Liebehere	Germany
MBI Fermantas	St.Leon-Rot, Germany
Merck	Darmstadt, Germany
Mettler-Toledo	Giessen, Germany
Millipore	Bedford, MA, USA
NEN Perkin Elmer	Boston, MA, USA
New England Biolabs	Beverly, USA
Nikon	Dusseldorf, Germany
Novex, Invitrogen	Carlsbad, CA, USA
Nunc	Wiesbaden, Germany
PAA Laboratories	Pasching, Austria
Packard GMI Inc	Ramsey, MN, USA
Pan Biotech	Aidenbach, Germany
Peqlab Biotechnology GmbH	Erlangen, Germany
Perkin-Elmer	Forster City, USA
Pharmacia Amersham	Buckinghamshire, UK
Pierce	Rockford, IL, USA
Promega	Madison, WI, USA
Qiagen	Hilden, Germany
Roche	Basel, Germany

Roth	Karlsruhe, Germany
Santa Cruz Biotechnology	Santacruz CA, USA
Sarstedt	Numbracht, Germany
Sartorius	Goettingen, Germany
Serva	Heidelberg, Germany
Siemens	Frankfurt, Germany
Sigma Aldrich	St.Louis, CA, USA
Stratagene	la Jolla, CA, USA
Terumo Europe	Leuven, Belgem
Thermoquest	Engelsbach, Germany
Visitron	Puchheim, Germany

2.1 Chemical Reagents

Agar	Roth
Agarose	Roth
Ampicillin	Roth
Acrylamide	Sigma
APS (ammonium persulfate)	Roth
Bacto Agar	Roth
Brilliant Blue G250	Sigma-aldrich
BSA (bovine serum albumin)	Roth
ATP (deoxyadenosine triphosphate)	MBI Fermentas
dCTP (deoxycytidine triphosphate)	MBI Fermantas
DEPC (diethylpyrocarbonate)	Sigma
dGTP (deoxyguanosine triphosphate)	MBI Fermentas

DNA ladder (1kb)	MBI Fermentas
DTT (dithiotreitol)	Roth
dTTP (desoxythymidine triphosphate)	MBI Fermentas
Ethidium bromide	Roth
Formaldehyde 37%	Roth
Formamide	Roth
HEPES	Roth
Roti marker	Roth
N, N, N'N, Tetramethylethylendiamine TEMED	Sigma
PEG (polyethyleneglycol)	ATCC
PMSF (phenyl-methly-sulfonylfluoride)	Merk
Precision Protein Marker prestained	BioRad
Glutathione Sepharose 4Bbeads	Amersham
Salmon sperm DNA	Sigma
Streptavidin sepharose beads	Amersham
Calmodulin sepharose beads	Amersham
Triton X-100	Roth
Tryptone	Merck
Trasylol	Bayer
Tween20	Roth
Yeast extract	Roth
Trypan Blue	Sigma
β-Mercaptoethanol	Roth
EDTA	Roth
10x Trypsin/EDTA, 0.5%/0.25%w/v)	Biochrom

Bacto tryptone	Roth
Bacto Yeast extract	Roth
Dimethlysulfoxide (DMSO)	Roth
DMEM	Biochrom
Fetal calf serum (FCS)	Biochrom
MOPS	Roth

2.2 Kits

Nucleic Acid & Protein Purification Nucleospin extract II	Macherey & Nagel
pGEM T easy cloning kit	Promega
Plasmid Maxi kit	Qiagen
Plasmid Midi Kit	Qiagen
Rneasy Mini Kit	Qiagen
cDNA Isolation kit	Qiagen
Jet Nick ‘‘Probe Purification after Labelling’’	Genomed
Megaprime Labelling	Amersham

2.3 Antibodies

Mouse monoclonal anti-Thoc1 (p84N5	Gene Tex inc
Monoclonal anti –FMIP (F6d)	(Mancini et al:2007)
Mouse monoclonal anti-Myc (9E10)	Santacruzbiotechnology

2.4 Enzymes

Dnase I, Rnase free	Qiagen
T4 DNA Ligase	MBI Fermentas
Restriction Enzymes	New England Biolabs, MBI Fermentas, Roche

2.5 E.coli strains

DH5α - F^-, recA1, endA1, gyrA96 (nal^+), thi, hsdR17, supE44, relA1 (Gibco BRL)

HB101- F^-, hsd20 (r-B-m-B), recA13, ara-14, proA2, lacY1, galk-2, rpsl20 (str_R), xyl-5 mtl-1, supE44 (Boyer & Roulland- Dussoix, 1969, Bolivar et al, 1977)

JM101- F^-, ▲ (lac-pro), proAB, supE, rK^+, mK^+, thi/F, traD36, laclqZ, ▲M15

2.6 Cell Lines

HEK293	Human embryonic kidney (HEK-293) fibroblast derived packaging cell line CRL-1573, ATCC
MEF	Mouse embryonic fibroblast
Hela	Human cervix carcinoma (Hela) (ATCC, Cat.No-CCL-2)

2.7 Plasmids

pCDNA3	Invitrogen
pGEM T Easy	Promega
pNTAP	Stratagene
pGEX 2T	Pharmacia

2.8 List of Primers used for RTPCR

Gene	Sequence 5′-3′
ß-Actin	**Forward-** AACACCCCAGCCATGTACGTAG **Reverse-** GTGTTGGCATAGAGGTCTTTACGG
Fmip	**Forward-** TCTGCCTTTTCACCTGGAAG **Reverse-** CTCGGTACTTTTCTGCCAGC
Hoxb3	**Forward-** CCACCTACTACGACAACACC **Reverse -**TTGCCTCGACTCTTTCATCC
Top3b	**Forward-** GGAGATTGCACAGATGTTTTTAAAC **Reverse-** TTCTGTCCGTGGGTAGCTGATATAGC
Tfrc	**Forward-** TGGATTCATGAGTGGCTACCTGG **Reverse-** GTTCATCTCGCCAGACTTTGCTG
Bcl2	**Forward-** TCGCTACCGTCGTGACTTC **Reverse-** AAACAGAGGTCGCATGCTG
Cbx2	**Forward-** GTAGTCCCAAAGCCCAGTCAG **Reverse-** CAAGTGCCTACATCAGCTTGC
Sox15	**Forward-** CGGCGTAAGAGCAAAAACTC **Reverse –**TGGGATCACTCTGAGGGAAG
Pou6f2	**Forward-** ATAGCTGGACAAGTCAGTAAGCC **Reverse-** TCCTCGCTGTCATTTGATTCC
Runx1	**Forward-** GCAGGCAACGATGAAAACTACT **Reverse-** GCAACTTGTGGCGGATTTGTA
IL7	**Forward-** TTCCTCCACTGATCCTTGTTCT **Reverse-** AGCAGCTTCCTTTGTATCATCAC
Tnrc15	**Forward-** CGCCGACTGGAAGAGAACC **Reverse-** TTGCTGTGTTAGACTGCTGAC
Ptgfr	**Forward-** CTGGACTCATCGCAAACACAA **Reverse-** AGGAAGCCTTTGACTTCTGTCTA
Zranb3	**Forward-** GCAGTCATCGAAAGCAAGTCT **Reverse-** CTGCACTGTCCGATTCGGT
Kcna7	**Forward-** GAAAGCTCAAGAGATCCACGG **Reverse-** GCGGGTAAAAATAGCATGGAAAG

Cryl1	**Forward-** AGGAGTGTGTTCCAGAGAACC **Reverse-** TGGTGGATTGACAGGATGAGC
Ret	**Forward-** GCATGTCAGACCCGAACTGG **Reverse-** CGCTGAGGGTGAAACCATCC
Gys1	**Forward-** CGCTGGAAGGGTGAGCTTT **Reverse-** GAAGTGGGCAACCACATACG
Ptprs	**Forward-** GTGGTGTCTGTGGTGGGTC **Reverse-** CTCTCTGATAAACCTGGGTGGT
Fosl1	**Forward-** ATGTACCGAGACTACGGGGAA **Reverse-** CTGCTGCTGTCGATGCTTG
Cops5	**Forward-** GCTTCCGGGAGTGGTATGG **Reverse-** CGCCGCCAGGATTTCTTGT
Id2	**Forward-** GTCCGGTGAGGTCCGTTAG **Reverse**- TGTAGAGCAGACTCATCGGGT
Arf6	**Forward-** GGTGGGCTTCAACGTGGAG **Reverse-** CGGTGTAGTAATGCCGCCAG
Twsg1	**Forward-** ACTGTGTCGGTATGTGCAACC **Reverse-** GGAGACGATGTTCCAGTTCAG
Mt2	**Forward-** GCCTGCAAATGCAAACAATG **Reverse-** AGCTGCACTTGTCGGAAGC
Plp1	**Forward-** TGAGCGCAACGGTAACAGG **Reverse-** TTCCCAAACAATGACACACCC
Dnaj	**Forward-** TTCGACCGCTATGGAGAGGAA **Reverse-** CACCGAAGAACTCAGCAAACA
Traf3	**Forward-** CAGCCTAACCCACCCCTAAAG **Reverse-**TCTTCCACCGTCTTCACAAAC
Thoc7	**Forward-** GTGACTGACGACGAAGTTATACG **Reverse-** ACTGGCTATACCCTTCCTCTTG

2.9 Radionucleotides

[^{32}P]-dCTP (3000 Ci/mmol)	Hartmann, Amersham

2.10 Equipments

Adjustable air-displacement pipettes	Gilson
Biomax MS films	Kodak
Centrifuge Biofuge	Heraeus
Centrifuge Megafuge 1.0 R	Heraeus
CO_2 incubator Hera Cell	Heraeus
Digital camera	Nikon
Dounce homogenizer	Braun
Exposition chamber Hypercassette	Amersham
Hyperfilm ECL	Amersham Pharmacia
Incubator Function Line	Heraeus
Laminar flow hood Hera Safe	Heraeus
Light microscope Wilovert A	Hund
Magnetmixer Variomag	H+P Labortechnik
Nitrocellulose membrane	Schleider & Schuell
Nylon Membrane	Amersham Pharmacia
PCR machine T3 thermocycler	Biometra
pH meter	Radiometer
Orbital Shaker	Forma Scientific
Refgrigerator 80°C Freezer	Forma Scientific
Rotor SS-34	Sorvall
Scintillation Counter	Packard
Ultracentrifuge L7-55	Beckman
UV-Bank UV Transilluminator 2000	Bio-Rad
Vortex Genie 2	Scientific industries
Phosphoimager Fujix BAS1000	Fuji

2.11 Other Materials

24-well plates	Nunc
6-well plates	Nunc
Blotting paper	Schleicher & Schuell
Cannules (1.2x40mm, 0.45x12mm)	Terumo Europe
Cell culture flasks	Nunc
Cell Culture Petridishes	Nunc
Chromatographic paper, Whatman 3mm	Machery-Nagel
Eppendorf tubes	Sarstedt
Filmcassette Coenex high plus	Dupont
Filmcasstte	Siemens
Cryotubes, Nalgene	Nalgene, Falcon
Petridishes flasks 15cm	Greiner
Petridishes for cell culture	Greiner
Plastic 15ml tubes	Falcon
Plastic Tubes 50ml	Falcon
Polyfect	Qiagen
Sterile filters	Schleicher & Schuell
Sterile filter	Nalgene
Sterile filte 0.22um	Sarstedt
X-ray films X-OMAT	Kodak

3 METHODS

3.1 Culture of E.coli

E.coli cultures were grown according to standard protocols in 2YT medium supplemented with antibiotics needed for selection of plasmids.

2YT medium: Yeast extract - 1% (w/v)
Tryptone- 1.6% (w/v)
Nacl- 0.5% (w/v)

2YT Agar: 2YT medium + agar- 2% (w/v)

Antibiotics: Ampicillin- 100µg/ml
Kanamycin- 25µg/ml
Chloramphenicol- 10µg/ml

3.2 Maintenance of bacterial strains

Strains were stored as glycerol stocks (2YT medium, 20% v/v glycerol) at -80°C. An aliquot of the stock was streaked on to 2YT agar plates containing the appropriate antibiotics and incubated overnight at 37°C. Plates were stored upto 6 weeks at 4°C.

3.3 Preparation of competent bacteria (Calcium chloride method)

A single colony was inoculated into 2ml 2YT medium and incubated overnight at 37°C. 500µl of overnight culture was inoculated into 200ml of LB medium and incubated on a shaker at 37°C for 4-6hrs until OD reaches OD_{600} = 0.4-0.6. The culture was transferred to sterile centrifuge tubes and centrifuged at 4000rpm for 10min at 4°C. The supernatant removed and the cell pellet was kept on ice. The cell pellet was resuspended in 10ml of ice cold sterile 50mM $CaCl_2$, and incubated for 30min on ice. After centrifugation at 5000rpm for 10min at 4°C the cells were resuspended in 5ml of ice cold 50mM $CaCl_2$ containing 10%

glycerol. Aliquot of 100-150µl were made and incubated on ice for 2hrs. The suspension was frozen in liquid nitrogen and stored at -80°C.

3.4 Transformation of E.coli

To 100-150µl of competent *E.coli* (DH5α, HB101, or JM101) cells either 50-100ng of plasmid DNA or 10µl of ligation mixture were added and incubated for 30min on ice. Heat shock was given for 1.5 min at 42°C and cells were incubated on ice for 2min. 200µl of 2T medium was added to the bacteria and incubated at 37°C for 60min. Aliquots were plated on 2YT plates containing the appropriate antibiotic. Plates were incubated at 37°C overnight.

3.5 Plasmid preparation

For isolation of plasmid DNA, mini/midi prep was performed according to the QIAGEN plasmid prep kit following manufactures protocol.

3.6 Enzymatic modification of DNA

3.6.1 Digestion of DNA by restriction endonucleases.

DNA was incubated with appropriate enzymes and buffer for 2-24hrs at recommended temperature according to the manufacturers protocol.

Each 20µl digestion reaction contains:

DNA:	1-2µg
10×buffer:	2µl
restriction enzyme:	1unit

Water to make up the final volume

3.6.2 Ligation of DNA fragments

Ligation of DNA fragments was performed by mixing 50-100ng of vector DNA with three fold to eightfold molar excess of insert DNA.

1µl of T4 DNA ligase (1unit/µl) and 2µl of 10x ligation buffer were added and the reaction mix volume was brought to 20µl. The reaction was incubated for 3-12hrs at 4°C. The ligation mixture was used directly for transformation without any further purification.

Each 20µl ligation reaction contains:

vector DNA:	50-100ng
DNA fragment:	150-800ng
10× ligation buffer:	2µl
T4 DNA ligase (1U/µl):	1µl
total volume	20 µl

3.7 DNA electrophoresis

The size and purity of DNA was analyzed by agarose gel electrophoresis. Concentration of agarose used for analysis is inversely proportional to the size of the DNA of interest, that is, the larger the DNA the lower the concentration of agarose.

Agarose concentration (% [w/v])	Separation area (Kb)
0.6	1-20
0.9	0.5-7
1.2	0.4-6
1.5	0.2-4
2.0	0.1-3

DNA fragments were separated in horizontal electrophoresis chambers (5×7.5cm or 11×14cm) using agarose gels. Agarose gels were prepared by heating 1% (w/v) agarose in 1xTAE buffer (25ml for 5×7.5cm and 80ml for 11×14cm gels). The gel was covered with

1xTAE buffer, the DNA samples were mixed with sample buffer and loaded in agarose gel pockets. The gel was run at constant voltage (10V/cm gel length) until the loading dye had reached the end of the gel. The gel was the immersed in water containing 0.5µg/ml ethidium bromide for 15-20min at room temperature and further developed using a UV light imaging at 254nm.

1% agarose gel:	0.25g agarose
(5×7.5cm chamber)	25ml TAE buffer.
1% agarose gel:	0.8g agarose
(11×14cm chamber)	80ml TAE buffer.
1× TAE buffer:	40mM Tris, pH 8
	5mM Na Acetate
	1mM EDTA
6× loading buffer:	0.25% (w/v) bromophenol blue
	30% (v/v) glycerol in water

3.8 DNA purification

3. 8.1 DNA extraction from agarose gels

The DNA gel extraction kit (Nucleospin) was used for isolation and purification of DNA fragments from agarose gels. Ethidium bromide stained gels were illuminated with UV light and the appropriate DNA band was excised out from the gel using a clean scalpel and transferred into an eppendorf tube. The DNA fragment was purified following the manufacturers protocol.

3.8.2 Determination of DNA and RNA concentration

The DNA concentration was determined using Nanodrop ND-1000 spectrophotometer. DNA was diluted in water and the absorbance was measured at 260nm. Absorbance or optical

density (OD) of 1 at 260nm corresponds to ~50µg/ml of double stranded DNA or ~40µg/ml of single stranded DNA and RNA. The ratio between the readings at 260nm and 280nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 and 2.0, respectively. Any contamination would yield values less than mentioned above.

3.9 Protein extraction from mammalian cells.

To harvest cells, medium was removed and cells were washed with PBS then lysed by using appropriate lysis buffer (200-500µl/5cm petridishes). Lysates were transferred to a microcentrifuge tubes and centrifuged for 15min at 4°C. The clear supernatant containing protein extracts were stored at -20°C.

Whole cell lysates were prepared by direct lysis in laemli buffer. Cells were first washed with PBS, pelleted and then resuspended in 160µl 2x SDS sample buffer. The tubes were incubated at room temperature and centrifuged for 10 min at 13000rpm. Aliquots from the supernatants were analyzed by SDS-PAGE followed by immunoblotting analysis.

4 x SDS loading buffer:	1ml	4xSDS-solution
	320µl	0.1% bromophenol blue
	50µl	β- mercaptoethanol.

3.10 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) is a method for separation of proteins according to their molecular mass (Laemmli, 1970). In the present work gels were casted using biometra equipment and were 1.5mm thick. Depending on the size of the protein to be analyzed the pore size of the polymerized gel was altered (usually7-15%) by adjusting the concentration acrylamide. Mostly 11% gels were used in the present work. Separating and stacking gels were prepared according to protocols published by

Sambrook *et al* (1989). After complete polymerization of the gel, the chamber was assembled as described by the manufacturer's protocol. Protein samples were mixed with laemmli sample buffer, denatured at 95°C for 5-10 min centrifuged and directly loaded onto the gels. The gel was run at constant current at 10mA for the stacking gel and then at 20mA for the separating gel. The molecular weight of proteins was estimated by running 5µl of pre-stained marker proteins. The run was stopped when the bromophenol blue dye had reached the end of the gel. The gel was then either stained or subjected to western blotting.

	11% Separating gel	**5% Stacking gel:**
Acrylamide	2.2ml	0.334ml
1M Tris-HCL pH8.8	2.5ml	-------
1MTris HCl pH 6.8	----	0.25ml
Water	1.55ml	1.4ml
20%SDS	30µl	10µl
TEMED	6µl	3µl
10%APS	20µl	10µl

1x SDS running buffer:

Tris	3.03g
Glycine	14.22g
SDS	1g

Water to final volume of 1 litre.

4x SDS loading buffer:

4x SDS solution	1ml
0.1% bromophenol blue	320µl
β-mercaptoethanol	50µl

3.11 Western Blot (Semi-Dry)

Proteins were separated by 11% SDS-PAGE and transferred to polyvinyldendifluorid membrane (PVDF, Macherey & Negel) using the Bio-Rad Trans-Blot semi-Dry apparatus. For proteins transfer, the current was set to 0.8mA/cm^2 gel size for 30min. The gel was sandwiched between 3mm whatman papers cut exactly to the size of gel and soaked in anode or cathode buffer.

Cathode (-)

3 pieces of whatman paper soaked in cathode buffer

SDS polyacrylamide gel

PVDF membrane soaked in ddH2O

1 piece of whatman paper soaked in anode buffer II

2pieces of Whatman paper soaked in anode buffer I

Anode (+)

After transfer the membrane was placed in blocking buffer and incubated for 1hr at room temperature. After 1hr the primary antibody was diluted in TBST and added to the membrane, and incubated overnight at room temperature. Membrane was then washed thrice with TBST for 10-15min and was further incubated for 1hr at room temperature with horse radish peroxidise (HRP) coupled secondary antibody diluted in TBST. The unbound secondary antibody was washed away with TBST (3times for 10-15min) and the blot was incubated with chemiluminescence substrate solution (pierce). Protein antibody complexes was detected using LAS.

Anode buffer I (pH 10.4): 300mM Tris

20% (v/v) methanol

pH was adjusted before addition of methanol.

Anode buffer I (pH 10.4): 25mM Tris

20% (v/v) methanol

pH was adjusted before addition of methanol

Cathode buffer (pH 9.4): 40mM 6-amino-n-Hexanacid

25mM Tris

20% (v/v) methanol

pH was adjusted before addition of methanol.

Tris- buffered saline (TBS): 10mM Tris –HCL pH 7.4

150mM Nacl

TBST: 0.1% (v/v) Tween 20 in TBS

Blocking buffer: 2% BSA (bovine serum albumin) in TBST

Stripping of PVDF Membranes.

Removal of antibodies from a blot was done under mild conditions. After washing the membrane 3times in TBST, the blot was incubated in SDS stripping buffer for 30mins, followed by washing with TBST. The membrane was incubated with new antibody as described above, starting from the blocking step.

SDS stripping buffer: 16mM Tris-Cl pH 6.8

2% SDS

10mM β-mercaptoethanol

3.12 Detremination of protein concentration (Bradford)

The Bradford dye binding assay is a colorimetric assay for measuring total protein concentration (Bradford 1976). It involves the binding of coomassie Brilliant blue to protein.

3.13 Staining of protein gels.

Coomassie staining

Proteins were separated by SDS-PAGE, and protein gels were incubated in coomassie staining solution with gentle shaking at room temperature for 2hrs or overnight. To remove Unspecific staining was removed by incubating gels in destaining solution for 2-3hrs at room temperature.

Staining solution: 0.1% (w/v) coomassie brilliant blue R250
45% (v/v) methanol
10 % (v/v) acetic acid

Destaining solution: 45% (v/v) methanol
10% (v/v) acetic acid.

3.14 GST pull – down assays.

3.14.1 Expression and purification of recombinant GST-fusion protein in E.coli.

The E.coli strain DH5α were transformed with the expression plasmid expressing GST tagged protein and cultured in 50ml of 2YT medium with ampicillin antibiotic overnight at 37°C. The overnight culture was diluted 1:500 in 100ml of 2YT medium containing the antibiotic and cultured at 37°C until cell density reaches OD 600 of 0.3. To induce expression of the recombinant protein 1mM IPTG was added and the culture was incubated for 2-6hrs at 37°C. Cells from a 100ml bacterial culture was harvested at 4000rpm and washed once with ice cold PBS. All the following steps were performed on ice. The cell pellet was resuspended in 4ml lysis buffer. The suspension was sonicated (8 times, each 30sec, amplitude 50%Branson sonifier). The cell extracts were seperated by centrifugation at 5000rpm for 15mins at 4°C (centrifuge, Hettich).

The clear supernatant was transferred to a new tube and incubated overnight at 4°C with 50µl of glutathione sepharose 4B beads (Amersham). Next day beads were washed 3 times and

resuspended in 1ml washing buffer (1:2 ratio of beads and washing buffer), 8-10ul of the 50% beads slurry was separated by SDS –PAGE and bead bound proteins were stained with coomassie to determine the amount of immobilized proteins. Beads were either stored at 4°C untill further use or the bound proteins were eluted immediately.

For elution, beads were washed in 20mM tris-HCl, pH 8.0 containing protease inhibitors, before incubating with 200µl elution buffer at 4°C for 2hrs or overnight. The elution step was repeated twice, and 7µl of the eluate was analyzed by SDS-PAGE followed by coomassie staining. The eluted fractions were dialyzed using micron columns (Millipore) and stored at -80°C.

GST lysis Buffer: 20mM Tris-HCl pH 7.5
0.1% Triton X -100
1mM PMSF
1% Trasylol
1% SDS

Washing Buffer: 20mM Tris –HCl pH 7.5
0.5% Triton X -100
15mM β-mercaptoethanol
1mM PMSF
2% Trasylol in PBS

Elution Buffer: 20mM Tris- HCl pH 7.5
20mM glutathione
1mM PMSF
1% Trasylol.

3.14.2 GST pull- down assay using HEK293, Hela, *MEF* Cell Lysates.

Beads slurry containing equal amount of GST tagged proteins (GST, GST-THOC5, GST-THOC7) was used for pull down assay. Beads were washed 3 times with lysis buffer, after the final wash the supernatant was removed and cell lysate transfected or untransfected was added and incubated at 4°C for 4hrs or overnight. The beads were then washed 3 times with buffer and centrifuged at 2000rpm for 2 min at 4°C. After the final wash the supernatant was removed and 20µl of 4xSDS loading buffer was added, the sample was boiled for 15min, chilled on ice and centrifuged at 13000rpm at 4°C. The supernatant was loaded onto 11% SDS-PAGE and analysed by western blotting using appropriate antibody.

3.15 Mammalian Tandem affinity purification

Tandem affinity purification (TAP) is a method to identify protein–protein interaction. It allows identification of all the components in a multisubunit complex that either directly or indirectly interacts with the protein of interest. The basic principle involves expression of Tap tag construct fused with the protein of interest at its amino or carboxy terminal. The Tap vector contains (SBP) streptavidin binding protein and Calmodulin binding proteins (CBP). The SBP tag allows the protein of interest and its interacting proteins to be captured through the tight binding of SBP to streptavidin resin and purified by eluting with excess biotin. The CBP tag allows a second purification step in which the CBP tag binds to calmodulin resin and released by EGTA through calcium (Ca^{2+}) chelation. The final eluate contains almost the proteins in the same complex with the protein of interest and can be further indentified using mass spectrometry. In this study one step TAP purification was performed using streptavidin beads.

3.15.1 Cloning of Tap-THOC7-myc

The interplay ^{TM} N-terminal mammalian TAP system pNTAP vector from stratagene was used (Figure 3.1). The vector was linearised using EcoRI and XhoI and the PCR product

(myc tagged THOC7 fragment) was then inserted into the linearised pNTAP vector. The insertion pntap-THOC7-myc construct was verified by DNA sequencing.

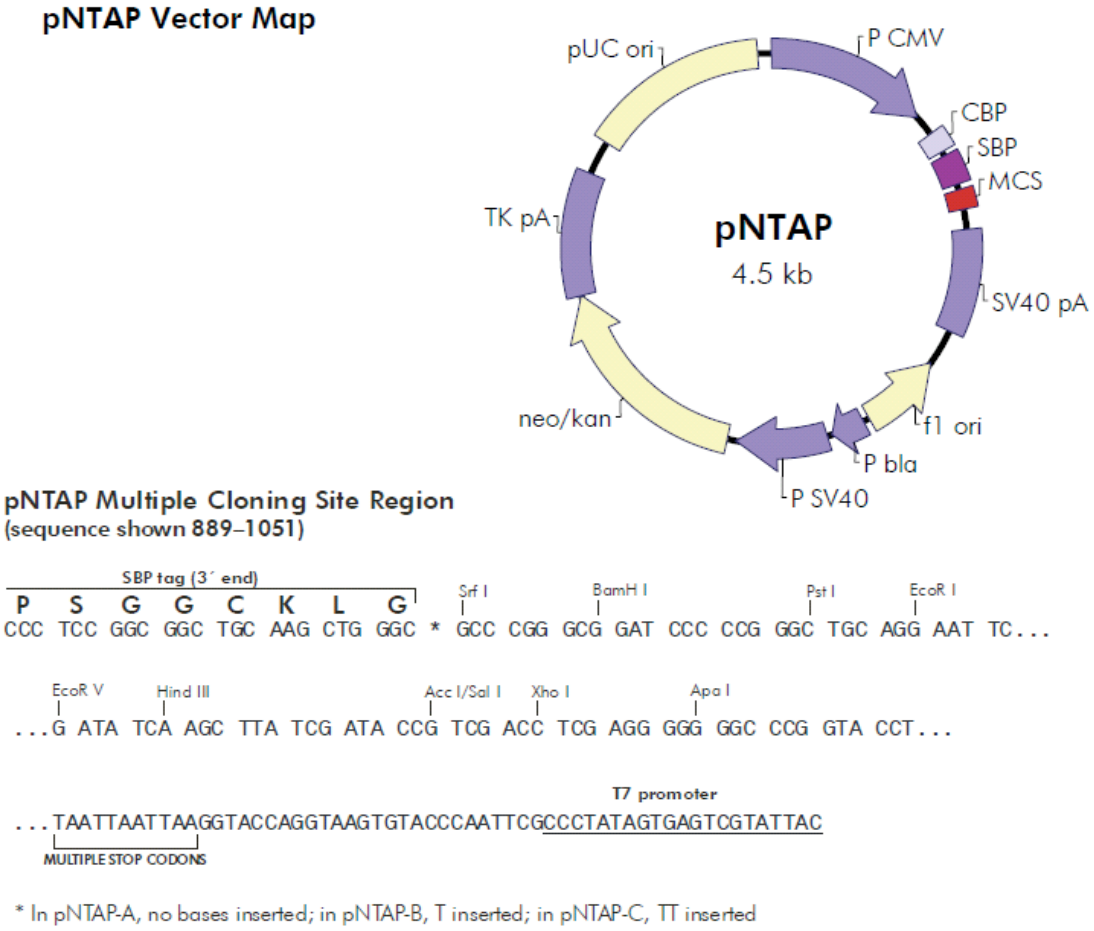


Figure 3.1 Circular map of pNTAP expression vector (Adapted from interplay^{TM} TAP purification kit protocol).

3.15.2 Establishment of mammalian Tandem affinity purification

HEK293 cells were transfected with TAP-THOC7-Myc construct or empty vector (pNTAP). The transfected cells were washed twice with ice cold PBS. Cells were lysed with lysis buffer and were harvested by scraping. To improve protein recovery freeze thaw method was done twice in liquid nitrogen and 37°C thermomixer.

The cell lysates were centrifuged for 15mins at maximum speed 13000rpm and the supernatant was collected into a fresh eppendorf tube. Streptavidin beads were washed twice with lysis buffer before adding the to the cell lysates and incubated at 4°C overnight (10µl cell lysates was stored separately). Next day beads were washed with lysis buffer and supernatant was removed completely. Sample buffer was added directly to the beads and were boiled at 95°C for 10mins. It was briefly centrifuged and was loaded in SDS polyacrylamide gel. Gel was stained with coomassie to check for proteins. Western blot was performed using myc antibody.

Lysis Buffer:

10 mM	Tris-Cl pH 8.0
150mM	NaCl
0.1%	NP40
1mM	PMSF
1%	Trasylol

Streptavidin binding buffer (SBB)

10mM	Tris – Cl pH 8.0
150mM	NaCl
0.1%	NP40
1mM	PMSF
1%	Trasylol
10mM	β-mercaptoethanol
0.5mM	EDTA

Streptavidin elution buffer (SEB)

10mM	Tris-Cl pH8.0
150mM	NaCl
0.1%	NP40
1mM	PMSF
1%	Trasylol
10mM	β-mercaptoethanol
0.5mM	EDTA
10mM	D- Biotin

Calmodulin binding buffer (CBB)

10mM	β-mercaptoethanol
10mM	Tris- Cl pH8.0
150mM	NaCl
1mM	Mg- acetate
1mM	imidazole
2mM	$CaCl_2$
0.1%	NP40

Calmodulin elution buffer (CEB)

10mM	Tris-Cl pH 8.0
150mM	NaCl
1mM	Mg-acetate
10mM	β-mercaptoethanol
1mM	imidazole
2mM	EDTA
0.1%	NP40

3.15.3 Purified protein separation and visualization

To analyse the results in TAP procedure the protein aliquots were visualized on a 11% SDS-PAGE. The 0.5 to 4µg of protein samples or 15µl beads were added to 2x SDS loading sample buffer. The samples were boiled for 12mins and loaded on 11% SDS gel. For the molecular weight markers, 1µl of protein standard (Bio-Rad) with a range of 10- 250 kDa were used. The gel was then stained using silver stain plus kit (Bio-Rad) or stained for 2hrs using coomassie stains (Sigma).

3.16 Mass Spectroscopy and Maldi-TOFF

The identification of low level proteins from the SDS-PAGE was performed in the laboratory of Prof. Tony Whetton at the university of Manchester.

Mass spectroscopy is a powerful tool used extensively in proteomics. In order to identify the major proteins present in the SDS-PAGE gel. Gel slices were cut and subjected to enzymatic digestion using trypsin, which typically generates peptides which can be analysed by mass spectroscopy. Separated peptides were eluated from the analytical column directly into the electrospray ion source of a QSTAR XL hybrid mass spectrometer. MS/MS data was acquired using an established information dependent acquisition (IDA) protocol enabling peptide sequence information to be obtained. The instrument was instructed to scan for potential peptide ions.

3.17 Cell culture

3.17.1 Cell culture and maintenance of cell lines.

Human embryonic kidney (HEK)293 cells, Hela cells, MEF (mouse embryonic fibroblast) cells were routinely cultured in 37°C, 5% CO_2 in dulbecco's modified eagle medium (DMEM; GIBCO) containing 10% fetal calf serum (FCS), 100µg/ml penicillin, 100µg/ml streptomycine and 2mM L-glutamine in PBS. Medium was changed after 2-3 days and the cells were passaged at a dilution 1:5 or 1:10 when the cells were 90 – 95 % confluent.

3.17.2 Passaging by trypsinization

For passaging, the cells were allowed to reach confluence and then the medium was discarded. The cells were washed carefully with PBS. An appropriate amount of trypsin (according to the size of the culture flask or petri dish) was added to the cells. The cells were incubated at 37°C for 2-3min until they detached from the surface of the culture vessel. Fresh

medium was immediately added to the cells and the cells were split into an appropriate number of culture flasks according to the need.

3.17.3 Counting the cells

The cell count was determined using the heamocytometer. An aliquot of the cell suspension obtained after trypsinizing the cells was diluted 1:1 with Trypan Blue (0.8% v/v in PBS). Trypan Blue is a cell permeable dye; while living cells are able to extrude the dye, dead cells are unable to do so and hence stain blue. To obtain an accurate count, the cells have to be uniformly distributed over the entire chamber. The chamber is divided into 9 squares. Each square has a surface area of $1mm^2$ and depth of the chamber is 0.1mm. To get the final count in cells/ml, first divide the total count by 0.1mm (chamber depth) then divide the result by the total surface area counted.

3.17.4 Transient transfection in MEF, Hela and HEK293 cells

Cells were grown till they are 70% confluent in 10cm petridishes. Before transfection the media is removed and fresh media is added to the plate. Polyfect reagent was used for the DNA transfer following manufacturers instructions. About 5µg of DNA was mixed in a total volume of 300µl DMEM without antibiotics. Polyfect 30µl was added to the mixture and was incubated at room temperature for 8-10mins to allow the formation of liposomes. After this incubation 1ml of DMEM medium with antibiotics is added to this reaction and transferred to the cells. The cells are incubated at 37°C for next 24hrs. The transfected cells were analyzed by western blotting for checking the expression of the construct.

3.17.5 Freezing and thawing of cells

Cells that are to be frozen are cultured until they reach confluence. Cells from a confluent 10cm flask were frozen as 2-4 vials. Each freezing ampoule was labeled with the name of the cell line and the date of freezing. Cells were pelleted after trypsinization, resuspended in 1ml

freezing medium per vial, immediately placed on ice and stored at -80°C overnight. Next day the vials were transferred to the liquid nitrogen container.

***Freezing medium*:** 90% FCS

10% DMSO

For thawing cells the cell line stored in liquid nitrogen was taken out and allowed to thaw in 37°C water bath. The cells were transferred to a $75cm^2$ cell culture flask containing 10ml medium. The cells were incubated for 4hrs under standard conditions (5% CO_2, 37°C) the medium was changed after 4hrs and the cells were allowed to grow till they are confluent.

3.18 Adenovirus infection

MEF cells were used for adenovirus infection. The cells were seeded one day before infection $1x10^4$cells /well for 24 well plates. The medium was removed from the plates and fresh medium was added to the plate (235µl). 1µl of virus stock (titre :1x 10^{10}IFU/ml) was added to each well, it was mixed and the plate was incubated at 37°C, 5%CO_2 incubator. 5hrs after infection fresh medium was added to each wells (800µl). Next day the media was changed from the plates and fresh medium was added to each wells. After 4 days the media was removed and the cells were washed with PBS and Lysed with RLN buffer, cytoplasmic RNA was isolated from the cells using qiagen RNA isolation kit.

Ad-CMV-GFP (Vector Biolabs)

Viral backbone	- Adenovirus-Type 5 (dE1/E3)
Promotor	- CMV
Transgene	- eGFP
Titre	- $1x10^{10}$IFU/ml
Storage buffer	- DMEM w/2%BSA &2.5%Glycerol

Ad-Cre –GFP (Vector Biolabs)

Viral backbone	- Adenovirus-Type 5 (dE1/E3)
Promotor	- CMV
Transgene	- Cre
Marker/Tag	- GFP
Titre	- $1x10^{10}$IFU/ml
Storage Buffer	- DMEM w/2%BSA&2.5% Glycerol

3.19 Isolation of Cytoplasmic RNA

Cytoplasmic RNA was isolated after four days from MEF cells infected with Ade-Cre (adenovirus carrying Cre recombinase) and Ade-GFP(adenovirus+GFP, control) Mock (uninfected) MEF cells. RLN buffer was used to lyse the cells. RNA Isolation protocol according to manufactures instructions. RNA concentration was measured in Nanodrop.

RLN Buffer

50mM	Tris, pH 8.0
140mM	NaCl
1.5mM	$MgCl_2$
1.06g/ml	0.5%(v/v)nonidet P-40

Just before use add: 1000U/ml RNase inhibitor(optional), 1mM DTT(optional)

3.19.1 Isolation of POLY A^+ RNA from total RNA using Dynabeads Oligo$(dT)_{25}$

Buffers required:

Binding Buffer (50ml)

20mM	Tris-HCl pH 7.5
1M	LiCl
2mM	EDTA
	RNAse free water

Washing Buffer (50ml)

10mM	Tris-HCl pH 7.5
0.15M	LiCl
1mM	EDTA
	RNAse free water

Elution Buffer (15ml)

10mM	Tris-HCl pH 7.5
	RNAse free water

To every100µg of total RNA, 200µl of oligodT-linked Dyna bead suspension was used in this isolation procedure. The beads were washed twice with washing buffer. The supernantant was discarded completely and the beads were resuspended in binding buffer. RNA was dissolved in equal volume of binding buffer and was incubate at 65°C for 5mins. The sample was shortly centrifuged for 30 seconds. RNA sample was mixed with the bead and left at RT for 5mins. The tube was now placed in a magnetic stand and the supernatant was removed. The sample was washed twice with washing buffer. The supernatant was completely removed after the second wash. The tube was removed from the magnetic stand and the elution buffer was added to the beads and incubated at 65°C for 2mins. Tubes were then placed back into the magnetic stand and the supernatant was now collected in a new sterile eppendorf tube. The poly A^+ RNA prep was stored at -80°C until further use.

3.19.2 RNA gels

Dependeing on the percentage of gel and the pore size required amount of agarose was dissolved in DEPC water and boiled. Accordingly 10X MOPS and formaldehyde solution were added and the gel was immediately poured into gel chambers.

Small 1% agarose gel:	0.25g agarose
(5x7.5cm)	18.5 ml DEPC water
	2.5 ml 10x MOPS
	4 ml formaldehyde 37% (v/v)
Big 1% agarose gel:	0.8 g agarose
(11x 14 cm)	59ml DEPC water
	8 ml 10x MOPS
	13 ml formaldehyde 37% (v/v)

10 x MOPS buffer: 0.4 M Morpholinpropanesulfonic acid

100mM Natrium acetate

10mM EDTA

For sample preparation

A master mix was prepared by adding

RNA - 5.5µl (5- 10 µg)

10x MOPS - 1µl

Formaldehyde - 3.5 µl

Formamide - 10 µl

Mixed by Pipetting up and down and incubated at 60°C for 15 min. The samples were stored on ice untill further use. Loading buffer mix was added to the samples and load on to the gel. The gel was run at 120V till the dye front migrated halfway down the gel.

3.20 Northern Transfer

The RNA gel was visualized under the transilluminator to check the presence of RNA. The gel was dipped in 10x SSC for 30 min, in the meantime the transfer apparatus was set up. The nylon membrane were cut to the size of gel to be transferred and were dipped in 10x SSC buffer for 30mins. After 30mins the transfer was set up was done, a long piece of whatman filter paper was placed on top of the tray such that both of its end were dipped in 10xSSC, the gel was carefully placed on top of the paper, nylon membrane was placed on top of the gel, 3 pieces of whatman filter paper which were cut according to the gel size was placed on top of membrane, stack of paper towels were placed on top, and a weight was placed over the whole set up (Figure 3.2). The transfer was allowed to take place for about 20hrs. Membranes was washed in 2x SSC for 2mins and baked for 2hrs at 80° C.

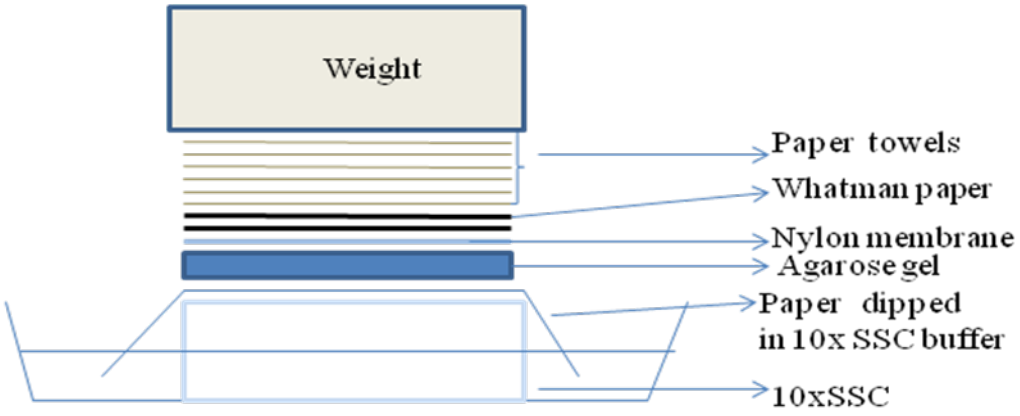


Figure 3.2 Transfer system for Northern blotting

3.20.1 Hybridization of Northern blots

The membrane was incubated in prehybridization solution for at least 6 hrs at 42° C. After incubation the denatured salmon sperm DNA (for blocking) and denatured radiolabelled probe was added to the tube, (the probe was radiolabelled by ^{32}P using megaprime labelling systems and was purified using jetnick kit protocol according to manufacturers instructions). the membrane was incubated O/N at 42 °C, next day the membrane is :-

a) washed twice with 2X SSC at room temperature for 5mins,

b) washed twice with 2X SSC and 1%SDS for 30mins at 60 °C,

c) washed twice with 0.1 X SSC for 30mins at room temperature.

After the last wash the membrane was dried in whatman filter paper and wrapped in fresh wrap. The membrane was placed along with X-ray films in a cassette and was stored in -80°C. The film is developed using a developer.

3.21 Microarray Analysis

For microarray analysis, the quality and integrity of the total RNA were confirmed using an Agilent Technology 2100 Bioanalyzer (Agilent Technology, Palo Alto, CA, USA). Biotin-labeled target synthesis was performed by Affymetrix (Memphis TN, USA). About 12.5 µg

of each biotinylated cRNA preparation were fragmented and placed in a hybridization cocktail that contained four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. All samples were hybridized to the same lot of Affymetrix MGU74A for 16h. The GeneChips were washed, stained with streptavidin-phycoerythrin, and read by using an Affymetrix GeneChip fluidic station and scanner. Analysis of microarray data was done by using the Affymetrix Microarray Suite 5.0, Affymetrix Micro DB 3.0, and Affymetrix Data Mining Tool 3.0. For normalization all array experiments were scaled to a target intensity of 150 (Kroger et al., 2007).

3.22 Ingenuity Pathway analysis (IPA)

Ingenuity Pathway Analysis (IPA) assigns biological functions to genes using the Ingenuity Pathways Knowledge Base. The knowledge base comprises over 200,000 full text articles and information about thousands of human, mouse, and rat genes (Calvano SE et al 2005). This information is used to form networks to create an 'interactome' of genes all involved in specific biological processes. IPA groups significant genes according to the biological processes in which they function. The program displays the genes' significance values, the other genes with which it interacts, and how the gene products directly or indirectly act on each other, including those not involved in the microarray analysis (Subramanian A et al, 2005). IPA was used to analyse the microarray results.

3.23 RT- PCR

Procedure for the reverse transcription reaction is mentioned below :-

10X buffer RT	2µl
dNTP mix (5mM each dNTP)	2µl
Oligo dT primer (10uM)	2µl
RNase Inhibitor (10 units/µl)	10 units (per 20 µl reaction)
Omniscript reverse transcriptase	1µl (4 units per 20 µl reaction)
	RNase free water varies
Template RNA	varies (upto 2µg per 20 µl reaction
Total volume	**20 µl**

A master mix is made by adding all the above components, the master mix is mixed and centrifuged briefly and is incubated for 60mins at 37°C.

3.24 Polymerase chain reaction(PCR). PCR master mix was purchased from pEQlab.

Master mix	10µl
Primer forward(10uM)	1µl
Primer reverse(10uM)	1µl
cDNA	1µl
water (adjust total vol.with water)	
total	20µl

PCR was carried out using an automated thermal cycler.

PCR program

1min 94°C(denaturation)

1min 60°C(annealing)

3min 72°C (extension)

PCR products were separated by agarose gel electrophoresis.

4 RESULTS

I) To identify THOC5 dependent mRNA by microarray using MEF cells from conditional THOC5 knockout mouse.

4.1 Conditional knockout of fmip from the mouse embryonic fibroblast.

Published work from our lab, using conditional THOC5/FMIP knockout mice has demonstrated that THOC5/FMIP is critical in embryo development and in haematopoiesis (Mancini et al 2010). Earlier studies in *Drosophila* suggested that more than 20% of the transcriptome was regulated by THO component (Rehwinkel et al 2004). In the same system it has also been shown that upon deletion of UAP56 and NXF1 (TAP) or p15 ~75% of *Drosophila* gene expression is affected (Herold A et al 2003). Hence we were interested in examining the effects of deletion of FMIP/THOC5, a member of the THO complex on the gene expression. FMIP *flox/flox* mice were created in our lab. A mouse embryonic fibroblast cell line was generated from the embryos of these mice. Initial efforts were to strategically delete *fmip* from the MEF cells using Cre-loxP system. Infection of these MEF cells with adenoviruses expressing cre-recombinase (Ade-Cre) resulted in excision of region containing exon IV and V of *fmip* thereby making it functionally equivalent to *fmip* null cells (Fig.4.1). As a control MEF cells were infected with adenoviruses expressing GFP. Kinetics of FMIP knockdown was tested both at protein level and mRNA level.

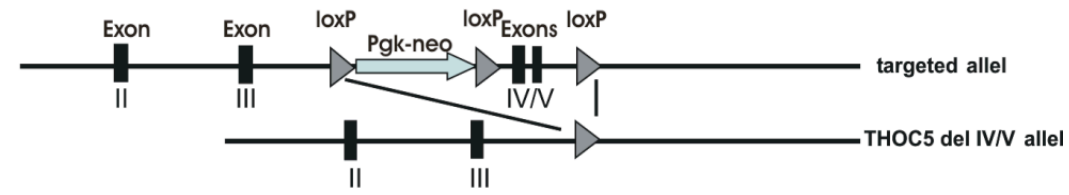


Figure 4.1 Schematic representation of *fmip* deletion. FMIP *flox/flox* mice were generated using Cre l*oxP* system. *loxP* sites were introduced flanking exon IV and exon V of FMIP gene. By infecting *MEF* cells by adenoviruses carrying cre recombinase these exon IV and V region of *fmip* will be floxed out and a truncated protein of about 83 amino acid is obtained.

4.2 Confirmation of FMIP deletion at protein level.

Knockdown of FMIP protein in infected MEF cells was tested by doing western blots. At 4 days of post-infection no FMIP protein was detectable in MEF cells infected with Ade-Cre while the levels of THOC5 protein expression remained unchanged in control MEF cells infected with Ade-GFP (Figure 4.2).

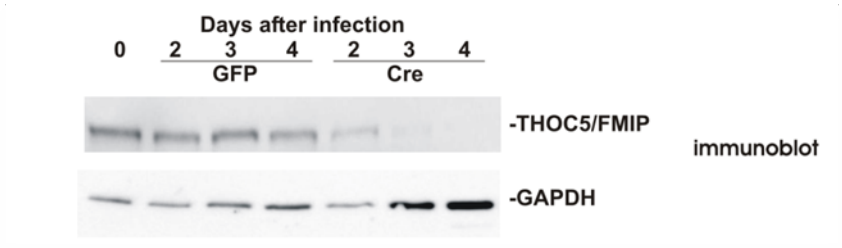


Figure 4.2 Western blot analysis to confirm *fmip* deletion. Adequately confluent MEF cultures were infected with Ade-Cre or Ade-GFP. On the specified days post-infection cell lysates from these cells were loaded on to the gel and were probed using anti-FMIP and GAPDH antibodies.

4.3 Confirmation of FMIP deletion at mRNA level.

Analysis of *fmip* mRNA level by northern blot analysis and RT-PCR provided further confirmation of *fmip* deletion. The cytoplasmic RNA was isolated from MEF cells infected with Ade-Cre and Ade-GFP at various time points post infection and was probed with FMIP and GAPDH probes. Downregulation of *fmip* mRNA was detected as early as day2 post-infection. RT-PCR analysis of cytoplasmic RNA from the MEF cells infected with Ade-Cre was performed using *fmip* specific primers. Primers selected were located in exon II (forward primer) and exon VII (reverse primer) region of *fmip* gene. The product detected in the *fmip* depleted fraction was shorter and had a length of 429bp, thus confirming the excision of exon IV and V from the *fmip* gene. MEF controls infected with Ade-GFP showed a *fmip* product of 643bp (Figure 4.3).

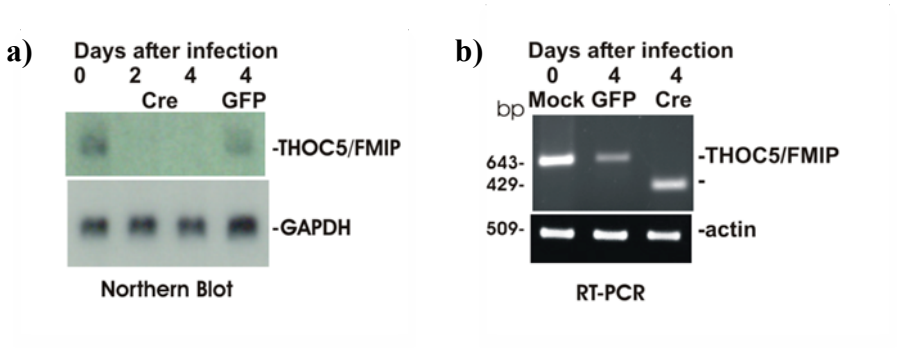


Figure 4.3 Testing *fmip* deletion (a) Northern blot analysis to confirm *fmip* deletion**.** Four days after infection of MEF cells with Ade-cre or Ade-GFP, cytoplasmic poly A+ RNA was isolated and northern blot analysis was performed. Nylon membrane was hybridized with THOC5 and GAPDH specific ^{32}P radiolabeled probes**. (b)** RT-PCR using FMIP specific primers was performed with the RNA isolated from MEF cells. Actin was used as control. cDNA was checked on agarose gel electrophoresis.

4.4 Gene array analysis of FMIP deleted MEF.

As mentioned above, deletion of certain members of THO complex severely affected the gene expression pattern in *Drosophila.* Hence we sought to examine if similar phenomenon was observed in MEF cells upon *fmip* deletion. A genome wide screening was performed to study the level of fraction of genes whose expression requires THOC5. Cytoplasmic fraction of RNA was isolated from the MEF cells infected with Ade-Cre or Ade-GFP and microarray analysis was done (Figure 4.4).

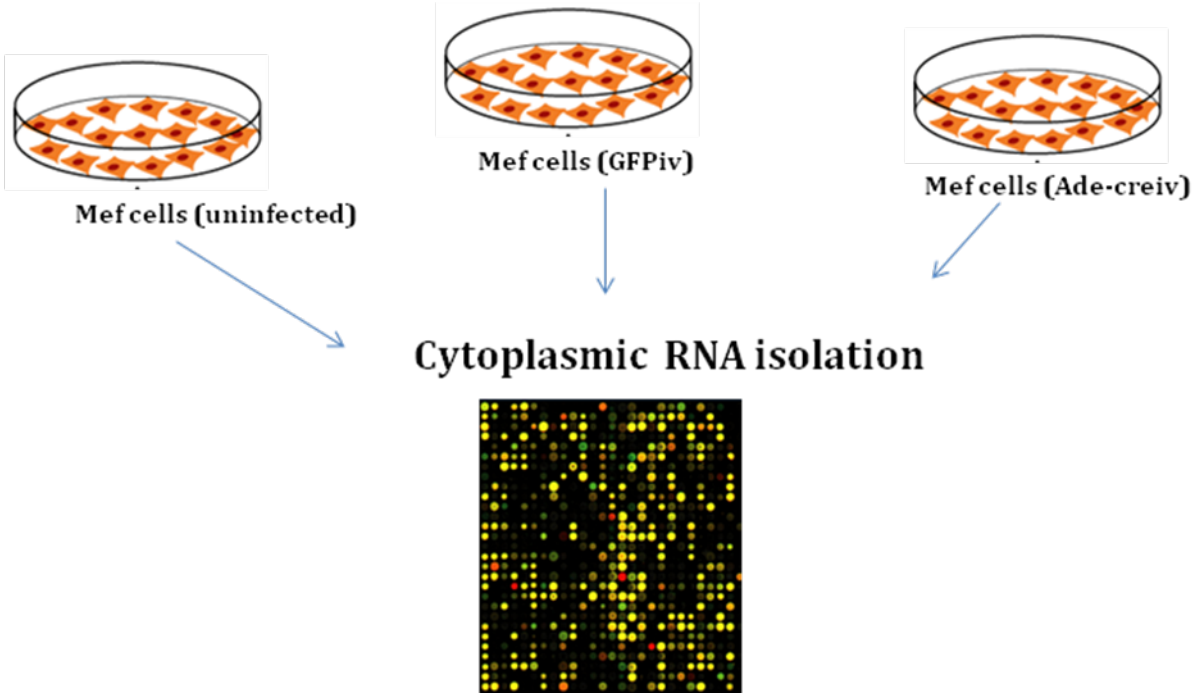


Figure 4.4 Schematic representation of samples subjected to microarray. All samples were hybridised to the same lot of Affymetrix MGU74A for 16 h.

Initially, genes that were down-regulated more than 3-fold by Ade-Cre infection, but were down regulated less than 1.5-fold by Ade-GFP control virus infection were chosen for further analysis. According to this criterion only 199 genes showed more than 3-fold down-regulation from the fraction that were FMIP depleted. This corresponds to 1% of the total genes analyzed. By selecting genes that were down regulated more than 2-fold, 559 genes were found. When searched for the function associated with these genes, it was observed that 50 genes among 559 genes were transcriptional regulators, indicating that not all 559 genes are regulated directly by THOC5/FMIP. Among the 199 genes which are down regulated more than 3-fold by depletion of THOC5/FMIP, 72 genes has known functions (Table 4.1). Looking for the exact functions of these 72 genes using ingenuity pathway analysis (IPA) we found that 44% genes among these are involved in cell differentiation and development (Table 4.2).

Table 4.1 Differentially expressed genes in THOC5/FMIP depleted MEF cells identified by cDNA microarray analysis.

Symbol	Gene name	Genbank ID	Molecular function	Fold change
AGPAT6	1-acylglycerol-3-phosphate O-acyltransferase 6	NM_018743	acyltransferase activity, glycerol-3-phosphate O-acyltransferase activity	-4.7
AKT1S1	AKT1 substrate 1	BM068933	Protein binding	-3.1
ARF6	ADP-ribosylation factor 6	BI248938	GTP binding, protein binding	-4.2
ARMC9	armadillo repeat containing 9	AK017564	protein-protein interactions	-14.5
ARNT	aryl hydrocarbon receptor nuclear translocator	AU022700	DNA binding, protein binding, receptor activity, transcription regulator activity	-6.2
ATOX1	ATX1 antioxidant protein 1 homolog (yeast)	BM210947	Copper ion binding, metal ion binding	-4
ATP6V1B2	ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B2	AI790362	ATP binding, hydrogen ion transporting ATP synthase activity, rotational mechanism, hydrolase activity	-3.3
BCL11B	B-cell CLL/lymphoma 11B	AK020296	Metal ion binding, nucleic acid binding, zinc ion binding	-6.8
CBX2	chromobox homolog 2	NM_007623	DNA binding chromatin binding protein binding	-5.8
CD274	CD274 molecule	NM_021893	Protein binding, receptor activity	-10
CHRNA1	cholinergic receptor, nicotinic, alpha 1 (muscle)	NM_007389	contributes to acetylcholine receptor activity, nicotinic acetylcholine-activated cation-selective channel activity	-4.1
COPS5	COP9 constitutive photomorphogenic homolog subunit 5	AI662452	Hydrolase activity, metal ion binding, metallopeptidase activity, transcription coactivator activity	-4.8
CRYL1	crystallin, lambda 1	AI482548	3-hydroxyacyl-CoA dehydrogenase activity	-11.2

DDX11	DEAD/H box polypeptide 11	BB133021	DNA repair helicase activity	-4.5
DIMT1L	DIM1 dimethyladenosine transferase 1-like (S. cerevisiae)	AK015145	RNA binding, methyltransferase activity, rRNA (adenine) methyltransferase activity, rRNA (adenine-N6, N6-)-dimethyltransferase activity, rRNA methyltransferase activity	-3
DOCK1	dedicator of cytokinesis 1	BB283533	GTP binding, GTPase binding, SH3 domain binding, guanyl-nucleotide exchange factor activity, protein binding	-3.7
ENY2	enhancer of yellow 2 homolog *(Drosophila*)	AV033253	Zinc finger domain binding	-7
FAM38B	family with sequence similarity 38, member B	BM220702	Molecular function	-4.3
FMO3	flavin containing monooxygenase 3	NM_008030	FAD binding, NADP or NADPH binding, oxidoreductase activity	-5.5
FOSL1	FOS-like antigen 1	U34245	DNA binding, protein dimerization activity, transcription factor activity	-4.8
GJA1	gap junction membrane channel protein alpha 1	M63801	SH3 domain binding , gap junction channel activity, PDZ domain binding	-3.1
GIGYF2	GRB10 interacting GYF protein 2	BB782031	Protein binding	-11.7
GRIK2	glutamate receptor, ionotropic, kainate 2	BB355480	extracellular-glutamate-gated ion channel activity, ion channel activity, ubiquitin protein ligase binding	-4
GRIN3B	glutamate receptor, ionotropic, N-methyl-D-aspartate 3B	NM_130455	extracellular-glutamate-gated ion channel activity, transporter activity	-4.8
HMOX1	heme oxygenase (decycling) 1	NM_010442	heme binding, heme oxygenase (decyclizing) activity, oxidoreductase activity, phospholipase D activity	-3.3

HOXB3	homeobox B3	X66177	DNA binding, transcription factor activity, transcription regulator activity	-5.2
ID2	inhibitor of DNA binding 2	NM_010496	Protein binding, transcription regulator activity, transcription repressor activity	
IL7	interleukin 7	NM_008371	Cytokine activity, growth factor activity, interleukin-7 receptor binding	-3.5
IL7R	interleukin 7 receptor	AI573431	Cytokine receptor activity	-7
INADL	InaD-like	AV287690	Protein binding	-3.6
KCNA7	potassium voltage-gated channel, shaker-related subfamily, member 7	NM_010596	Ion channel activity, postassium channel activity, protein binding, voltage-gated potassium channel activity	-8.9
LGALS2	lectin, galactoside-binding, soluble, 2	NM_025622	Galactoside binding, sugar binding	-5.8
LPAR4	lysophosphatidic acid receptor 4	BB297502	G-protein coupled receptor activity, lipid binding, signal transducer activity	-8.9
MT1E	metallothionein 1E	AA796766	Cadmium ion binding, copper ion binding, zinc ion binding	-3.5
MYRIP	myosin VIIA and Rab interacting protein	BB429683	Rab GTPase binding, actin binding, metal ion binding, myosin binding, protein binding/zinc ion binding	-6.3
PMS2	Postmeiotic segregation increased 2	AK014190	endonuclease activity, hydrolase activity, mismatched DNA binding	-7.8
POU6F2	POU class 6 homeobox 2	BB244362	Sequence-specific DNA binding, transcription factor activity	-15.6
PPP2R1B	protein phosphatase 2 regulatory subunit A, beta isoform	AI505185	Protein phosphatase activity	-10.2
PREB	prolactin regulatory element binding	AV362891	Protein binding, DNA binding, transcription factor activity, transcription activator activity	-3.1

PRKAR2A	protein kinase, cAMP-dependent, regulatory, type II, alpha	AV112640	cAMP-dependent protein kinase regulator activity, nucleotide binding, protein homodimerization activity	-3
PRMT6	protein arginine methyltransferase 6	BB233495	histone-arginine N-methyltransferase activity, protein methyltransferase activity	-14.2
PSMG4	proteasome (prosome, macropain) assembly chaperone 4	AV090264	Protein binding	-4.6
PTF1A	pancreas specific transcription factor, 1a	NM_018809	Sequence-specific DNA binding, protein binding, transcription regulator activity	-11.2
PTPRT	protein tyrosine phosphatase, receptor type, T	NM_021464	Alpha-actinin binding, catenin binding, hydrolase activity, phosphatase activity, receptor activity, thioester hydrolase activity	-5.3
RAMP3	receptor (G protein-coupled) activity modifying protein 3	NM_019511	Coreceptor, soluble ligand activity	-3.4
RPS25	ribosomal protein S25	BM729504	structural constituent of ribosome	-3
RSBN1	round spermatid basic protein 1	AI551821	Protein binding	-5.9
RUFY2	RUN and FYVE domain containing 2	AI852705	Zinc ion binding	-4.2
SERINC3	serine incorporator 3	NM_012032	Induction of apoptosis	-3.5
SIK1	salt-inducible kinase 1	NM_010831	ATP binding, Magnesium ion binding, protein kinase activity	-3.5
SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	M75135	D-glucose transmembrane transporter activity	-7.4
SLC39A6	solute carrier family 39 (zinc transporter), member 6	BB213740	metal ion transmembrane transporter activity	-3.2

SMC3	structural maintenance of chromosomes 3	AK005647	ATP- and protein-binding	-4.2
SOX9	SRY (sex determining region Y)-box 9	BC024958	Sequence-specific DNA binding, transcription factor activity	-3
SOX15	SRY (sex determining region Y)-box 15	AF182945	DNA-and Chromatin-binding, transcription factor activity	-5.1
SOX5 (includes EG:6660)	SRY (sex determining region Y)-box 5	BB018032	DNA-and Chromatin-binding, transcription factor activity	-4.5
STX8	syntaxin 8	AK011376	ubiquitin protein ligase binding	-3.3
SYN3	synapsin III	AV327590	ATP binding, catalytic activity	-52.7
TEX9	testis expressed 9	BB428851	Chromatin binding	-10
TFRC	transferrin receptor (p90, CD71)	AK011596	iron ion transmembrane transporter activity, HSP70 protein binding	-3.9
TMSB10	thymosin beta 10	AV148480	Actin monomer binding	-4.3
TOP3B	topoisomerase (DNA) III beta	BB107606	DNA topoisomerase type I activity	-11.2
TSFM (includes EG:10102)	Ts translation elongation factor, mitochondrial	AK020437	translation elongation factor activity	-25.2
TWSG1	twisted gastrulation homolog 1 *(Drosophila)*	AF292033	Protein binding	-11.5
USF1	upstream transcription factor 1	BB385241	Sequence-specific DNA binding, transcription factor activity	-6.4
VCAN	versican	AK017328	hyaluronic acid binding, calcium ion binding	-19.5
VRK1	vaccinia related kinase 1	AV341598	ATP binding, kinase activity	-4.3
WDR37	WD repeat domain 37	AK014128	Protein-protein interaction	-4.6
WIPI1	WD repeat domain, phosphoinositide interacting 1	BI251603	Receptor binding	-3.3

ZDHHC2	zinc finger, DHHC-type containing 2	BB342242	Zinc ion binding, methyltransferase activity	-9.5
ZFP119	zinc finger protein 119	AF242376	Metal ion binding, protein binding	-4.6
ZNF81	zinc finger protein 81	BB281667	Metal ion binding, transcription factor activity	-3.3

Table 4.2 THOC5/FMIP dependent genes are involved in differentiation and development.

Category	Genes
Hematological System Development and Function	ID2, GJA1, CHRNA1, CBX2, BCL11B, TWSG1, IL7, IL7R, HOXB3, HMOX1, LGALS2, USF1, CD274, GRIK2
Lymphoid Tissue Structure and development	IL7R, GJA1, ID2, BCL11B, IL7
Connective Tissue Development and Function	GJA1, ID2, COPS5, CBX2, TWSG1, IL7, VCAN, HOXB3, HMOX1, SOX9, MT1E, FOSL1, AGPAT6, SOX5
Skeletal and Muscular System Development and Function	HOXB3, HMOX1, ID2, GJA1, SOX9, SOX15, CBX2, FOSL1, RAMP3, TWSG1, IL7, SOX5
Digestive System Development and Function	ARF6, ID2, SOX9, ARNT
Hepatic System Development and Function	ARF6, MT1E, ARNT
Reproductive System development and Function	GJA1, ID2, SOX9, SOX15, FOSL1, AGPAT6, TOP3B
Embryonic Development	SOX9, CBX2, FOSL1, SLC2A3, ARNT, SOX5
Nervous System Development and Function	SOX9, ID2, GJA1, CHRNA1, POU6F2, SYN3, PTF1A, BCL11B, GRIK2, VCAN
Respiratory System Development and Function	HMOX1
Visual System Development and Function	PTF1A
Cardiovascular System Development and Function	HMOX1, HOXB3, ID2, SOX9, GJA1, VCAN, ARNT
Renal and Urological System Development and Function	GJA1, USF1
Hair and Skin Development and Function	GJA1, ID2, MYRIP, ATOX1
Endocrine System Development and Function	PTF1A

Furthermore, IPA analysis also revealed the down-regulation of certain interesting candidate genes upon FMIP deletion. These include HOXb3, which is important during mouse embryonic development, Sox15, which belongs to Sox genes family of transcription factors known to play a important role in the determination of cell fate during development.(Lee et al 2004) and GLUT3 glucose transporter gene *Slc2a3* that is essential for the development of early post-implanted embryos (S Schmidt et al 2009; Table 4.1, 4.2). Altogether this explains the embryonic lethal phenotype of THOC5/FMIP knockout mice.

4.5 Validation of microarray data with RT-PCR

The results obtained from the affimetrix gene analysis was further validated by RT-PCR. MEF cells were infected with Ade-Cre or Ade-GFP and at 4days post-infection the cytoplasmic RNA was isolated from the cell lysates. Certain genes with altered gene expression were selected and was screened by RT-PCR using specific primers. In accordance with the microarray data these genes showed either no expression or lower expression upon fmip depletion (Figure 4.5).

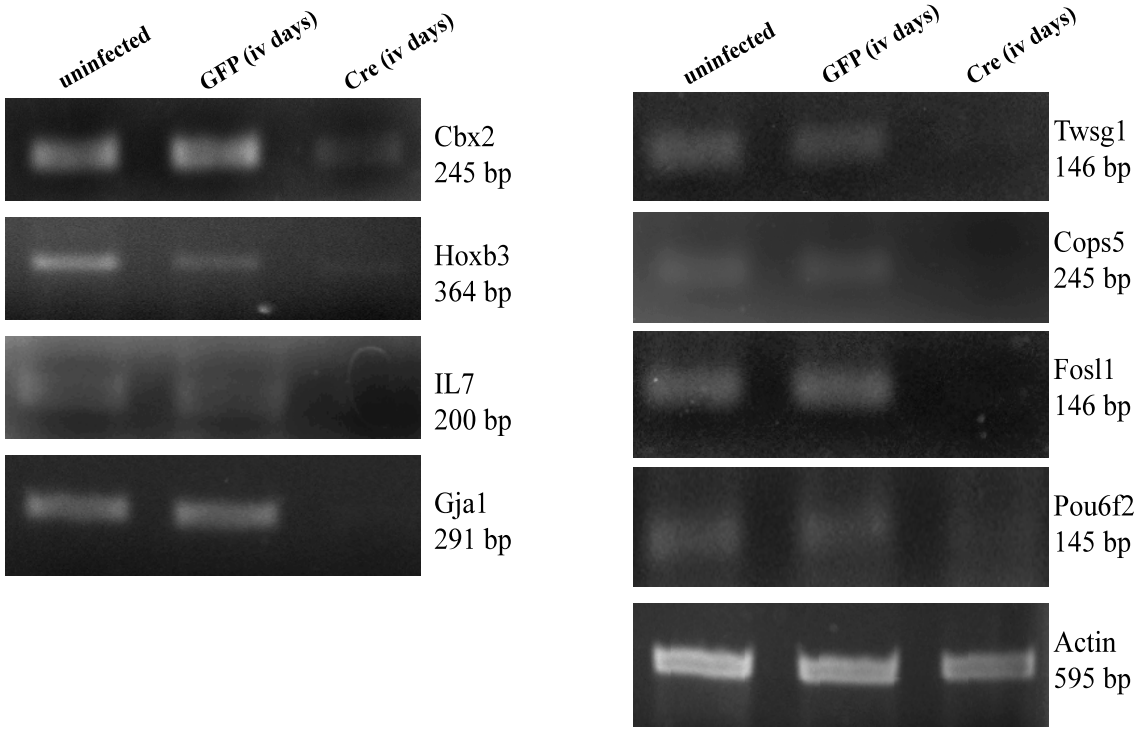


Figure 4.5 RT-PCR of downregulated genes RNA isolated from the MEF cells infected with Ade-Cre or Ade-GFP was screened by RT-PCR using specific primers for the genes that showed altered gene expression in affimetrix analysis.

We performed a literature search for the phenotype of the available knockout mouse of those genes that were downregulated upon FMIP depletion in order to confirm its role in regulation of genes involved in mouse development and haematopoiesis *in vivo*. Consistent with our previous experience most of these mice were either embryonic lethal or showed severe defects in growth and haematopoesis (Table 4.3). Taken together these data suggest that, THO complex FMIP also participate in the expression of genes that are involved in embryo development and haematopoesis.

Table 4.3. Phenotype of the knockout mice for the genes downregulated by THOC5/FMIP depletion.

Gene Symbol	Name	Phenotype/Knockout	Reference.
FMIP	Fms interacting protein	Embryonic lethal	Mancini et al BMC Biology 2010
Hoxb3	Homeobox 3	Inhibit multicellular organismal development	King-Hung Ko,et al 2007
Cbx2 M33	Chromobox homolog 2	M33 (-/-) mice show greatly retarded growth, homeotic transformations of the axial skeleton, failure to expand in vitro of several cell types including lymphocytes and fibroblasts.	Core N et al Development 1997
Glut3/Slc2A3	Glucose transporter	glut3-/- embryonic lethal	Ganguly et al 2007
Twsg1	Twisted gastrulation homolog 1	twsg-/- mice died within 4 weeks after birth, lymphopenia, forebrain defects including rostral truncations	Petryk A et al 2004
Fosl1	FOS-like antigen 1	osteopetrosis due to an early differentiation block in the osteoclast lineage	Koichi Matsuo, Nature genetics 2000
IL7	interleukin 7	IL7-/- lymphopenia	von Freeden-Jeffry U, J Exp Med 1995
Sox15	SRY (sex determining region Y)-box 15	Inhibit skeletal muscle regeneration.	Heon-Jin Lee et al Molecular & Cellular Biology 2004

II) To identify novel binding partners of THOC7 using tandem affinity purification followed by mass spectroscopy.

4.6 THO complex

THO complex is a member of TREX transcription export complex and is involved in mRNA processing and export. TREX complex contains UAP56, ALY, Tex1 and THO complex members THOC1, THOC2, THOC5, THOC6 and THOC7. The potential role of the individual members of THO complex is still unclear. If they function individually or in cooperation with other members of THO complex is the area of immense interest. This study was aimed at deciphering the potential proteins that interact with the members of THO complex which can therefore provide certain valuable clues regarding their function. To understand the biological functions of THOC5 and THOC7 was of specific interest. Overexpression studies performed in our lab has shown cytoplasmic localization of THOC7. Intriguingly, overexpression of THOC5 in cells resulted in nuclear localization of THOC7. Although we provide evidence for direct interaction with THOC5 is essential for nuclear import of THOC7 (El Bounkari et al 2009), it is still unclear if THOC5 is also required for THOC7 to exit the nucleus. Further we were interested in knowing if THOC5-THOC7 interaction resulted in synergistic or counteracting effects. This is based on the observation that overexpression of THOC5 enhanced cell proliferation while overexpression of THOC7 suppresses proliferation. Being a member of THO complex, the potential role of THOC7 in mRNA export is expected. To investigate if THOC7 exhibits any additional function other than mRNA export, it was decided to use THOC7 as a bait to trap the proteins that interact either directly or indirectly with THOC7. The binding partners were then characterized using mass spectrometry.

4.7 Isolation of new binding partners of THOC7 by using tandem affinity purification

The location of a particular protein and the partners with which they interact could provide valuable insights into the function of a protein. In this study the method of tandem affinity purification (TAP) was employed in order to screen the interacting partners of THOC7. TAP is an efficient method used to isolate proteins involved in a protein complex. Using the protein of interest as bait, all the proteins that interact with it was captured from the cell lysates and was later pulled down by a one-step affinity purification procedure.

TAP plasmid pNTAP was purchased from stratagene. Full length THOC7 gene was inserted in frame to the downstream of the TAP sequence. Antibodies that specifically detect THOC7 is unavailable at this moment. Hence THOC7 was expressed as a fusion to Myc at its C-terminal and this allowed the detection of THOC7 using antibodies against Myc. The entire gene cassette was cloned under the CMV promoter for its constitutive expression (Figure 4.6).

Figure 4.6 Scheme showing TAP tagged THOC7-Myc construct. CBP: calmodulin binding peptide, SBP: streptavidin binding peptide, Myc: Myc epitope.

To examine if the TAP tag constructs were in desired reading frame and are suitable for mammalian cell expression, HEK293 cells were transfected with Tap-THOC7-Myc plasmid or with empty vector (pNTAP). The cell lysates of the transfected HEK293 cells were prepared and the expression of the recombinant TAP-THOC7-Myc construct was analyzed by western blotting. Probing the membranes using anti-Myc antibody detected a specific band of 35kDa only in the cells that were transfected with TAP-THOC7 constructs. The weight of the

protein detected correlated with the sum of the weights of individual proteins (TAP-6kDa, THOC7-24kDa, MYC-4kDa) of fusion construct thereby confirming the expression of THOC7 in HEK293 cells (Figure 4.7).

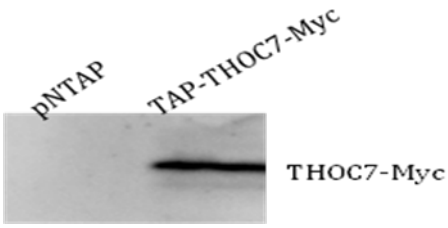


Figure 4.7 TAP-THOC7-Myc construct expression in Hek293 cells. Hek293 cells were seeded and next day transfected with pNTAP (empty vector) and TAP-THOC7-Myc construct. Cell lysates was prepared by adding lammeli buffer and lysates were loaded on gel and protein was detected by immunoblot using anti-Myc antibody.

Once the expression of THOC7 was confirmed, large-scale purification of THOC7 protein complexes from the transfected HEK293 cells was performed. Cell lysates from the transfected cells were incubated with streptavidin coated beads. These beads enriched TAP-THOC7 from the lysate by binding to its N-terminal streptavidin binding peptide. Along with the TAP-THOC7 fusion protein all other proteins that interact with THOC7 were also enriched by this method. The protein complexes bound to the beads were separated from the unbound fraction of cell lysate by centrifugation. To test if the bound fraction contained THOC7, a small portion of beads was subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue Brilliant G-250. In addition to the expected band of THOC7-MYC fusion protein, several other bands were detected in sample from TAP-THOC7 transfected cells that were not found in the control fraction (pNTAP transfected). This implicated the purification of THOC7 and its new binding partners. To further characterize the proteins that were complexed with THOC7 the remaining beads fraction was sent to Prof. Anthony D Whetton's lab in Manchester University for mass spectrometric analysis.

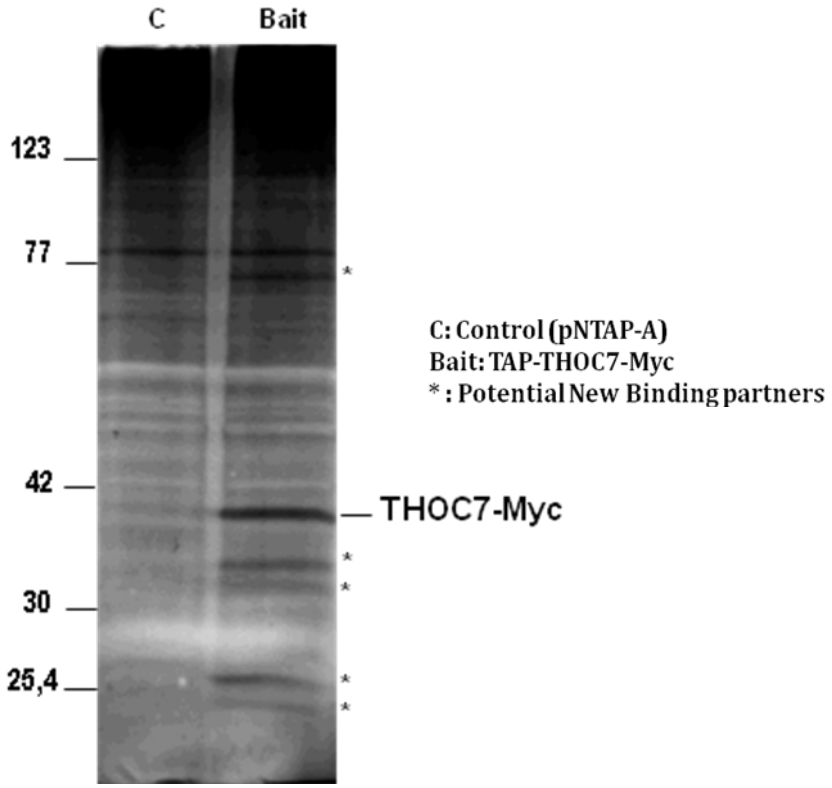


Figure 4.8 Silver staining of TAP-THOC7-Myc purified proteins. HEK293 cells were transfected with pNTAP (empty vector) and TAP-THOC7-MYC constructs. Cell lysates was prepared by adding lammeli buffer and proteins were purified by TAP method. The eluate from streptavidin beads were separated by SDS-page and stained by silver staining. As a protein size marker prestained protein standard was used. The asterix (*) represent the potential binding partners of THOC7.

4.8 Interacting partners identified by mass spectrometry

The proteins from the complex were resolved on SDS-PAGE and all the distinct bands were dissected from the gel. After trypsin digestion the proteins were analyzed by mass spectrometry.

About 94 proteins that interact with THOC7 were identified in mass spectrometric analysis (Table 4.4). The identified proteins were classified based on their location and function using ingenuity pathway analysis. Majority of interacting partners were identified as cytoplasmic

proteins (40%), while others included nuclear proteins (22%), mitochondrial proteins (13%), plasma membrane proteins (11%), and ribosomal proteins (9%) (Fig 4.9). When screened the MS results for the proteins that were involved in mRNA processing or export, 4 proteins identified were involved in mRNA processing and 2 proteins belonged to the nuclear pore complex. Novel putative interacting partners for THOC7 are:

- **NUP35,** that forms structural components of nuclear pore complex (NPC) or act as docking or interaction partners for transiently associated nuclear transport factors (Hawryluk-Gara L.A et al (2005). **NUP95,** member of nuclear pore complex and is required for proper nuclear pore assembly.
- **PABPC1** that has been implicated in governing the stability and translation of mRNA by binding to the 3′-poly (A) tail of mRNA.
- **CPSF6** Cleavage and polyadenylated specific factor 6, a 68kDa protein subunit of cleavage factor that is required during 3' RNA cleavage and polyadenylation of mRNA. Interaction of this factor with the RNA is one of the initial steps in the assembly of the 3' end processing complex and facilitates the recruitment of other processing factors.

Identification of these proteins as interacting partners suggest that potentially THOC7 is involved in translation and protein synthesis in cytoplasm.

Among the new interacting partners for THOC7 we found 5 proteins that are involved in translation such as EEF1A1, EEF1D, EIF2B2, EIF2S1 and EIF3H. It was interesting to find as many as 14 ribosomal proteins interacting wiith THOC7. The role of ribosomal proteins in rRNA processing, ribosome biogenesis and in translation has already been described (Blaha. G, and Nierhaus, K.H 2004). Altogether this set of data suggests that THOC7 may have some role in translation in cytoplasm.

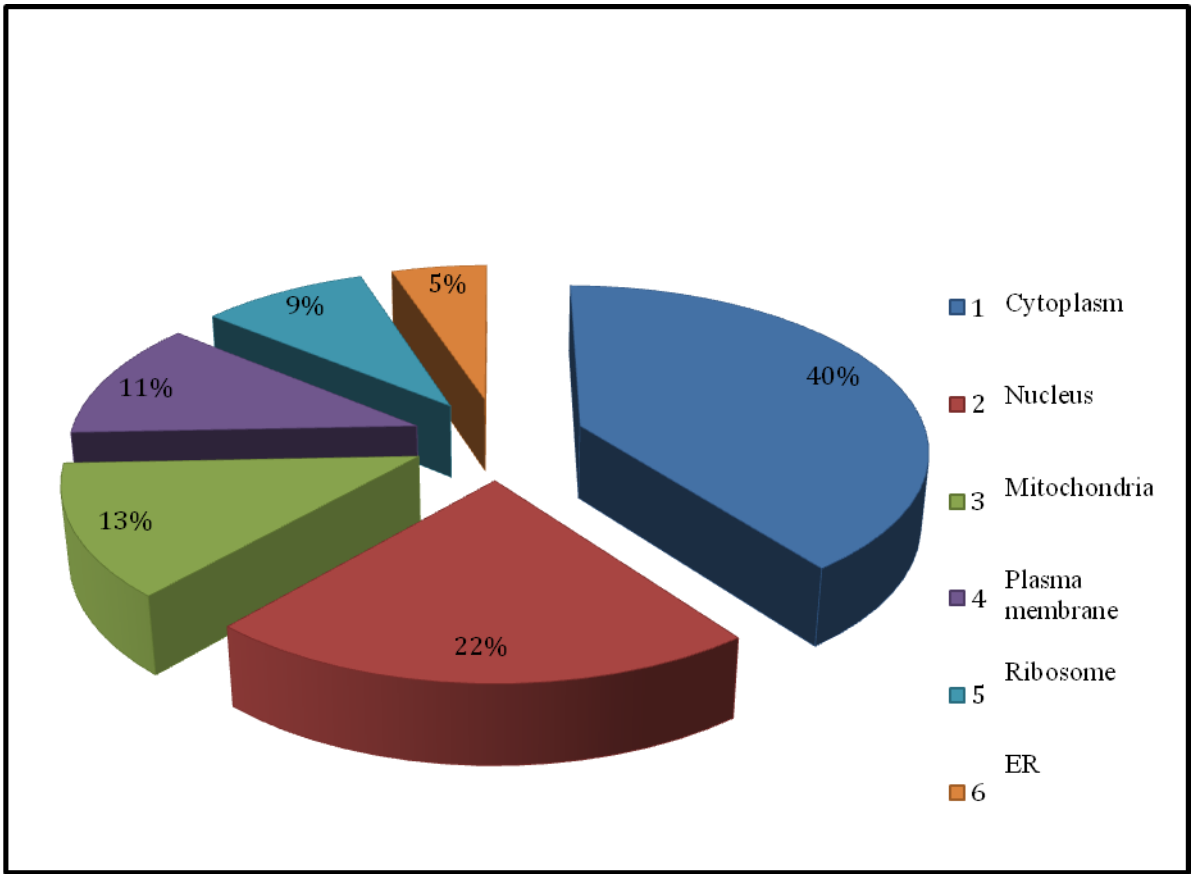


Figure 4.9 The pie diagram shows classification of identified proteins based on location. The protein hits obtained by mass spectrometry was grouped based on their location within the cell.

Table 4.4 Interacting partners of THOC7 identified by mass spectroscopy

	Symbol	Entrez Gene Name	Location	Possible function
1	ACAD11	acyl-Coenzyme A dehydrogenase family, member 11	Cytoplasm	Oxidation-reduction
2	ACTA1	actin, alpha 1, skeletal muscle	Cytoplasm	Protein binding
3	ACTA2	actin, alpha 2, smooth muscle, aorta	Cytoplasm	Protein binding
4	ACTG2	actin, gamma 2, smooth muscle, enteric	Cytoplasm	Protein binding
5	ADH7	alcoholdehydrogenase 7 (class IV), mu or sigma polypeptide	Cytoplasm	
6	ALDH18A1	aldehydedehydrogenase 18 family, member A1	Cytoplasm	
7	APOL2	apolipoprotein L, 2	Cytoplasm	
8	ARD1A	ARD1 homolog A, N-acetyltransferase (S. cerevisiae)	Nucleus	
9	ARD1B	ARD1 homolog B (S. cerevisiae)	unknown	
10	ATAD3A	ATPase family, AAA domain containing 3A	Nucleus	ATP binding
11	ATAD3B	ATPase family, AAA domain containing 3B	Nucleus	‘’
12	ATAD3C	ATPase family, AAA domain containing 3C	unknown	‘’
13	ATP5A1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	Cytoplasm	
14	ATP5C1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	Cytoplasm	
15	BTF3	basic transcription factor 3	Nucleus	transcription
16	BTN3A3	butyrophilin, subfamily 3, member A3	unknown	
17	CDK2	cyclin-dependent kinase 2	Nucleus	Cell cycle control
18	CDK3	cyclin-dependent kinase 3	Nucleus	Cell-cycle control
19	CPSF6	cleavage and polyadenylation specific factor 6, 68kDa	Nucleus	mRNA processing
20	DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6	Nucleus	transcription
21	EEF1A1	eukaryotic translation elongation factor 1 alpha 1	Cytoplasm	translation
22	EEF1D	eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)	Cytoplasm	translation
23	EIF2B2	eukaryotic translation initiation factor 2B, subunit 2 beta, 39kDa	Cytoplasm	translation
24	EIF2S1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	Cytoplasm	translation

25	EIF3H	eukaryotic translation initiation factor 3, subunit H	Cytoplasm	translation
26	EPRS	glutamyl-prolyl-tRNAsynthetase	Cytoplasm	
27	ERLIN2	ER lipid raft associated 2	Plasma Membrane	Protein binding
28	FDPS	farnesyldiphosphatesynthase (farnesyl pyrophosphate synthetasedimethylallyltranstransferase, geranyltranstransferase)	Cytoplasm	
29	GALK1	galactokinase 1	Cytoplasm	
30	GJA1	gap junction protein, alpha 1, 43kDa	Plasma Membrane	
31	GM12141	Chaperonin	unknown	
32	GNA13	guanine nucleotide binding protein (G protein), alpha 13	Plasma Membrane	
33	GNAO1	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O	Plasma Membrane	
34	GNAS	GNAS complex locus	Plasma Membrane	
35	GNAT1	guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1	Plasma Membrane	GTP binding
36	GNAT2	guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2	Plasma Membrane	
37	GNAT3	guanine nucleotide binding protein, alpha transducing 3	Plasma Membrane	
38	GOT2	glutamic-oxaloacetictransaminase 2, mitochondrial (aspartateaminotransferase 2)	Cytoplasm	
39	HIST1H1C	histone cluster 1, H1c	Nucleus	
40	HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 1	Cytoplasm	
41	HSPA1L	heat shock 70kDa protein 1-like	Cytoplasm	
42	HSPA7	heat shock 70kDa protein 7 (HSP70B)	unknown	
43	HSPD1	heat shock 60kDa protein 1 (chaperonin)	Cytoplasm	
44	IMMT	inner membraneprotein, mitochondrial (mitofilin)	Cytoplasm	
45	KARS	lysyl-tRNAsynthetase	Cytoplasm	

46	LMNA	lamin A/C	Nucleus	
47	MAT2A	methionineadenosyltransferase II, alpha	Cytoplasm	
48	MCCC1	methylcrotonoyl-Coenzyme A carboxylase 1 (alpha)	Cytoplasm	
49	MRPL13	mitochondrial ribosomal protein L13	Cytoplasm	
50	MYLK2	myosin light chain kinase 2	Cytoplasm	
51	NAPA	N-ethylmaleimide-sensitive factor attachment protein, alpha	Cytoplasm	
52	NDUFA9	NADH dehydrogenase (ubiquinone) 1 alphasubcomplex, 9, 39kDa	Cytoplasm	
53	NDUFS1	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa (NADH-coenzyme Q reductase)	Cytoplasm	
54	NPM1	nucleophosmin (nucleolarphosphoprotein B23, numatrin)	Nucleus	
55	NSF	N-ethylmaleimide-sensitive factor	Cytoplasm	
56	NUP35	nucleoporin 35kDa	Nucleus	NPC component
57	NUP93	nucleoporin 93kDa	Nucleus	NPC component
58	PABPC1	poly(A) binding protein, cytoplasmic 1	Cytoplasm	mRNA processing
59	PABPC4	poly(A) binding protein, cytoplasmic 4 (inducible form)	Cytoplasm	mRNA processing
60	PHGDH	phosphoglyceratedehydrogenase	Cytoplasm	
61	POLR1C	polymerase (RNA) I polypeptide C, 30kDa	Nucleus	
62	POLR1D	polymerase (RNA) I polypeptide D, 16kDa	Nucleus	
63	QARS	glutaminyl-tRNAsynthetase	Cytoplasm	tRNA synthesis
64	RFC2	replication factor C (activator 1) 2, 40kDa	Nucleus	DNA replication
65	RPL13	ribosomal protein L13	Cytoplasm	Protein synthesis
66	RPL17	ribosomal protein L17	Cytoplasm	Protein synthesis
67	RPL19	ribosomal protein L19	Cytoplasm	Protein Synthesis
68	RPL23	ribosomal protein L23	Cytoplasm	Protein Synthesis
69	RPL27A	ribosomal protein L27a	Nucleus	Protein synthesis
70	RPL6	ribosomal protein L6	Cytoplasm	Protein synthesis
71	RPL7	ribosomal protein L7	Cytoplasm	Protein synthesis
72	RPL7A	ribosomal protein L7A	Nucleus	Protein synthesis
73	RPS25	ribosomal protein S25	Cytoplasm	Protein synthesis
74	RPS4Y1	ribosomal protein S4, Y-linked 1	Cytoplasm	Protein synthesis
75	RPS4Y2	ribosomal protein S4, Y-linked 2	unknown	Protein synthesis
76	RPS8	ribosomal protein S8	Cytoplasm	Protein synthesis
77	RPS9	ribosomal protein S9	Cytoplasm	Protein synthesis

78	SEC13	SEC13 homolog (S. cerevisiae)	Cytoplasm	
79	SEC23A	Sec23 homolog A (S. cerevisiae)	Cytoplasm	
80	SEPT9	septin 9	Cytoplasm	
81	SNX2	sortingnexin 2	Cytoplasm	
82	SNX9	sortingnexin 9	Cytoplasm	
83	STOML2	stomatin (EPB72)-like 2	Plasma Membrane	
84	THOC5	THO complex 5	Nuclear/Cytoplasm	mRNA processing
85	THOC6	THO complex 6 homolog *(Drosophila)*	Nucleus	mRNA processing
86	TTC35	tetratricopeptide repeat domain 35	Nucleus	
87	TUBB3	tubulin, beta 3	Cytoplasm	
88	TUBB4	tubulin, beta 4	Cytoplasm	
89	TUBB6	tubulin, beta 6	Cytoplasm	
90	VPS35	vacuolar protein sorting 35 homolog (S. cerevisiae)	Cytoplasm	
91	ZNF607	zinc finger protein 607	Nucleus	
92	MRPL17	mitochondrial ribosomal protein L17	Cytoplasm	Translation
93	RPL13A	ribosomal protein L13a	Cytoplasm	Translation
94	NCF2	neutrophilcytosolic factor 2	Cytoplasm	

4.9 Common binding partners of THOC5 and THOC7

In the lab similar mass spectrometric analysis was performed to identify interacting partners of THOC5. One of the interacting partners of THOC5 that was identified in this analysis was THOC7. Hence we were interested in fishing out the candidates that were interacting with both THOC5 and THOC7. For this purpose a comparative analysis of the MS data of THOC5 and THOC7 was performed. The analysis showed that among the total interacting partners of THOC5, 73 were nuclear proteins, while THOC7 interacted only with 17 nuclear proteins. It is known that THOC5 is a nuclear-cytoplasmic shuttling protein whereas THOC7 *per se* is a cytoplasmic protein. Only when THOC7 interacts with THOC5 or other nuclear shuttling proteins it is translocated to nucleus. This could be a possible explanation for the few hits of nuclear proteins identified in the MS analysis of THOC7. Another interesting observation was

the detection of nuclear pore complex proteins in THOC7 MS that were absent in THOC5 MS result. This suggests that apart from THOC5, THOC7 might also interact with other nuclear- cytoplasmic shuttling protein that helps in their translocation between nucleus and cytoplasm. When compared for the interaction with other members of THO complex it was identified that THOC5 interacted with THOC1, THOC2, THOC3, THOC4 and THOC7 whereas THOC7 interacted only with THOC5 and THOC6. (Table 4.5).

Table 4.5 Common binding partners of THOC5 and THOC7

S.nr	*Symbol*	*Name*	*Location*	*Function*
1	AIFM1	Apoptosis inducing factor, mitochondrion associated-1	Cytoplasm	Enzyme
2	ALDH18A1	Aldehydedehydrogenase 18 family, member A1	Cytoplasm	Kinase
3	ATP5B	ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide	Cytoplasm	transporter
4	CCT3	Chaperonin containing TCP1, subunit3 (gamma)	Cytoplasm	other
5	DYNC1I2	Dynein, cytoplasmic1, intermediate chain2	Cytoplasm	other
6	EEF1A1	Eukaryotic translation initiation factor 1 alpha 1	Cytoplasm	Translation regulator
7	EIF2S1	Eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	Cytoplasm	Translation regulator
8	ERLIN1	ER lipid raft associated 1	Plasma membrane	other
9	GNA13	Guanine nucleotide binding protein (G protein), alpha 13	Plasma membrane	enzyme
10	LMNA	Lamin A/C	Nucleus	other
11	MCCC1	Methylcrotonoyl-CoAcarboxylase 1 alpha	Cytoplasm	enzyme
12	MCM7	Minichromosome maintenance complex component 7.	Nucleus	Enzyme
13	NDUFS1	NADH dehydrogenase (ubiquinone) F-S protein 1, 75kDa (NADH-coenzyme Q reductase)	Cytoplasm	enzyme
14	PHGDH	Phosphoglyceratedehydrogenase	Cytoplasm	enzyme
15	POLR1C	Polymerase (RNA)I polypeptide C, 30kDa	Nucleus	Other
16	RFC2	Replication factor C (activator 1)2, 40kDa	Nucleus	Other
17	RPL19	Ribosomal protein L19	Cytoplasm	other

18	RPLP0	Ribosomal protein large P0	Cytoplasm	Other
19	RPS6	Ribosomal protein S6	Cytoplasm	Other
20	TUBB4	Tubulin, beta 4	Cytoplasm	Other

The MS analysis revealed that THOC7 interacted only with THOC5 and THOC6 but not with other members of THO complex. When overexpressed THOC7 interacts with THOC5 and not with THOC1 in cytoplasm. To confirm these findings, TAP purification of protein complexes from the Hek293 cells transfected with pNTAP-THOC7-Myc or empty vector pNTAP was performed. Cell lysates were incubated with streptavidin beads and after centrifugation both, the beads fraction and the supernatants were analysed by western blots using Myc, THOC1, and THOC5 antibodies. When probed with THOC5 antibody, specific bands were detected in the bead-bound fraction as well as in the unbound fraction of the cells transfected with pNTAP-THOC7-Myc. On the other hand THOC1 signal was detected only in the unbound fraction and not in the bead bound fraction of pNTAp-THOC7-Myc transfected cells. In the cells transfected with pNTAP signals of THOC1 and THOC5 was detected only in the unbound fraction (Figure 4.10).

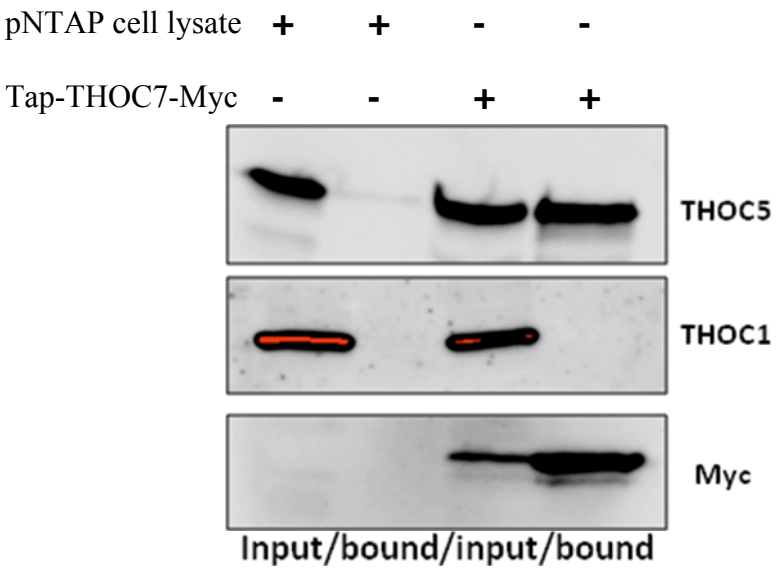


Figure 4.10 Western blot analysis THOC7 interaction with other THO members. Hek293 cells were transfected with pNTAP or pNTAP-THOC7-Myc. The lysates prepared from these cells were incubated with streptavidin beads. The bound proteins were separated by 11% SDS-PAGE and western blot analysis was done using THOC5, THOC1 and Myc antibody.

Taken together these results further confirm the observations made in the MS analysis and suggest that THOC7 interacts with THOC5 and not with THOC1 in cytoplasm. Therefore it is speculated that THOC7 might play a role in translation and protein synthesis independent of THOC1.

5 DISCUSSION

Cell differentiation is paralleled by a timely ordered expression of a set of genes. Most genes are regulated at the transcriptional level. However, several epigenetic mechanisms exist that can greatly influence gene expression. For instance, recent data has shown that regulatory RNAs and certain RNA-binding proteins serve as developmental regulators (Biedermann et al., 2010). Depending on the signals issued these regulators can alter mRNA stability, localization, translation etc. that ultimately influence pattern of gene expression in a cell specific manner. In response to differentiation and DNA damage signals, THOC5/FMIP, a member of the mRNA export complex is also modified post-translationally. Stimulation of myeloid cells with growth factor M-CSF causes phosphorylation of tyrosine residue 255 of THOC5/FMIP (Pierce et al., 2008; Tamura et al., 1999) where as insulin signalling for adipocyte differentiation leads to threonine 328 phosphorylation (Gridley et al., 2005). In contrast the phosphorylation of THOC5/FMIP by protein kinase C impairs the THOC5/FMIP nuclear/cytoplasm shuttling (Mancini et al., 2004). DNA damage leads to phosphorylation of THOC5/FMIP by ATM kinase (Matsuoka et al., 2007). All these facts suggest a major role of THO complex in fine-tuning differentiation processes dependent upon the extracellular cues.

5.1 THO complex and mRNA export

THO complex is involved in mRNA export yet its actual role in this process remains unclear. Initial evidence relating THO complex to mRNA export was provided by Cheng *et al* where they showed the association of THO complex with the cap binding protein (CBP) 80, a nuclear factor that is involved in bulk poly (A)+ RNA export (Cheng et al., 2006). However, recent data show that the depletion of THOC5 does not affect on bulk poly (A)+ RNA export, but severely affected Hsp70 mRNA export in Hela cells (Katahira et al., 2009). Further it has been shown that in *Drosophila* only 20% of all genes were down-regulated by the knockdown of THO members THOC1 and THOC2 (Rehwinkel et al., 2004). At the same time about 80%

of all genes were down-regulated when NXF1 (TAP in mammals), UAP56, or p15 that are involved in bulk poly A+ RNA were depleted (Herold et al., 2001; Herold et al., 2003). This suggest that THO complex has influence only on certain set of genes.

In this study THOC5/FMIP was conditionally depleted from the MEF cells and a microarray analysis was performed to identify the percentage of genes influenced by THOC5/FMIP depletion. The array data suggested that only 2.9% of the total genes were affected by THOC5/FMIP depletion. Among the 559 genes down-regulated 9% of the genes belong to the gene family of transcription regulators. Hence there is a possibility that not all the genes that are down-regulated upon THOC5/FMIP depletion are directly dependent on THO complex. This could rather be an indirect effect of down-regulation of transcription regulator that might be crucial in the expression of certain genes. Interestingly many of the genes, such as Hoxb3, CBX2, Gja1, IL7, Pou6f2, Glut3, Sox15 and Twsg1 are involved in cell differentiation and development. These data indicate that the role of the THO complex is limited to a subset of genes. How the THO complex selects a subset of mRNA still remains to be elucidated. Understanding the relevance of THO complex in differentiation, development and cell proliferation can provide a better understanding of its functional role.

5.2 THOC5 involved in early embryo development

Among the down regulated genes more than 44% of them are involved in the differentiation and development process. Earlier our attempts to generate THOC5/FMIP knockout mice failed as it was embryonic lethal. Later it was known that THOC1 and THOC5/FMIP are required for viability of the early mouse embryo (Wang et al., 2006; Wang et al., 2009; Mancini et al., 2010) although the actual reason for this was unclear at that time. Here, the array analysis revealed that among those genes that were affected by THOC5/FMIP depletion, 6 genes are involved in embryo development (Table 4.2). In particular, the expression of a solute carrier family 2 member 3 (Slc2a3 or Glut3) that facilitated glucose

transport, was reduced by 87%. There were reports showing that mutation in *glut3* gene causes early pregnancy loss and fetal growth restriction (Ganguly et al., 2007). Hence we believe that down-regulation of this gene in FMIP deficient mice could be the reason for its embryonic lethality.

5.3 THOC5 essential for cell differentiation

Indeed, THO depletion in differentiating cells showed severe defects in cell differentiation. In addition, we have shown previously that overexpression of THOC5/FMIP enhances muscle differentiation of C2C12 cells and down regulation of THOC5/FMIP suppressed muscle differentiation (Mancini et al., 2007). Supporting these findings, we have observed down regulation of 12 genes that are involved in skeletal and muscle development upon THOC5/FMIP depletion (Table 4.2). Our previous study also showed that hematopoietic differentiation was severely impaired by THOC5/FMIP depletion in adult mice (Mancini et al., 2010). Although this effect was not due to arrested cell growth but was rather due to increased apoptosis of differentiating hematopoietic cells. Similar observation was also made with the deletion of THOC1, another major conserved component of THO complex, as this deletion caused apoptosis in transformed cells, but not in normal fibroblasts (Li et al., 2007). These findings implicate a potential role of THO members not only in cell growth and differentiation but also in cell survival as lack of THO components leads to cell death. On the other hand, we show here that THOC5/FMIP deficient fibroblasts just cease growing. Similarly, yeast THO null mutants are sick and slow growers (Chavez et al., 2001; Garcia-Rubio et al., 2008), and THOC1 and/or THOC2 depletion has a negative effect on growth rate of *Drosophila* cell line (Rehwinkel et al., 2004). The bulk of these data shows that the THO complex has different roles in distinct cell types.

5.4 THOC7 forms a complex with THOC5 and THOC6 but not with THOC1

The THO complex was initially isolated from Hela nuclear extract (Masuda et al 2005). How ever interaction of each individual member of THO complex is yet to be characterised. Thus far interaction between only THOC1-THOC5 and THOC7-THOC5 has been proven(El.bounkari et al 2009). Earlier we have shown that THOC7 in association with THOC5 (FMIP) is translocated to the nucleus and interacts with mRNA processing machinery. Over expression of THOC7 revealed its cytoplasmic localization. Since no information regarding THO complex in cytoplasm is known we used an approach of tandem affinity purification followed by mass spectrometry to identify proteins that interact with THOC7 which can provide us valuable clues to predict its function.

A total of 94 proteins were identified using this method (Table 4.4). Among the interacting partners identified for THOC7 majority of the proteins are localized in cytoplasm further supporting the overexpression of THOC7. When screened for its association with other members of the THO complex it was found that THOC7 interacted only with THOC5/FMIP and THOC6. Apart from interacting with certain members of THO complex, the newly identified interacting partners of THOC7 consists of proteins involved in mRNA processing, nuclear pore complex, protein synthesis etc.

5.5 Potential role of THOC7 in mRNA processing and export

Proteins such as CPSF6 (cleavage and poladenylated specific factor6) that is involved in 3' end processing and polyadenylation and PABP1 (poly A binding protein1) that increases the mRNA stability were also found to interact with THOC7. Additionally, some of the novel interacting partners identified were the proteins associated with nuclear pore complex such as NUP35 and NUP93, which is a main feature of proteins involved in mRNA export.

Association of THOC7 with the members of TREX complex, proteins involved in mRNA processing and nuclear pore complex suggests a potential role of THOC7 in mRNA processing and export.

5.6 THOC7/THOC5 might couple mRNA export with translation

Interestingly, 14 ribosomal proteins were identified to interact with THOC7. It is known from the literature that ribosomal proteins play a crucial role in rRNA processing, protein assembly, RNA folding, transport of the ribosomal precursors etc. which are the key events in ribosome biogenesis and in translation(Blaha. G, and Nierhaus, K.H 2004). Taken together these findings suggest that THOC7 might form an interface between mRNA export and translation.

Similar studies were performed to identify the interacting partners of THOC5 and as mentioned above THOC5 also interacted with THOC7 (El.bounkari et al 2009). To examine if this interaction led to a synergistic effect or had a counteracting influence on each other we compared the mass spectrometry results of THOC5 and THOC7. The comparitive analysis revealed only a few common interacting partners for both proteins (Table 4.6). While majority of hits obtained for THOC5 were proteins involved in mRNA processing, THOC7 associated mainly with the translation machinery in the cytoplasm. One of the interesting candidate among the common interacting proteins for THOC5 and THOC7 was eEF1A1 (eukaryotic translation elongation factor 1 alpha 1) that is involved in the delivery of all amino acyl–tRNAs to the ribosome (Mateyak et al 2010). A recent report has shown that in addition to delivery of tRNAs, eEF1A1 also functions in nuclear export of proteins by recognizing transcription dependent-nuclear export motif(TD-NEM, $DxGx_2Dx_2L$) (M.Kacho et al 2008). When the sequence was analyzed for both THOC7 and THOC5 it revealed that only THOC5 but not THOC7 contained such motif. This is an interesting observation as it

explains the relavance of THOC5-THOC7 interaction. THOC7 which is a cytoplasmic protein uses THOC5 that contain both NLS and NEM, as a vehicle to shuttle between nucleus and cytoplasm. Although eEF1A is being pulled down by both THOC5 and THOC7, exact role of THOC5 and THOC7 in translation/protein synthesis is not clear. To address this question, further studies involving depletion of THOC7 would be needed.

6 CONCLUSION

The results of this study reveals that depletion of THOC5/FMIP gene influences expression of only 72 functionally known genes, indicating that the relevance of the THO function is not a consequence of a general genome-wide role, but its role is limited to a subset of genes. However, about 44% of down-regulated genes are involved in cell differentiation and development. Since THOC5/FMIP is regulated by extracellular signalling, it may play an important role in fine tuning the differentiation and development process.

THOC7, a member of THO complex localized in cytoplasm shuttles in to the nucleus in association with THOC5. Cytoplasmic localization of THOC7 is further supported by the fact that majority of the interacting partners identified for THOC7 were cytoplasmic proteins with few proteins belonging to mRNA processing machinery and nuclear pore complex. Furthermore, although 6 members of THO complex forms a complex in the nucleus, only THOC7, THOC5, THOC6 are detected in cytoplasm and not THOC1, THOC2, and THOC3. Hence THOC7 in association with THO complex might be involved in coupling mRNA export with translation.

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8 List of figures

8.1 List of Tables

8.2 Abbreviations

2YT	modified luria- bertani broth
AA	amino acid
Ade-Cre	adenovirus carrying cre recombinase
Ade- GFP	adenovirus carrying GFP
ADP	adenosine diphosphate
Amp	ampicillin
App.	Approximately
APS	ammonium persulfate
ATP	adenosine-5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
Ci	Curie
C-terminal	carboxy terminal
DMEM	dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E.coli	Escherichia coil
EDTA	Ethylemediaminetetraacetic acid
ER	endoplasmic reticulum
FCS	Fetal calf serum
Fig.	Figure
FITC	Fluoresceine- isothiocyanate
FMIP	FMS interacting protein
GAPDH	glutamate dehydrogenase
GFP	green fluorescent protein
GST	glutathione-S-transferase
GTP	guanine triphosphate
HEPES	N-2-hydroxyethylenepiperazine-N-2-ethanesulfonic acid
hnRNP	heterogenous nuclear ribonucleoprotein
IP	immunoprecipitation
IPTG	isopropyl β –D- thiogalactopyranoside

Kb	kilo basepairs
kDa	kilodalton
M	molar
mM	milimolar
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
NLS	nuclear localisation signal
NES	nuclear export signal
NP-40	nonident P-40
nt	nucleotide
N-terminal	Amino terminal
OD	optical density
OD600	optical density at 600nm
PBS	phosphate buffer saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNP	ribonucleoprotein
Rpm	rounds per minute
rRNA	ribosomal RNA
RT	room temperature
SDS	sodium dodecylsulfate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	saline sodium citrate buffer
TAP	Tandem affinity purification
TBE	Tris-EDTA-borate buffer
TEMED	N, N, N, N'-tetramethyl-ethylene diamine
THOC	THO complex subunit
TREX	transcription export complex
Tris	Tris(hydroxymethyl)-amino-methane
v/v	volume/volume
Vol	volume
w/v	weight/volume
wt	wild type
X-gal	5-bromo-4-chloro-3-indoyl-β-D- galactopyranoside

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