Characterization of Fms-interacting protein (FMIP), a novel substrate for tyrosine kinase: Connection between receptor tyrosine kinase signaling and mRNA-processing

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Abstract

FMIP was originally identified as a binding partner of c-fms tyrosine kinase, a macrophage colony stimulating factor receptor (M-CSFR, also termed CSF1R) (Tamura et al., 1999).

Data obrtained by the mammalian TAP-tag purification of hFMIP revealed that the nuclear/cytoplasmic shuttling protein FMIP is a member of THO complex, termed THO complex subunit 5 (THOC5), that interacts with c-fms receptor tyrosine kinase. Most of isolated proteins are involved in mRNA processing and splicing. This finding shows a new connection between receptor tyrosine kinase signalling and mRNA processing. However, little is known about the role of THO complex in mRNA splicing and the dynamic assembly of THO complex. This work shows a possible assembly of THO complex.

In the present work the uncharacterized protein THOC7 (NIF3L1 BP) was identified as a novel and direct putative binding partner of FMIP/THOC5. The mapping of this interaction revealed that the residue 50-137 within THOC7 amino acid sequence is required for THOC7-FMIP direct association, whereas the N-terminal domain (1-199) of FMIP is responsible for this interaction. GST-pulldown assays using *Xenopus laevis* oocytes extracts indicates that THOC7-FMIP association is probably conserved in different species. Subcellular localisation studies demonstrate that the direct interaction between THOC7 and FMIP/THOC5 leads to the translocation of THOC7 into the nucleus, indicating that nuclear import of THOC7 is mediated by FMIP/THOC5.

Interestingly, both proteins (THOC7 and FMIP) show different effects on cell growth and differentiation. Ectopic expression of THOC7 slows down the cell growth; in contrast the overexpression of FMIP enhances the cell proliferation. Remarkably, the overexpression of THOC7 in multipotent C2C12 mesenchymal progenitor cells showed a similar phenotype observed by downregulation of

endogenous FMIP using siRNA in the same cells (Mancini et al., 2007), indicating that THOC7 acts as a negative regulator of FMIP/THOC5 by mediating adipocyte differentiation.

The nuclear microinjection of GST-THOC7 into *Xenopus* oocytes reveals that overexpression of THOC7 in the nucleus appears to be toxic for the oocytes. This effect prevented the analysis of the intron-containing adenovirus major late (AdML) mRNA export in this system. Interestingly, the overexpression of THOC7 in mammalian cells induces caspase-3 activation. In contrast, the overexpression of FMIP shows no effect on the caspase-3 activation. Surprisingly, the coexpression of both proteins leads to a reduction of caspase-3 activation. The same results were obtained upon overexpression of a generated THOC7 mutant containing an artificial nuclear localisation signal (NLS-THOC7), which is located in the nucleus. This finding indicates that FMIP-mediated nuclear import of THOC7 is involved in the regulation of caspase-3 activation.

In addition to THOC7-FMIP interaction, the death domain containing protein p84N5/THOC1 was identified as a novel binding partner of FMIP. The C-terminal domain of FMIP (559-683) is required for this interaction, indicating that FMIP is a common interacting partner of THOC1 and THOC7. Cellular fractionation studies demonstrate that the trimer complex THOC7-FMIP-THOC1 is located in the nucleus.

In summary, the present study provides for the first time new connection between receptor tyrosine kinase and mRNA processing and new insights into the role played by THO complex subunits (THOC7, FMIP/THOC5, and THOC1) in cell growth, differentiation and apoptosis.

Key words: c-Fms interacting protein (FMIP), Rezeptor tyrosine kinase, Transkription Export complex (TREX), THO complex, mRNA processing

Zusammenfassung

FMIP wurde zuerst als Substrat und Bindungspartner der Tyrosinkinase c-Fms identifiziert, auch bekannt als M-CSFR1 (Tamura et al., 1999).

Mit Hilfe einer Tandem-Affinitätsaufreinigung (Tandem Affinity Purification/TAP) von FMIP allerdings konnte eine große Zahl an Proteinen isoliert werden, die an der mRNA-Prozessierung beteiligt sind. Dadurch stellte sich heraus, dass das nukleär-zytoplasmatische Shuttle-Protein FMIP nichts anders als THOC5 und damit ein Mitglied des sogenannten THO-Komplexes ist. Diese Ergebnisse zeigen zum ersten Mal eine Verbindung zwischen extrazellulären Signalen von Rezeptortyrosinkinasen und der mRNA-Prozessierung. Trotzdem ist wenig über die exakte Rolle des THO Komplexes bekannt. Diese Arbeitbeschäftigt sich mit der möglichen Zusammensetzung dieses Komplexes.

THOC7 wurde als direkter Bindungspartner von FMIP/THOC5 identifiziert. Durch Herstellung verschiedener Mutanten und anschließende GST-Pulldowns wurde einerseits gezeigt, dass die N-terminale Domäne von FMIP(1-199AA) an THOC7 bindet, andererseits ist die Aminosäuresequenz (50-137AA) in THOC7 die Bindungsstelle von FMIP. Außerdem bindet humanes gereinigtes GST-THOC7 an *Xenopus Laevis* FMIP/THOC5. Dies deutet darauf hin, dass diese Interaktion möglicherweise in verschiedenen Spezies erhalten ist. Mit Experimenten zur intrazellulären Lokalisation ließ sich demonstrieren, dass die direkte Interaktion zwischen THOC7 und FMIP für die Translokation von THOC7 in den Kern verantwortlich ist.

Interessanterweise zeigten beide Proteine (THOC7 und FMIP) gegensätzliche Effekte auf Zellwachstum und Zelldifferenzierung. Während die Überexpression von THOC7 zu einer Verlangsamung des Zellwachstums führt, zeigt sich bei Überexpression von FMIP eine Beschleunigung des Zellwachstums. Außerdem führt die Überexpression von THOC7 in den multipotenten mesenchymalen Progenitor-Zellen C2C12 zur Adipozyten-Differenzierung. Interessanterweise

verursacht siRNA gegen FMIP einen ähnlichen Phänotyp (Mancini et al., 2007). Dies deutet darauf hin, dass THOC7 ein negativer Regulator von FMIP bei der Regulierung der Adipozyten-Differenzierung sein könnte.

Nukleäre Mikroinjektion von GST-THOC7 in *Xenopus*-Oozyten zeigte, dass die Überexpression von THOC7 im Kern toxisch zu sein scheint. Dieser Effekt verhinderte die Durchführung eines mRNA-Exportassays von AdML (Adenovirus Major Late) in *Xenopus*-Oozyten. Interessanterweise führt die Expression von THOC7 in Säugerzellen zur Aktivierung von Caspase-3. Im Gegensatz dazu zeigt die Überexpression von FMIP keinen deutlichen Effekt auf die Caspase-3-Aktivierung. Die Co-Expression von THOC7 und FMIP führt dagegen zu einer Reduzierung der Caspase-3-Aktivierung. Das gleiche Ergebnis ließ sich bei Expression einer THOC7-Mutante (NLS-THOC7) beobachten, die durch ein künstlich eingefügtes NLS im Kern lokalisiert ist. Dies deutet darauf hin, dass der FMIP-vermittelte Import von THOC7 in den Zellkern eine deutliche Rolle bei der Regulierung der Caspase-3-Aktivierung spielen könnte.

Neben der direkten Assoziation von THOC7 und FMIP wurde das eine „Death Domain“ enthaltende Protein p84N5/THOC1 als neuer Bindungspartner von FMIP identifiziert. Dabei bindet die C-terminale Domäne (559-683) von FMIP an THOC1. Zellfraktionierungsexperimente zeigten, dass der THOC7-FMIP-THOC1-Komplex im Kern lokalisiert ist.

Zusammenfassend zeigt diese Arbeit zum ersten Mal eine Verbindung zwischen extrazellulärem Rezeptortyrosinkinase-Signal und mRNA-Processierung, sowie die Rolle von THO-Komplex-Untereinheiten (THOC7, FMIP und THOC1) in Zellwachstum, Zelldifferenzierung und Apoptose.

Schlagwörter: c-Fms interagierendes protein (FMIP), Rezeptor Tyrosinekinase, Transkription Export Komplex (TREX), THO Komplex, mRNA-Prozessierung

1 INTRODUCTION

Unlike prokaryotes, eukaryotic cells segregate the vast majority of their RNA and protein synthesis into two distinct cellular compartments, i.e. the nucleus and the cytoplasm. This division necessitates nucleocytoplasmic transport pathways that can rapidly and specifically transport newly made macromolecules from their site of synthesis to their site of use. In addition, this subdivision also implies the existence of communication systems that can allow the nucleus and cytoplasm to respond in concert to changes affecting the cell. It has, in fact, recently become clear that regulated changes in the subcellular localization of specific proteins form a key component of the cellular response to the activation of many signaling pathways (Hood and Silver, 1999).

Gene expression encompasses a multitude of different steps including transcription; 5' capping, splicing, cleavage, and polyadenylation of the mRNA; packaging of the mRNA into a mature mRNP; nuclear export of the mRNP; and finally translation of the message into the encoded protein by the ribosome. It has been shown in recent years that the single steps of gene expression are physically and functionally linked (Maniatis and Reed 2002; Proudfoot et al., 2002).

During expression of protein-coding genes, pre-mRNAs are transcribed in the nucleus and undergo several RNA processing steps, including capping, splicing, and polyadenylation. The mature mRNA is then exported from the nucleus to the cytoplasm for translation. Many of the steps in gene expression are coupled to each other via an extensive network of physical and functional interactions among the cellular machines that carry out each step of gene expression (Hirose and Manley 2000; Bentley 2002; Maniatis and Reed 2002; Reed 2003). This extensive coupling is likely to function as a quality control mechanism by ensuring that each step occurs efficiently only if the proper contacts are made to the other steps in the pathway.

In eukaryotic cells, intracellular transport pathways are modulated as a function of differentiation, development, and cell cycle in response to a variety of stimuli. However, the molecular mechanisms ensuring this plasticity remain poorly characterized and probably affect both transport machineries and cargoes.

1.1 THO/TREX complex

In eukaryotes, mRNAs are synthesized and processed in the nucleus before they are transported through the nuclear pores into the cytoplasm, where they are translated into proteins. Nuclear export of most mRNAs is mediated by the conserved transcription export (TREX) complex.

RNA export factor 1 (REF1)/Aly (Yra1 in yeast) is an adapter that is recruited to mRNA by specific transcription elongation complexes that include the DEAD-box RNA helicase UAP56 (Sub2 in yeast) (Straber et al., 2002; Luo et al., 2001). The association of THO complex with the mRNA export factors Sub2 (UAP56 in human) and Yra1 (Aly/REF in human) form the multi-subunit TREX (Transcription/Export) complex (Fischer et al., 2002; Jimeno et al., 2002). In addition to Sub2 (UAP56 in human) and Yra1 (Aly in human), the TREX complex also contains Tex1 and the THO proteins (Jimeno et al., 2002; Reed and Cheng, 2005; Masuda et al., 2005). The TREX complex is conserved in yeast, human and Drosophila, and seems to be a key player in the transport of mRNA from the nucleus to the cytoplasm.

A central component of TREX is the THO complex. This was first identified in yeast as a four-protein complex containing proteins encoded by Hrp1 (90kDa) and Tho2 (160kDa), two genes previously identified by hyper-rcombination mutations (Aguilera and Klein, 1990; Piruat and Aguilera, 1998), and thp2 (40kDa), and Mft1 (60kDa) (Chavez et al., 2000). Two of these proteins (Hrp1 and Tho2) are conserved from yeast to human (Reed and Cheng 2005). The *Drosophila* counterpart of the THO complex was also recently identified and contains homologs of Tho2 and Hpr1 as well as three additional proteins, THOC5, THOC6,

and THOC7, which do not have apparent yeast homologs (Rehwinkel et al., 2004). GST-UAP56 and GST-Aly pull-downs from RNase-treated HeLa nuclear extract followed by Mass spectrometry revealed the identification of the human counterpart of the yeast TREX complex (Straesser et al., 2002; Masuda et al., 2005).

Human THO complex contains six proteins namely hHpr1, hTho2, fSAP79 (THOC5 in drosophila), hTex1 (THOC3 in drosophila), fSAP35 (THOC6 in drosophila) and fSAP24 (THOC7 in drosophila) (Straesser et al., 2002; Masuda et al., 2005) (Figure 1.1). However, the characterisation and the dynamic assembly of this complex are still unknown.

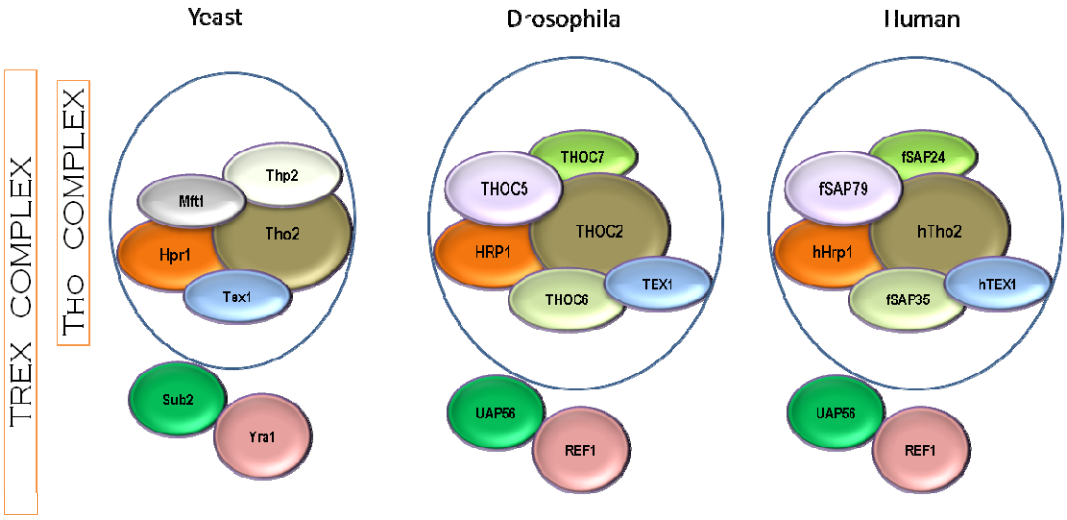

Fig. 1.1: Conserved TREX complex subunits in *S. cerevisiae, D. melanogaster, and H. sapiens*. (Adapted from Reed and Cheng, 2005)

The most studies for identification and characterisation of the THO complex were done in yeast. The *Saccharomyces cerevisiae* multisubunit THO complex, which has been identified as a possible elongation component, has been associated with many aspects of RNA and DNA metabolism (Fan et al., 1996; Prado et al., 1997; Fan et al., 2001; Jimeno et al., 2002). The chromatin immunoprecipitation (ChIP) experiments in yeast indicate that the THO complex is recruited to actively transcribed genes, suggesting that it plays a role in transcription elongation

(Straesser et al., 2002; Abruzzi et al., 2004; Kim et al., 2004). Null mutants of the THO subunits in yeast cells causes pleiotropic phenotypes including temperature sensitivity, increased transcription-dependent Hyper-recombination, transcription elongation impairment and nuclear accumulation of polyadenylated (polyA+) RNAs under heat-shock conditions (Chavez et al., 2000; Jimeno et al., 2002; Straesser et al., 2002; Libri et al., 2002; Piruat et al., 1998; Rondon et al., 2003; Schneiter et al., 1999; West et al., 2000). Furthermore the THO components are not essential for viability (Chavez et al. 2000). This studies in yeast provide strong evidence linking the THO/TREX complex to transcription elongation and the co-transcriptional recruitement of the mRNA export machinery (Reed and Cheng 2005). Moreover, in yeast THO complex is dependent on the frequency of RNAPII transcription initiation, indicating the importance of THO in transcription and the maintenance of genome stability (Jimeno et al., 2008).

In *Drosophila*, Depletion of dTHO complex subunits by RNA interference affects the expression and export of only very few *D. melanogaster* transcripts, indicating that most mRNAs in drosophila are exported independently of THO complex (Rehwinkel et al., 2004). In contrast to yeast THO complex, *D. melanogaster* THO is required for cell proliferation (Rehwinkel et al., 2004).

In yeast the THO/TREX complex is recruited to active genes and travels the whole length of the gene with RNA polymerase II (Straesser et al., 2002; Abruzzi et al., 2004; Kim et al., 2004), whereas in mammals the THO/TREX complex does not appear to be directly linked to the transcription machinery, but instead seems to be recruited to mRNA during splicing (Masuda et al., 2005). A proteomic analysis indicates that all THO/TREX components are present in the human spliceosome (Zhou et al., 2002). Masuda et al. show that hTHO components associate with spliced mRNA, but not with unspliced pre-mRNA (Masuda et al., 2005). Although, the role played by THO complex is still unknown. Recent studies show that the human THO/TREX complex is recruited in a splicing- and cap-dependent manner to a region near the 5´ end of the mRNA, suggesting an alternative model in which

UAP56, Aly/REF and cap-binding protein CBP80 are associated with a spliced mRNA not at the exon-exon junction complex (EJC), but rather as part of the TREX complex, which is recruited to the 5´ end of the mRNA (Cheng et al., 2006). In this model, it is the bound TREX complex that is required for efficient mRNA export and which might interact with TAP/NXF1. Once assembled, the TREX complex recruits the heterodimer TAP/p15 as an export factor. TAP interacts with nucleoporins directly and may thus allow bound transcripts to enter and eventually pass through the nuclear pores (Figure 1.2).

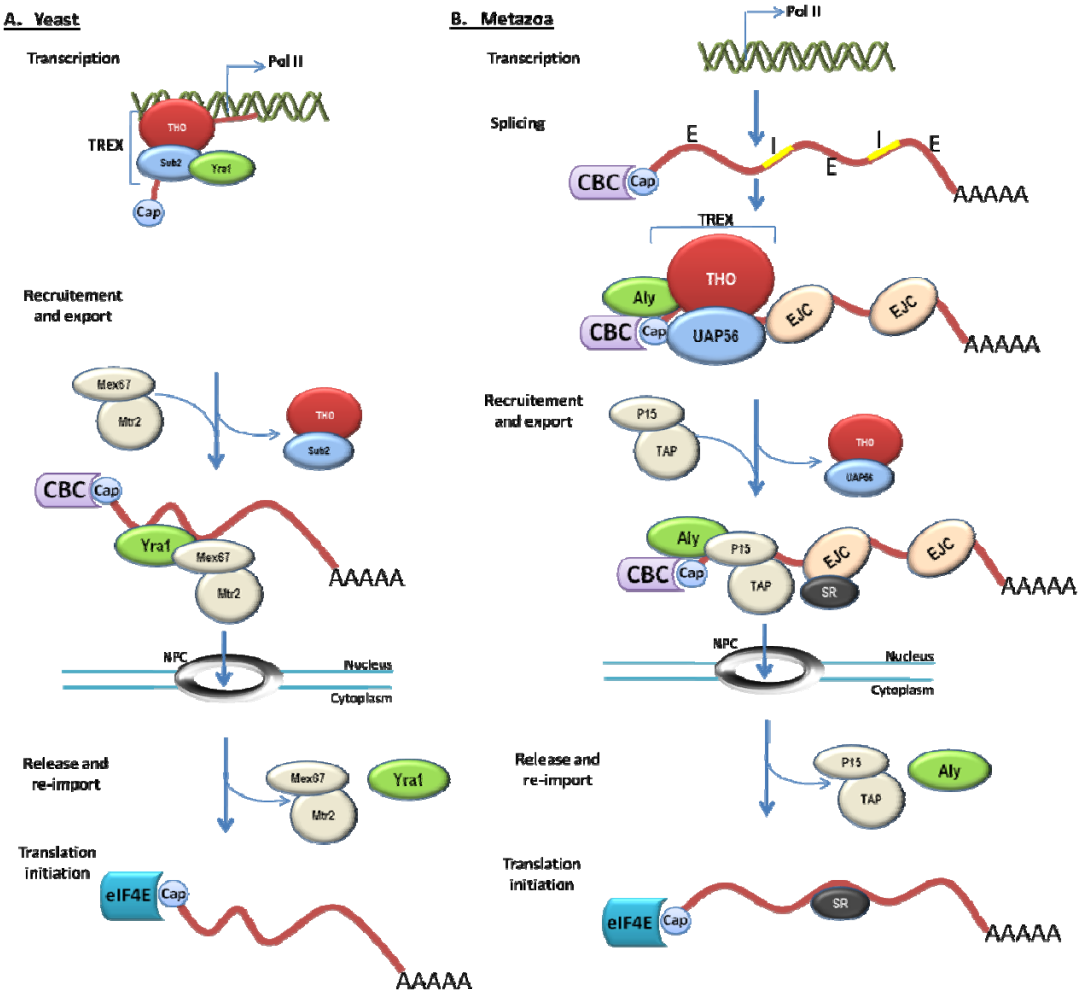

Fig. 1.2: Transcription-coupled (yeast) or splicing-coupled (Metazoa) mRNA export models. A) Yeast model coupling transcription and export machinery: During the transcription mediated by the Polymerase II (Pol II), the generated nascent transcript associates with the THO/TREX complex (THO subcomplex, and Yra1 and sub2). After association with the cap-binding complex (CBC), Mex67-Mtr2 binds to Yra1 to allow the mRNA export through the nuclear pore complex (NPC). After export, the transport factors are released from mRNA and re-imported into the nucleus. mRNA is then subjected to the translation-initiation machinery via 5´cap that associates the initiation factor eIF4A and other additional initiation factors. **B)** Metazoa model coupling splicing and export machinery: For metazoa, several mRNA export models have been described. In this model, mRNA export is cap-dependent and is coupled with splicing, but not with transcription, via THO/TREX complex. In metazoans the abundant introns are typically very large in comparison with exons. The mRNA export in this model recruits yeast homolgs mRNA-export factors (TAP-p15 and Aly/REF1). NPC: nuclear pore complex, CBC: cap binding complex, EJC: exon-junction complex (EJC), Pol II: RNA polymerase II, THO: THO complex, E: exon, I: intron; TREX, transcription-coupled export complex (Adapted from Köhler and Hurt, 2007).

1.2 Fms intracting protein (FMIP)

Fms interacting protein (FMIP) was originally identified in yeast two-hybrid screening as a binding partner of c-fms tyrosine kinase, a macrophage colony stimulating factor receptor (M-CSFR, also termed CSF1R) (Tamura et al., 1999). The N-terminal amino acid residue (1-144) of FMIP is required for FMIP/Fms interaction. Furthermore, this association is enhanced upon M-CSF stimulation, and thereby requiring activated Fms molecules (Tamura et al., 1999). FMIP contains a putative nuclear localisation signal (NLS); a Fms interacting domain, a WW binding domain, a putative PEST domain, a putative leucine zipper and three LXXLL motifs. Interestingly, Mouse and human FMIP show 89.6% homology at the nucleotide level and 95.6% homology at the amino acid level (Tamura et al., 1999) (Figure 1.3).

FMIP is a nuclear/cytoplasmic shuttling protein and is mainly detected in nucleus (Mancini et al., 2004). The translocation of FMIP from cytoplasm into the nucleus is mediated by the activation of protein kinase C (PKC), which phosphorylates the two serines (S5, S6) adjacent to the nuclear localisation signal NLS (**KKRK**) located at the amino acid position 7-10 (Mancini et al., 20004). The overexpression of FMIP in the bipotential mouse myeloid progenitor cell line, FDC-P1Mac11, causes M-CSF induced differentiation into granulocytes, suggesting that the expression of FMIP seems to play a role in the regulation of granulocyte/macrophage differentiation (Tamura et al., 1999). FMIP overexpression affects M-CSF signalling and proliferation. Furthermore the phosphorylation of the regulatory serines (S5 and S6) blocks the inhibitory effect on M-CSF mediated cell survival and differentiation, indicating that PKC mediated phosphorylation of FMIP on serines 5 and 6 seems to play a key role in promoting differentiation upon M-CSF stimulation in FDC-P1Mac11 cells (Mancini et al., 2004). FMIP was detected to be highly phosphorylated upon insulin stimulation in adipocyte cell line, 3T3-L1 cells, and thereby seems to be a substrate for serine/threonine kinases (Gridley et al., 2005).

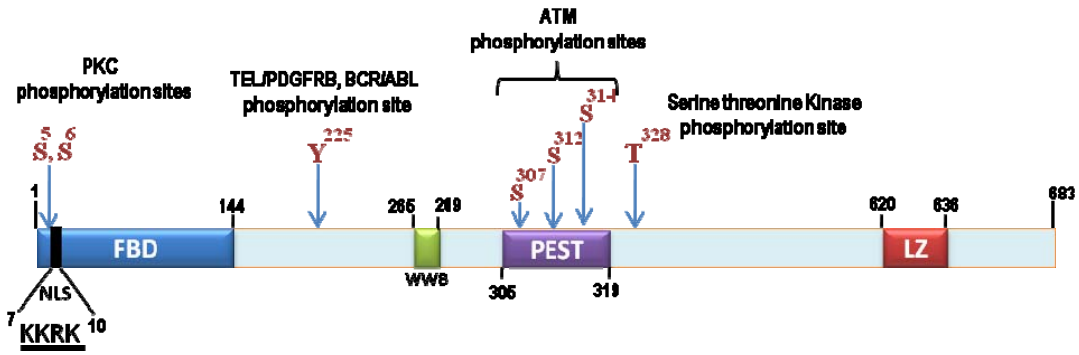

Fig. 1.3: Scheme of different functionally domains and phosphorylation sites in FMIP sequence. FBD: Fms tyrosine kinase (CSFR1) binding domain; NLS: nuclear localisation signal; LZ: leucine zipper, PEST: PEST domain; WWB: binding site for proteins with ww-motif; $S^5$and S^6: Serines, phosphorylated by protein Kinase C (PKC); Y^{225}: Tyrosine phosphorylated by TEL/PDGF receptor or BCR/ABL; S^{307}, S^{312}, S^{314}: Serines phosphorylated by ataxia telangiectasia mutated (ATM) kinase; T^{228}: Threonine phosphorylated by Serine threonine kinase.

Recently, ectopic overexpression of FMIP in the multipotent C2C12 mesenchymal progenitor cells was found to be involved in the adipocyte differentiation induced by insulin, dexamethasone and 3-isobutyl-1-methylxanthine (IBMX); whereas, downregulation of endogenous FMIP in the same cell line impairs muscle and accelerates adipocyte differentiation, respectively (Mancini et al., 2007). The influence of FMIP on adipocyte differentiation in C2C12 cells is mediated by downregulation of CCAAT/enhancer binding protein alpha (C/EBPα) (a transcription factor required for adipocyte differentiation) at the mRNA level, indicating that FMIP abrogates the adipocyte differentiation via C/EBPalpha (Mancini et al., 2007) (Figure 1.4).

More recently FMIP was found to be a substrate of an activated protein tyrosine kinase named TEL/PDGFRB, which is associated with chronic myeolomonocytic leukaemia (CMML)-a disease in which enhanced production of monocytic cells is observed (Pierce et al., 2008). TEL/PGFRB phosphorylates FMIP at the tyrosine 225 (Y225). TEL/PDGFRB potentiates the overexpression and phosphorylation of FMIP

in FDCP-Mix cells. The elevated expression of FMIP increased the expression of phosphatidylinositol 3.4.5 triphosphate (PIP3), and thereby leading to apoptosis suppression (Pierce et al., 2008). Furthermore, FMIP seems to be a target for other leukaemogenic tyrosine kinases, such as BCR/ABL, KITmutant (D816V) and NPM/ALK, indicating that FMIP may be a potential target that affects inositol lipid metabolism and transcription factors known to be disregulated in lymphoma and leukaemia (Pierce et al., 2008).

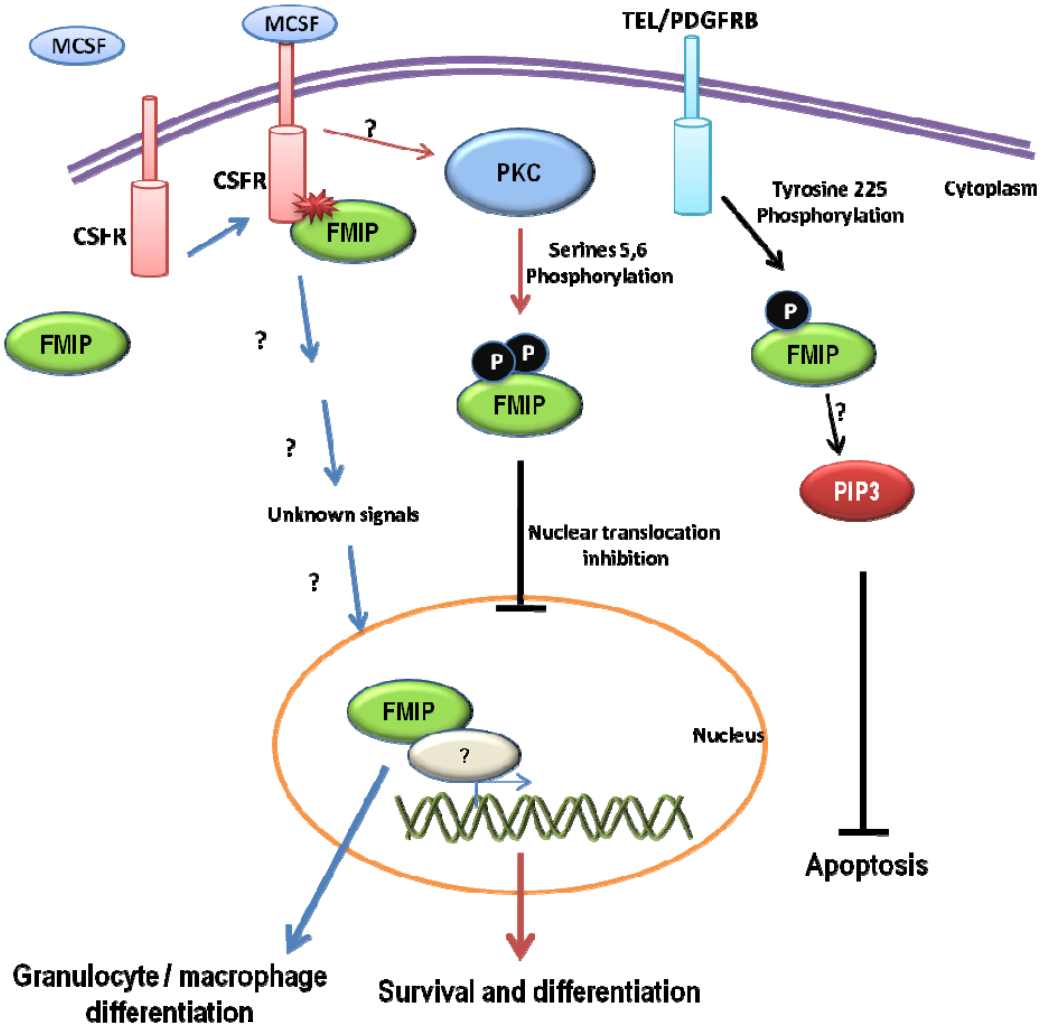

Fig. 1.4: Involvement of FMIP in different biological events such as cell proliferation, differentiation and survival. CSF1R: Macrophage colony stimulating factor receptor (MCSF) 1 receptor (c-Fms tyrosine kinase); MCSF: Macrophage colony stimulating factor; PKC: Proteine

kinase C; TEL/PDGFB: TEL/platelet-derived growth factor beta receptor; PIP3: phosphatidyl inositol 3.4.5-triphosphate. (For details see text)

1.3 THO subunit complex 7 (THOC7)/ NF3L1 BP

THO subunit complex 7 (THOC7), also termed NIF3L1 binding protein 1 (NIF3L1 BP1), was originally identified as a binding partner of Ngg1 interacting factor 3 like1 (NIF3L1) (Tascou et al., 2003). Mouse and human THOC7/NIF3L1 BP1 show 90% homology at the nucleotide level and 97% homology at the amino acid level. THOC7/NIF3L1 BP1 is a protein with a molecular weight of 24 kDa, which contains a putative leucine zipper domain located at the C-terminal region of the sequence. The mammalian two-hybrid system revealed that the putative leucine zipper domain is required for the interaction between NIF3L1 and Full-length THOC7/NIF3L1 BP1 (1-204aa) as well as the THOC7/NIF3L1 BP1 (1-137aa) splice variant (Tascou et al., 2003) (Figure 1.5).

THOC7 is ubiquitously expressed in different tissues. Subcellular localisation studies revealed that THOC7/NIF3L1 BP1 is detected mainly in the cytoplasm, but also present in the nucleus, furthermore THOC7/NIF3L1 BP1 and its interacting partner NIF3L1 are co-localized only in the cytoplasm, indicating that the association between both proteins occurs in the cytoplasm (Tascou et al., 2003). THOC7/NIF3L1 BP1 (FLJ23445 (Acc Nr.BF179247)) was also found to interact with the mediator subunit complex 8 (Med-8), which acts as a co-activator required for activation of RNA polymerase II by DNA binding trans-activators, in yeast two hybrid system (Sato et al., 2003).

Recently, THOC7/NIF3L1BP1 was found to be involved in mouse spermatogenesis during ontogenesis (Giuffrida et al., 2005). The NIF3L1 BP1/THOC7 expression increased progressively during germ cell maturation, reaching a maximal expression in the adult mouse, at which point all spermatogenic cell types are present, suggesting that NIF3L1 BP1/THOC7 is somehow involved in the initial fundamental steps of germinal cell maturation (Giuffrida et al., 2005).

NIF3L1 BP1/THOC7 was also identified as a member of THO complex, THO subunit complex 7. This protein was detected in *drosophila* (THOC7) and human (fSAP24) THO complex, but not in yeast THO complex (Rehwinkel et al., 2004; Masuda et al., 2005).

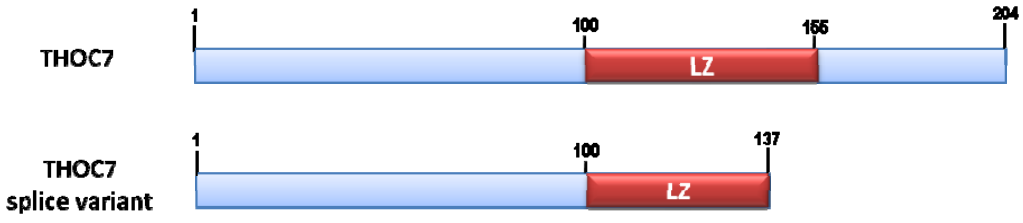

Fig. 1.5: Schematic illustration of full length and splice variant of THOC7. LZ: leucine zipper

1.4 THO subunit complex 1 (THOC1) / p84N5

TOHC1/p84N5 (also termed N5 protein) was originally identified as binding partner of retinoblastoma tumor suppressor protein (Rb) (Durfee et al., 1994). The N5 gene and its encoded protein are referred to as hHpr1 and p84N5. The official symbol as indicated in the human genome data base is Thoc1. p84N5 was also shown to be part of the THO complex, THO subunit complex1 (Straesser et al., 2002).

The predicted primary sequence of THOC1/p84N5 has significant similarity of the death domains of several well characterized proteins involved in apoptotic signalling (Feinstein et al., 1995). Expression of THOC1/p84N5 potently induces apoptosis in several tumor cell lines. This effect requires the death domain located in the C-terminal region (Doostzadeh-Cizeron et al., 1999). THOC1/p84N5 associates with the N-terminal domain of Rb (Durfee et al., 1994), and thereby inhibiting the THOC1-mediated apoptosis (Doostzadeh-Cizeron et al., 1999). THOC1/p84N5 localized predominantly within the nucleus of interphase cells and its expression can induce a G2/M cell cycle arrest (Doostzadeh-Cizeron et al.,

2001). The THOC1/p84N5 nuclear localisation signal located in C-terminal region is required for THOC1-induced apoptosis, classifying THOC1/p84N5 as the first death domain containing apoptotic signalling protein that functions within the nucleus (Evans et al., 2002).

Further studies characterized a novel spliced version of THOC1/p84N5 (Gasparri et al., 2004) (figure.). The THOC1/p84N5 short isoform (p84N5s) encodes for a protein lacking the death domain (DD) and the pRB-binding domain. This isoform localizes within the cytoplasm, was never found in the nucleus, and does not retain the ability to induce apoptosis (Gasparri et al., 2004). THOC1/p84N5 is highly expressed in breast tumors. Small interfering RNA studies of THOC1/p84N5 in breast cancer cell lines leads to inhibition of cellular proliferation and abrogation of mRNA export, indicating that THOC1/p84N5 seems to play a significant role in metastatic breast cancers (Guo et al., 2005). Moreover, THOC1/p84N5 is required for transcriptional elongation of some human genes, and physically associates with elongating RNA polymerase II and export factor UAP56 (Li et al., 2005). THOC1/p84N5 is required for viability of the early mouse embryo, suggesting that THOC1/p84N5 is essential for early embryonic development in the mouse (Wang et al., 2006).

THOC1/p84N5 is highly expressed in oncogene-transformed human cells (Li et al., 2007). Furthermore, loss of THOC1/p84N5 in cancer cell lines and oncogene-transformed human or mouse fibroblasts inhibits cell accumulation through induction of apoptosis and subsequent loss in cell viability. In contrast, loss of THOC1/p84N5 in normal human or mouse cells has no effect on viability (Li et al., 2007). More recently, it has been shown that THOC1/p84N5 leads to caspase-6 activation upon adeno-associated virus (AAV) infection (Garner et al, 2007).

1.5 Caspase-3 mediated cell death

Caspases are **c**ystein-rich **asp**artic-specific prot**eases**, which are activated specifically in apoptotic cells (Fadeel et al., 2000). Caspases are expressed as inactive pro-enzymes in the cells and their activation is induced by cleavage of the proenzymes (Cohen, 1997). Caspases in their proenzyme form contain three domains: an amino terminal prodomain; a large subunit containing the active site cysteine within a conserved QACXG motif; and a carboxy terminal small subunit (Fig. 6). Currently, there are 14 different known caspases, of which 12 (caspases 1-10, 12, 14) are present in human cells and at least 10 (caspases 1-3, 6-12) are present in rat cells (Van de Craen et al., 1998; Wolf and Green, 1999; Fischer et al., 2002). The mammalian apoptotic caspases have been classified as either intiator caspases (caspases 2, 8, 9 and 10) or effector caspases (caspases 3, 6 and 7). The prodomain (N-terminal domain) of initiator caspases is relatively long (over 100 amino acids) and can include a death domain (DD) or caspase recruitement domain (CARD). The effector caspases have short prodomains, the function of which is to prevent premature protease activation in healthy cells (Cohen, 1997; Chan and Mattson, 1999; Chang and Yang, 2000).

Caspases need to be cleaved to achieve their enzymatic activity. Prior to activation, a prodomain and linker area are cleaved from the procaspases producing large and small subunits that further dimerize with another cleaved caspase molecule and form an active heterotetrameric structure (Figure 1.6A). Once acticvated, caspases cleave their substrates typically after conserved aspartate residues and are responsible for most of the biochemical and morphological features of apoptotic cell death.

Caspase 3 is the major effector caspase (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Tewari et al., 1995), which activated by both intrinsic and extrinsic cell signalling pathways (Budihardjo et al., 1999; Grutter, 2000) (Figure 1.6B). Active Caspase3 cleaves poly (ADP-ribose) polymerase (PARP), inhibitor of caspase

activated DNAse (ICAD), catalytic subunit of the DNA-dependent protein kinase (DNA-PK), delta isoform of the protein kinase C (PKCδ) and other caspases (e.g., caspase 6 and 7) (Tewari et al., 1995; Hirahata et al., 1998; Sakahira et al., 1999; Seimon-DeVeries et al., 2007). Caspase 3 expression is highest during embryonic stages and downregulated thereafter (Shimohama et al., 20001). Caspase 3 expression is increased in human brain in Alzheimer's disease, Hntington's disease, Parkinson's disease and temporal lobe epilepsy (Masliah et al., 1998; Shimohama et al., 1999; Hartmann et al., 2000; Henshall et al., 2000b; Love et al., 2000).

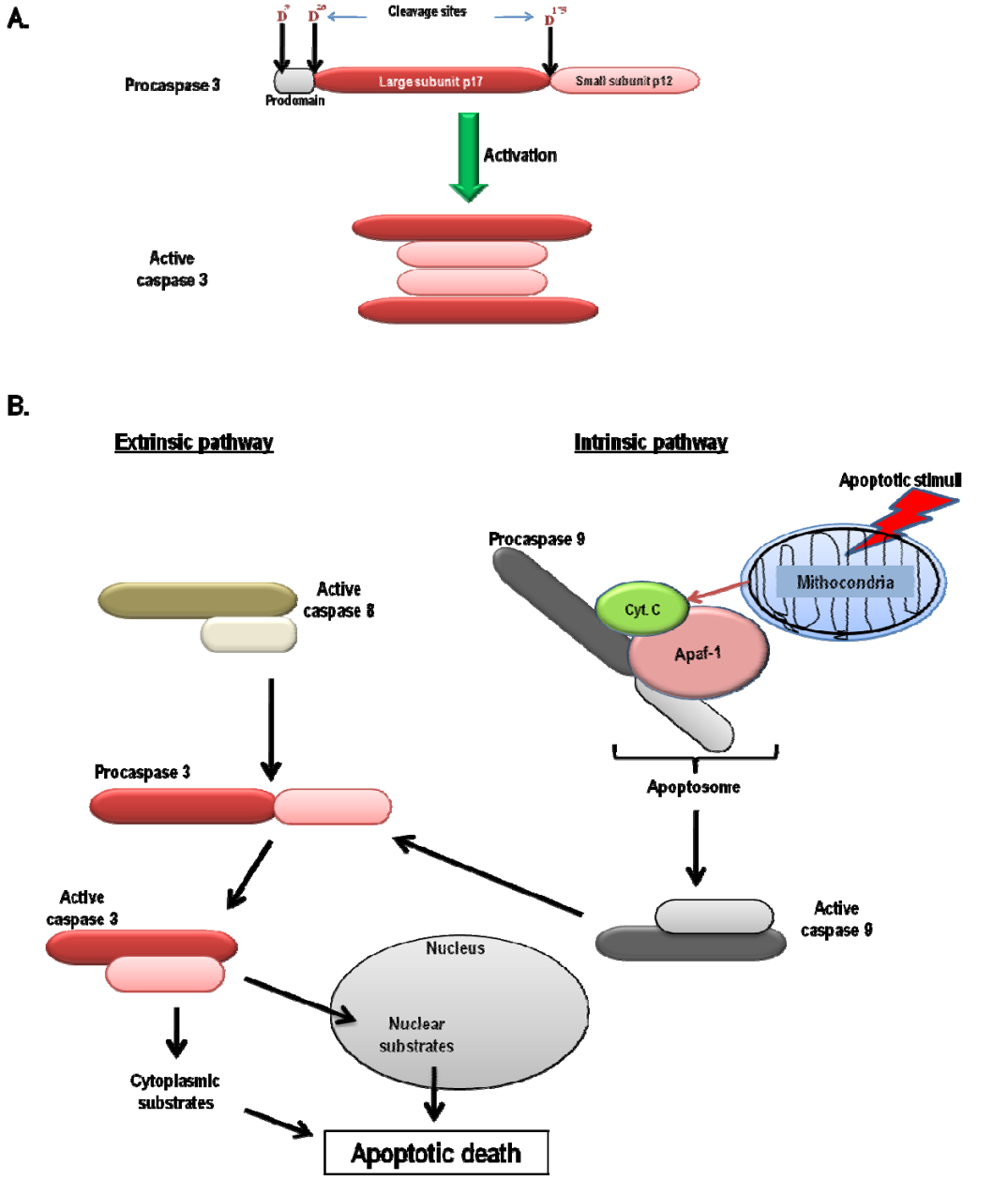

Fig. 1.6: Schematic illustration of caspase3 activation and caspase-mediated cell death pathway. A) Proteolytic activation of caspase3. The activation of caspase3 recruits two aspartate (D^{28}, D^{175}) cleavage events. The resultant functional caspase3 is a Tetramer of two large and two small subunits, thus the active caspase3 molecule has two sites for substrate binding and cleavage. **B)** The extrinsic and the intrinsic apoptosis signaling. The auto-activation of caspase8 mediated by death receptor signalling leads to activation of caspase3 and subsequently activation of downstream signals that induces apoptotic death. The activation of the intrinsic pathway results in the release of cytochrom c (Cyt. C), and in the formation of the apoptosome complex consisting of Apaf1 and pro-caspase9. Pro-caspase9 is processed by auto-catalysis. Upon activation of the downstream effector caspase3 the disassembly of the cell occurs in the execution phase of apoptosis (Modified and adapted from Cohen, 1997; Hunter et al., 2007).

1.6 Aim of this thesis

Macrophage colony-stimulating factor receptor (M-CSFR), the product of c-fms proto-oncogene, belongs to the receptor tyrosine kinase family. M-CSFR and its ligand M-CSF have been shown to be involved in the activation, survival, proliferation, and differentiation of mature cells of the monocyte/macrophage lineage, as well as acting as well as acting as a proinflamatory cytokines (Tushinski et al., 1982; Metcalf et al., 1986; Becker et al., 1987; Elliott etal., 1989; Young et al., 1990). It has been established that the aberrant expression and/or mutation of M-CSF and M-CSFR is implicated in the pathogenesis of several solid tumors as well as haematopoietic malignancies (Janowska-Wieczoreck et al., 1991; Burthem et al., 1994; McGlynn et al., 1998; Maher et al., 1998). Recently, a new binding partner of M-CSF receptor was identified as Fms interacting protein (FMIP) (Tamura et al., 1999). However, the biological role of FMIP is still unclear. Hence, this work broadly set out to perform the following aims:

- Identification of novel interacting proteins of FMIP by the mammalian tandem affinity purification followed by proteomics analysis and by GST-pulldown assays and co-immunoprecipitation.

- Subcellular localisation studies of the putative FMIP interacting proteins in mammalian cells.

2 MATERIALS

All standard chemicals for commonly used 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	Göttingen, 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, Bermany
Invitrogen,	Carlsbad, CA, USA
Kodak,	Rochester, NY, USA
Kühn & Bayer	Nidderau, Gernmany
Liebehere	Germany
MBI Fermentas	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	Düsseldorf, 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, Switzerland
Roth	Karlsruhe, Germany
Santa Cruz Biotechnology	Santa Cruz, CA, USA
Sarstedt	Nümbracht, Germany
Sartorius	Goettingen, Germany
Serva	Heidelberg, Germany
Siemens	Frankfurt/M., Germany
Sigma-Aldrich	St. Louis, MO, USA
Stratagene	La Jolla, CA, USA
Terumo Europe	Leuven, Belgem
Thermoquest	Engelsbach, Germany
Visitron	Puchheim, Germany

2.1 Chemical reagents

Agar	Roth
Agarose	Roth
Ampicilline	Roth
Acrylamide	Sigma
	Roth
APS (ammonium peroxodisulfat)	Roth
Bacto-Agar	Roth
Brilliant Blue G250	Sigma-Aldrich
Bromphenol blue	Roth
BSA (bovine serum albumin) fraction	Roth
ATP (deoxyadenosine triphosphate)	MBI Fermentas
dCTP (desoxycytidine triphosphate)	MBI Fermentas
DEPC (diethylpyrocarbonate)	Sigma
dGTP (desoxyguanosine triphosphate)	MBI Fermentas
Diethylpyrocarbonate DEPC	SIGMA
DNA ladder (1 kb)	MBI Fermentas
DTT (dithiotreitol)	Roth
dTTP (desoxythymidine triphosphate)	MBI Fermentas
Ethidium bromide	Roth
Ethidium bromide	Roth
D-Biotin	Roth
Formaldehyde 37%	Roth
Formamide	Roth
DNA marker	MBI Fermentas
HEPES	Roth

Roti Marker	Roth
N,N,N',N',-Tetramethylethylendiamine TEMED	SIGMA
PEG (polyethyleneglycol)	ATCC
peqGOLD TriFast™	PEQLAB
PMSF (phenyl-methyl-sulfonylfluoride)	Merk
Precision Protein Marker, prestained	BioRad
Protein A/G-PLUS agarose	Santa Cruz Biotechnology
Protein G Plus agarose	Santa Cruz Biotechnology
Glutathione -Sepharose-4Bbeads	Amersham
Salmon sperm DNA	SIGMA
Sodium-m-periodate	Sigma-Aldrich
Streptavidin sepharose beads	Amersham
Calmodulin sepharose beads	Amersham
D-Biotin	Roth
TEMED (tetramethylethylenedimine)	Sigma
Triton X-100	Roth
Tryptone	Merck
Trasylol	Bayer
Tween 20	Roth
Yeast Extract	Roth
X-gal	Merk
Neomycine® (G418)	Gibco BRL
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
Dimethylsulfoxid (DMSO)	Roth
DMEM	Biochrom
Fetal Calf Serum (FCS)	Biochrom

2.2 Kits

Invisorb Spin Plasmid Mini Two Kit	Invitek
pGEM® T easy cloning kit	Promega
Plasmid Maxi Kit	Qiagen
Plasmid Midi Kit	Qiagen
PlusOne™ Silver Staining Kit Protein	Biorad
PVDF Immobilon™-P membrane	Millipore
QiaEx II Agarose Gel Extraction Kit	Qiagen
Quick spin columns	Roche
Riboprobe *in vitro* Transcription Systems	Promega
RNeasy Mini Kit	Qiagen
DNA-Sequenzier-Kit BigDye™Terminator,	Perkin-Elmer

Jet Nick "Probe Purification after Labeling"	Genomed
Megaprime labeling RNP	Amersham

2.3 Antibodies

Mouse monoclonal anti-THOC1 (p84N5)	Gene Tex inc.
Monoclonal anti- FMIP (F6d)	(Mancini et al., 2007)
Mouse monoclonal anti-GAPDH	Santa Cruzbiotechnology
Mouse monoclonal anti-Myc (9E10)	Santa Cruzbiotechnology
Mouse monoclonal anti-GFP	Santa Cruzbiotechnology
Mouse monoclonal anti-GST	Santa Cruzbiotechnology
Anti-mouse IgG TRITC conjugate	Sigma-Aldrich
Anti-rabbit IgG FITC conjugate	Sigma-Aldrich
Anti- mouse Flag (M2)	Sigma-Aldrich
Rabbit polyclonal anti-caspase-3	Cell signaling technology

2.4 Enzymes

DNase I, RNase-free	Qiagen
Protease K	MBI Fermentas
Pfu Polymeras	Promega
Restriction enzymes various,	MBI Fermentas New England Biolabs Roche
T4 DNA Ligase	MBI Fermentas
Taq Polymerase	MBI Fermentas
Trypsin (5x)	Gibco BRL
Ribonuclease Inhibitor	MBI Fermentas
Protease K	MBI Fermentas

2.5 *E. coli* strains

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

HB101: F^-, hsdS20 (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^-,Δ(lac-pro), proAB, supE, rK^+, mK^+, thi/F, traD36, lacIqZ, ΔM15

2.6 Cell lines

C2C12 mouse myoblast cell line ATCC Number: CRL-1772 Organism: Mus musculus (mouse)

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

2.7 Plasmids

pCDNA3.1	Invitrogen
pCDNA Myc-HisA	Invitrogen
pEGFP-N3	Clontech
pGEM® T Easy	Promega
pNTAP®	Stratagene
pGEX 5X-1	Pharmacia
pGEX-2T	Pharmacia
pBS-Ad1	was kindly provided from Prof. Elisa Izauralde (MPI,Tübingen, Germany)
pBStRNA	was kindly provided from Prof. Elisa Izauralde (MPI, Tübingen, Germany)
pBSU6s	was kindly provided from Prof. Tomas Pieler (Georg-August Universität Göttingen)

2.8 cDNAs

THOC7- Full length cDNA Clone	IRAT p970D0486D	RZPD, Germany
BAT1- Full length cDNA Clone	IRAU p969C0816D	RZPD, Germany
THOC4- Full length cDNA Clone	IRAU p969B0727D	RZPD, Germany
THOC6- Full length cDNA Clone	IRAT p970A1277D	RZPD, Germany
THOC1- Full length cDNA Clone	IRAU p969H0452D	RZPD, Germany
THOC3- Full length cDNA Clone	IRAT p970B034D	RZPD, Germany
THOC2- Full length cDNA Clone	IRAK p961G11119Q	RZPD, Germany

2.9 Oligonucleotide

Custom made, gene specific oligonucleotides were ordered from Operon, Germany.

2.9.1 General Oligonucleotides

SP6	5'-ATT TAG GTG ACA CTA TAG AA-3'
T3	5'-AAT TAA CCC TCA CTA AAG GG-3'
T7	5'-GTA ATA CGA CTC ACT ATA GGG C-3'

2.9.2 Primers

Name	Sequence
EcoFMIPp	**5'** AGGAATTCATCAGAATCGAGCAAAAAACGGAAG**-3'**
FMIPMycHisEnd	**5'**-AACTCGAGTCAATGATGATGATGATTGATGGTC GAC**-3'**
THOC7GST-NLS	**5'**-GGGATCCGCAAGCGAAAGAGAGGAGCCGTGACTGAC GACGACGAAGTT**-3'**
THOC3 GSTp	**5'**-GGGATCCCCGGCATGGCGGTCCCCGCTGCAGCC**-3'**
THOC3GSTn	**5'**-TGTCGACAGAATCATTAGGAAGCCCAAACAG**-3'**
THOC6GSTp	**5'**-GGGATCCCCGATATGACCATCTTCTCCCAGAGCGT**-3'**
THOC6GSTn	**5'**-TGTCGACGAAGGACAGGGAGAAGGCTCGGTAACC**-3'**
THOC7GSTp	**5'**-GGGATCCCCGCCATGGGAGCCGTGACTGACGAC **-3'**
THOC7GSTn	**5'**-TGTCGACTGGCTTAGGATCTGTTTCCATGCT**-3'**
SF2GSTp	**5'**-GGGATCCCCGCCATGTCGGGAGGTGGTGTGATTCG**-3'**
SF2GSTn	**5'**-TGTCGACTGTACGAGAGCGAGATCTGCTATGACGG**-3'**
THOC7 (66)n	**5'**-TGTCGACGCCCATTGAAAATTCACATTGAGAC**-3'**
THOC7(137)n	**5'**-TGTCGACCTTTAATGTCTCATGCCTGTCTGG**-3'**
THOC137p	**5'**-GGGATCCCCGAACTAGAGGCTCTGGGAAAAG**-3'**
THOC6MycN	**5'**-TAAGCTTGAAGGACAGGGAGAAGGCTCGGTAAC**-3'**C
THOC7MycN	**5'**-TAAGCTTTGGCTTAGGATCTGTTTCCATGCT**-3'**

2.9.3 Radionucleotides and radiochemicals

$\gamma[^{32}P]$-ATP (3000 Ci/mmol)	Hartmann Amersham
$\alpha[^{32}P]$-dCTP (3000 Ci/mmol)	Hartmann Amersham
$[^{35}S]$ Trans-^{35}S-Label® (Methionin/Cystein)	Hartmann Amersham

2.10 Equipement

Adjustable air-displacement pipettes Pipetman®	Gilson
Biomax MS films	Kodak
Centricon-10 vials	Amicon
Centrifuge Biofuge	Heraeus
Centrifuge Megafuge 1.0 R	Heraeus
Centrifuge Sorvall RC 5B	Du Pont
CO_2-incubator Hera Cell	Heraeus
Digital Camera Digital	Nikon
Dounce homogenizer	Braun
Exposition chamber Hypercassette™	Amersham
Hyperfilm ECL Amersham	Pharmacia
Incubatior Function Line	Heraeus
Laminar flow hood Hera Safe	Heraeus

Light microscope Wilovert A	Hund
Magnetmixer Variomag®	H+P Labortechnik
Nitrocellulose membrane	Schleicher &Schuell
Nylon membrane (HyBond N)	Amersham Pharmacia
PCR machine T3 Thermal cycler	Biometra
pH-meter	Radiometer
Orbital Shaker	Forma Scientific
Refrigerator -86C Freezer	Forma Scientific
Rotor SS-34	Sorvall
γ-Sintillation 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-Plattes	Nunc
6-Well-Plattes	Nunc
Blotting paper	Schleicher & Schuell
Cannules (1.2×40mm, 0.45×12mm)	Terumo Europe
Cell culture flasks	Nunc
Cell culture Petri's dishes	Nunc
Chromatographic-paper, Whatman 3MM	Machery-Nagel
Dynal MPC®-E Mixer	Dynal
Eppendorf tubes	Sarstedt
Filmkassette Cronex high plus	DuPont
Filmkassette ohne Verstärkerfolie	Siemens
Hybond-N Nylon membrane	Amersham Biosciences, UK
Kryotubes, Nalgene	Nalgene Falcon
Nitrocellulose membrane	Schleich and Sch¨ull, Dassel
Nitrocellulose-Membran	Schleicher und Schuell
One way-Sterilfilter (0,2μm)	Schleicher & Schuell
Petri's dishes flasks 15 cm	Greiner
Petri's dishesfor cellculture	Greiner
Plastic 15ml tubes	Falcon
Plastic 50ml tubes	Falcon
PolyFect™	Qiagen
Steril filters	Schleicher & Schuell
Sterilfilter	Nalgene
Sterilfilter 0,22 μm	Sarstedt
X-ray films X-OMAT™	Kodak
Zentrifugengefäße 15ml	Sarstedt

3 METHODS

3.1 Molecular biology methods

3.1.1 Culture of *E. coli*

E. coli cultures were grown according to standard protocols in 2YT-medium supplemented with ampicillin, kanamycin or chloramphenicol if needed for selection of plasmids.

2YT-medium:	1% (w/v)	yeast extrakt
	1.6 % (w/v)	trypton
	0.5% (w/v)	NaCl
2YT-plates:	2YT-Medium	
	2% (w/v) Agar	
Added antibiotics:		
	100 µg/mL	ampicillin
	25 µg/mL	Kanamycin
	10 µg/mL	Chloramphenicol

3.1.2 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 crossed out on 2YT-medium containing the appropriate antibiotics and incubated overnight at 37°C. Plates were stored up to 6 weeks at 4°C.

3.1.3 Preparation of competent bacteria (Calcium chlorid-methode)

A single colony was inoculated into 2 ml 2YT-medium and incubated overnight on a shaker at 37°C. 500µl overnight culture was inoculated into 200 ml of LB medium and incubated on a shaker at 37°C for 4-6 h until OD_{600} =0.4-0.6. The culture was transferred to sterile centrifuge tubes and centrifuged 4000 rpm (Hettich universal-320R), 10 min at 4°C. The supernatant was poured off and the cells were

kept on ice. Then the cell pellet was resuspended in 10 ml of ice cold sterile 50 mM $CaCl_2$ and incubated for 30 min on ice. After centrifugation at 5000 rpm (Hettich universal-320R), 10 min at 4°C the cells were resuspended in 5 ml of ice cold 50 mM $CaCl_2$ containing 10% Glycerol. Aliquot of 100-150 µl were incubated in micorcentrifuge tubes for 2 h on ice. Subsequently, the suspension was frozen in liquid nitrogen and stored at –80°C.

3.1.4 Transformation of *E. coli*

To 100-150µl of competent *E.coli* (DH5-α, HB101 orJM101) cells either 50-100 ng of plasmid DNA or 10 µl of ligation mixture were added and incubated for 20 min on ice. After a heat shock (1.5 min, 42°C) and successive incubation on ice (2 min), 200 µl of 2YT-medium was added to the bacteria and incubated at 37°C for 60 min with gently shaking. Aliquots of transformed cells were plated on 2YT-plates containing the appropriate antibiotic. Plates were incubated at 37°C overnight.

3.1.5 Plasmid preparation

3.1.5.1 Plasmid Miniprepation

Preparation of small-scales of plasmid DNA was performed according to the QIAprep Spin® Miniprep Kit manufacture's protocol.

3.1.5.2 Plasmid Midipreparation

Preparation of large-scales of plasmid DNA was performed according to the QIAGEN Plasmid Midi (Maxi) Kit and/or Pure Link Kit™ (invitrogen) manufacture's protocols.

3.1.6 Enzymatic modification of DNA

3.1.6.1 Digestion of DNA by restriction endonucleases

DNA was incubated with the recommended amount of appropriate enzymes in the recommended buffer for 2-24 h at recommended temperature according to the manufacturer's protocol. 20µl Reaction typically contains:

0.2-1 µg DNA
Water to a final volume of 20µl
2µl of the appropriate 10x digestion buffer
1 Unit of restriction enzyme

Total volume 20µl

3.1.6.2 Ligation of DNA fragments

Ligation of DNA fragments was performed by mixing 50-100 ng vector DNA with threefold to eightfold molar excess of insert DNA. 1 µl of T4 DNA ligase (1 Unit/µl) and 2 µl of 10xligation buffer were added and the reaction mix was brought to a final volume of 20 µl. The reaction was incubated for 3-12h at 4°C or at 16°C. The reaction mixture was used directly for transformation without any further purification.

50-100 ng vectorDNA
150-800 ng DNA fragment
2µl 10x ligation buffer
1µl T4-DNA ligase (1U/µl)

Total volume 20µl

3.1.7 DNA electrophoresis

DNA fragments were separated in horizontal electrophoresis chambers (5x7.5cm or 11x14cm) using agarose gels.

Agarose gels were prepared by heating 1% (w/v) agarose in 1x TAE buffer (25ml for 5x7.5cm and 80ml for 11x14cm gels). The gel was covered with 1x TBE buffer and the DNA samples were mixed with sample buffer and pipetted in the sample pockets. The gel was run at constant voltage (10 V/cm gel length) until the bromophenol blue dye had reached the end of the gel. Finally gels were immersed in water containing 0.5µg/ml Ethidium bromide for 15-30min at room temperature (RT) and documented using a UV-light imaging at 254nm.

1% agarose gel: (5x7.5cm chamber)	0.25 g agarose 25 ml TAE- buffer
1% agarose gel: (11x14cm chamber)	0.8 g agarose 80 ml TAE- buffer
1x TAE- buffer:	40 mM Tris, pH8 5 mM Na-Acetate 1 mM EDTA
6x loading buffer:	0.25 % (w/v) bromophenol blue 30% (v/v) Glycerol in water

3.1.8 DNA purification

3.1.8.1 Purification of DNA (PCR) fragments

Purification of DNA fragments was performed using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacture's protocol.

3.1.8.2 Extraction of DNA from agarose gels

The DNA Gel Extraction Kit (Millipore) 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 from the gel with a clean scalpel and transferred into an Eppendorf tube. The DNA fragment was purified following the manufacture's protocol.

3.1.9 Determination of DNA and RNA concentration

The concentration of the isolated DNA or RNA and the ratio of absorbance at 260 nm to 280 nm (A260/A280 ratio) were measured with the NanoDrop ND-1000 spectrophotometer. A ratio of A260/A280 between 1.8 and 2 monitored a sufficient purity of the DNA or RNA preparation.

3.1.10 Polymerase chain reaction (PCR)

Reaction components were prepared as below:

20-50 ng plasmid DNA or cDNA
2µl 10mM dNTP mix
1.5µl 50mM $MgCl_2$
5µl 10x PCR-buffer
2µl 100µM forward primer
2µl 100µM reverse primer
Water to a final volume of 50µl
5min incubation at 95°C (initial denaturation)
1Unit of Taq DNA-polymerase

Total volume 50µl

PCR was carried out using an automated thermal cycler.

PCR program:

30 cycles:

1 min 94°C (denaturation)

1 min 65°C (annealing)
3 min 72°C (extension)

PCR products were either separated by agarose gel electrophoresis, excised and subsequently purified with the DNA Gel Extraction Kit (Millipore) or directly purified with the PCR Purification Kit.

3.1.11 DNA sequencing

DNA fragments and DNA plasmids were sequenced using the facility of GATC-Biotech Company.

3.1.12 *In vitro* transcription of AdML, U6snRNA and tRNA

For nuclear microinjection into *Xenopus* oocytes, mRNAs were transcribed *in vitro* from linearized plasmids using *in vitro* transkription kit (Promega). Plasmids were linearized with the appropriate restriction enzyme. Plasmids pU6 and ptRNA were linearised with *Dra*I and *Sal*I, respectively, then transcribed using T7 RNA polymerase, whereas plasmid pBS-Ad1 (pAdML) was linearised with *Sau*3A (*Mbo*I) and transcribed using T3 RNA polymerase. Linearized DNA was precipitated by adding 0.1 volumes of 3 M NaAC, pH 5.2 and 2 volumes of Ethanol at -20°C for 15 min and resuspended in ddH2O to final concentration of 0.5-1 µg/µl.
Synthesis of AdML pre-mRNAs was primed with the m^7G(5´)ppp(5´)G cap Analog (Ambion) dinucleotide, whereas U6snRNA was primed with G(ppp)G cap analog (NEB).

Reaction components were prepared as below:

	AdML	**U6**	**tRNA**
Template	1µg	1µg	1µg
5x tr-buffer	5µl	5µl	5µl
100mM DTT	2µl	2µl	2µl
RNasin (40U/µl)	1µl	1µl	1µl

rATP 10mM	1µl	1µl	1µl
rCTP 10mM	1µl	1µl	1µl
rGTP 10mM	0,5µl	0,5µl	1µl
m7G-cap 10mM	0,75µl	-----	----
G-cap analog 10mM	-----	0.75µl	----
[^{32}P]-UTP	2.5µl	2.5µl	2.5µl
RNase Free water	5.5µl	5µl	5µl
T7-RNApolymerase	----	1µl	1µl
T3-RNA polymerase	1µl	----	----

	Total volume 25µl		

The reaction mixture were placed into a thermal cycler and incubated for 90 min at 37°C. After completion, 1µl Dnase (invitrogene) was added to the reaction and incubated again for 30min at 37°C. RNA was purified with RNeasy (Qiagen) according to the manufacturer´s protocol. The purified transcripts were checked on an 8% polyacrylamide gel containing 7 M urea.

3.1.13 RNA purification

3.1.13.1 RNA cleanup kit

For the RNA microinjection into *Xenopus laevis* oocytes RNA was purified using RNeasy clean up kit (Qiagen) following the manufacturer's protocol.

3.1.13.2 Phenol/chloroform extraction

125µl Rnase free water was added to a Reaction mixture. Then 150µl of phenol/chloroform/isoamyl alcohol was added and vortexed and incubated for 5min at RT. After 10min centrifugation at 13000 rpm in a bench top centrifuge, the topaqueous layer was recovered into a new tube, and 150µl chloroform/isoamyl alcohol added. This was vortexed and centrifuged as previously. The aqueous layer was mixed with 300µl 100% ethanol and precipitated at -20°C for at least 2h (or overnight). The RNA was polluted at 13000rpm at 4°C for 30min, and then washed

with 200µl 75% ethanol. The pellet was dried and resuspended in appropriate volume of RNase free water.

3.1.14 *In vitro* coupled transcription/ translation of [^{35}S]-FMIP

The TNT T7 Quick coupled Rabbit Reticulocyte Transkrption/Translation System was purchased from Promega (Madison, WI, USA). This was rapidly thawed by handwarming and placed on ice. The reaction mixture as outlined below was assembled in a 0.5ml PCR tube, and mixed by gentle pipetting.

40µ	TNT Quick master mix
2µl	[^{35}S]-methionine
2µl	Plasmide DNA template (0.5µg/µl)
4µl	Nuclease free water
---	---
Total volume: 50µl	

The tube was the placed into a thermal cycler (Icycler, Bio-Rad) and programmed to run on cycle for 90 min at 30°C. After completion, a 2µl aliquot of the reaction was mixed with 20µl of 3x SDS loading buffer. The remainder of the solution was stored at -20°C. The sample for analysis was heat denatured at 95°C for 5 min, and chilled on ice. The sample as then loaded onto an 11% SDS-PAGE. The gel was then carefully placed onto a sheet of Whatman paper and dried at 80°C for 2h under vacuum using a gel dryer. Once dried, the gel was exposed to a phosphor screen for 2-3h and scanned using the phophorimager (Fujifilm BAS-1500) or using autoradiography (Kodak BioMax light film).

3.1.15 Urea-denaturing polyacrylamide gel

Denaturing polyacrylamide gels are used for the separation of radiolabelled RNA extracted from microinjected *Xenous Laevis* oocytes. Denatured RNA migrates

through these gels at a rate that is almost completely independent of its base composition and sequence.

Gel plates of 18 x 16 cm2 were washed thoroughly and rinsed with double distilled water, followed by ethanol and air-dried. One of the plates facing the gel matrix was treated with siliconizing fluid (Sigmacote) to ease the removal of the gel in-between the glass plates without tearing the gel. The Sigmacote was completely wiped dry and the plates were assembled and secured by clamps. Aproximatively 100 ml of 5% gel solution was prepared with the following components:

16.6 ml	Acryl amide 16.6 ml
20ml	5x TBE 20 ml
7 M	urea
62.7 ml	Distilled water
0.7 ml	10% APS
35 µl	TEMED

Total volume: 100µl

After polymerisation, the glass plates were secured into the electrophoresis system and samples were loaded. 3 µl of 6x loading buffer was added to each sample, and the samples were incubated at 95°C for 10 min, cooled on ice and loaded into the wells. Gels were run at 5 V/cm, measured as the distance between the electrodes, in 1x TBE buffer. Gels were then dried at 80°C for 2h under vacuum using a gel dryer (BioRad model 543). Once dried, radiolabelled RNAs were detected using autoradiography (Kodak BioMax light film), and/or a Phosphorimager (Fujifilm BAS-1500).

3.2 Protein biochemical methods

3.2.1 Protein extraction from mammalian cells

To harvest cells, medium was aspirated; cells were rinsed twice with PBS then lysed by addition of the apropiate lysis buffer (200-500µl per 5cm Petri dishes). Lysates were transferred to sterile 1.5ml microcentrifuge tubes and centrifuged for

15 min at 4°C. The cleared supernatant containing protein extracts were stordat -20°C.

Whole cell lysates were obtained as well by direct lysis in laemli buffer. Each $5x10^5$ - $1x10^6$ cells were first washed with PBS, pelleted and then resuspended in 160µl 2x SDS sample buffer. The tubes were vortexed for 30s and Protein extracts were denaturated at 95°C for 10 minutes, cooled to room temperature and centrifuged for 10min at 13000rpm. Alipquots from the supernatants were analysed by SDS-PAGE followed by immunoblotting analysis.

4x SDS loading buffer:

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

3.2.2 TCA/Acetone precipitation

Three volumes of ice-cold TCA/Aceton (1:8) were added to the extracted cytoplasmic fractions of oocytes. The mixture was mixed and incubated at -20°C for 1-2h, but preferably overnight. After incubation, the samples were centrifuged at maximum speed for 20min. The supernatant was removed carefully and washed twice with ice-cold Acetone. The pellet was dried (but not overdried) and boiled in 2xSDS buffer. Aliquots were analyzed by immunoblotting.

3.2.3 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (sodium dodecyl sulphate – polyacrylamide gel electrophoresis) is a method used for separation of proteins according to their molecular mass (Laemmli, 1970). In the present work gels were mainly poured using Biometra equipment and were 1.5mm thick. By variation of acrylamide concentration the

pore size of the polymerized gel were varied (usually 7-15%) according to the size of the protein to be analysed. 11%SDS gels were used in the present work. Separating and stacking gels were prepared according to Sambrook *et al.* (1989). After complete polymerization of the gel, the chamber was assembled as described by the manufacture'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 10 mA for the stacking gel and then for the separation gel at 20 mA. The molecular weight of proteins was estimated by running 5µl of pre-stained (Dual colour standards, Bio-Rad) or non-stained (Unstained protein standard, Roth) marker proteins. The gel run was stopped when the bromophenol blue dye had reached the end of the gel. Gels were then either stained or subjected to Western Blotting.

	11% separating gel:	**5% stacking gel:**
Acrylamide	2.2 ml	0.334ml
1M Tris-HCl pH8.8	2.5 ml	-------
1M Tris-HCl pH6.8	-------	0.25 ml
Water	1.55 ml	1.4 ml
20% SDS	30 µl	10 µl
TEMED	6 µl	3 µl
10%APS	20 µl	10µl

1x SDS running buffer:

3.03g Tris

14.22g glycine

1g SDS

Water to a final volume of 1L

4x SDS loading buffer:

1ml 4xSDS-solution

320µl 0.1% bromophenol blue

50µl β-mercaptoethanol

4x SD-solution:

40ml 20% SDS

40ml Glycerol

16ml 1M Tris-HCl, pH6,8

4ml water

3.2.4 Western blot (semi-dry)

Proteins were separated by 11% SDS-PAGE and transferred to Polyvinylidendifluorid membrane (PVDF, Macherey & Nagel) using the Bio-Rad ”Trans-Blot Semi Dry Apparatus” For Proteins transfer, the current was set to 0.8 mA/cm2 gel size for 90 min. Gels were sandwiched between gel-sized-Whatman 3 MM papers soaked in anode or cathode buffers as follows:

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

2 pieces of Whatman paper soaked in anode buffer I

+

Anode

After transfer, the membrane was then briefly washed with TBST and incubated in blocking buffer for 1h at room temperature. Incubation with the primary antibody diluted in blocking buffer occurred overnight at 4°C. After three 10-15 min washes with blocking buffer, the membrane was incubated with horse radish peroxidase (HRP)-coupled secondary antibody, diluted 1:10 000 in blocking buffer, for one hour at room temperature. The blot was washed three times 10-15 min with TBST

and incubated with chemiluminescence substrate solution (Pierce). Protein-antibody complexes were detected using Enhanced Chemi-Luminescence (ECL, Fujitzu) according to the manufacturer's instructions.

Anode buffer I pH 10.4

300 mM Tris

20% (v/v) methanol

(pH was adjusted before addition of methanol)

Anode buffer II pH 10.4

25 mM Tris

20% (v/v) methanol

(pH was adjusted before addition of methanol)

Cathode buffer pH 9.4

40 mM 6-amino-n-Hexanacid

25 mM Tris

20% (v/v) methanol

(pH was adjusted before addition of methanol)

Tris-buffered saline (TBS)

10 mM Tris-HCl pH 7.4

150 mM NaCl

TBST

0.1% (v/v) Tween-20

in TBS

Blocking buffer

2% BSA (bovinefraktionV (Roth)) powder

in TBST

3.2.5 Stripping of PVDF membranes

Removal of antibodies from a blot was done under mild conditions. After washing the membrane three times in TBST, the blot was incubated in SDS stripping solution for 30 min, tightly closed, on a wheel at 40-50°C, followed by several washes in TBST. Incubation of the membrane with the new antibody was done as described above, starting from the blocking step again.

SDS stripping solution

16 mM Tris-Cl pH 6.8

2% SDS

100 mM -mercaptoethanol

3.2.6 Determination 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. Roti quant 5x (Roth) is diluted with water in ratio 1:4. For each sample (50 µl) and incubated at RT for 5 min. Samples are placed in 96-well plate in duplicates (250 µl per well) and set on the ELISA reader. Absorbance is measured at a wavelength of 590nm. Samples to be measured are diluted appropriately and if the concentration of the protein is unknown several samples of various dilutions are made

Protein standards are prepared in the same buffer as the samples to be assayed. A convenient standard curve is made using bovine serum albumin (BSA) with concentrations of 0, 20, 40, 50, 60, 80 and 100µg/ml for the standard assay. Using protein standards a graph of absorbance at 590nm, the results are used to graph the standard curve from which unknown protein concentration is determined.

3.2.7 Staining of protein gels

3.2.7.1 Coomassie staining

Proteins were separated by SDS-PAGE, and protein gels were incubated in coomassie staining solution with gentle agitation at RT for 2h or overnight. To remove unspecific staining, gels were incubated in destaining solution for 2-3h at RT.

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

3.2.7.2 Silver staining

Purification of DNA fragments was performed using the Silver Stain Plus Kit (Bio Rad) following to the manufacturer's protocol.

3.2.8 Immunoprecipitation

HECK293 cells were transiently transfected with Myc-tagged FMIP, Myc- tagged FMIP deletion mutants or Myc-tagged THOC7. After 24 h, the medium was removed, and cells were washed three times with ice cold PBS. The cells were then lysed on ice for 15 min with 500µl immunoprecipitation lysis buffer. The cell lysate was collected and centrifuged at 13000rpm for 15min at 4°C. The pellet was discarded and the cleared supernatant was incubated with antibodies (Monoclonal anti-Myc (9E10) (Santa Cruz Biotechnology) and monoclonal anti-p84N5/THOC1 (Gene Tex)) for 1h at 4°C by swinging head-over-tail. After incubation, 20µl of protein-A sepharose (Santa Cruz biotechnology) was added to capture the complex

and further incubated at 4°C overnight. The immunprecipitates were washed three times with 500µl immunoprecipitation lysis buffer and mixed with 20µl of 4xSDS sample loading buffer. The samples were boiled for 10 min at 95°C and subjected to SDS-PAGE ad Western blott.

Immunoprecipitation lysis buffer

50mM Tris-HCl (pH7.5)

75mM NaCl

1% Triton X-100

1mM PMSF

2% Trasylol

3.2.9 GST pull-down assays

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

The *E. coli* strain DH-5α were transformed with the expression plasmid expressing GST tagged protein and cultured in 50 ml of 2YT-medium with the ampicillin antibiotic overnight at 37°C. The overnight culture was diluted 1:500 in 100 ml of 2YT-medium containing the antibiotic and cultured at 37°C until cell density reached OD600 of 0.3. To induce expression of the recombinant protein 1 mM IPTG was added and the culture was incubated for 6h at 37°C.

Cells from a 100 ml bacterial culture were 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 containing 0.1% (v/v) Triton X-100 and protease inhibitors, and incubated on ice for 10 min. The suspension was sonicated (8 times, each 30 sec, amplitude 50%, Branson sonifier). The cell extracts were clarified by centrifugation at 5000 rpm for 15 min at 4°C (centrifuge, Hettich). The cleared supernatant was transferred to a new tube and incubated overnight at 4°C with 70 µl of Glutathione-Sepharose 4B beads (Amersham). On the next day,

beads were washed 3 times and finally resuspended in 1ml washing Buffer (1:2 ratio of beads and washing buffer). 8-10 µl of the 50% 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 or bead-bound proteins were eluted.

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

GST-Lysis Buffer

20mM Tris-HCl pH7.5

0.1% Triton X-100

1mM PMSF

1% Trasylol

Washing buffer

20mM Tris-HCl pH 7.5

0.5% Triton X-100

15mM -Mercaptoethanol

1mM PMSF

2% Trasylol

In PBS

Elution Buffer

20mM Tris-HCl pH7.5

20mM Gluthation

1mM PMSF

1% Trasylol

3.2.9.2 GST pull-down assay using HECK293, Hela and C2C12 cells extract

Beads slurry containing equal amount of GST-tagged proteins (GST, GST-THOC3, GST-THOC6, GST-THOC7, and GST-SF2) was used for each pull down assay. These were washed 3 times with 200µl of GST-binding buffer. After the final wash, the supernatant was removed and 200µl of transfected or untransfected Cell lysates was added and incubated at 4°C for 4h or over night. The beads were then washed 3 times with 500µl pull down buffer, centrifuging at 2000rpm for 2min at 4°C between successive washes. After the final wash, the supernatant was removed and 20µl of water and 10µl of 4xSDS loading buffer was added. This was incubated at 95°C for 15 min, chilled on ice and centrifuged at 13000rpm at 4°C. The supernatant was loaded onto 11% SDS-PAGE and analysed by western blotting using the appropriate antibody.

GST-binding buffer

20mM Tris-HCl pH7.5

150mM NaCl

0.1% Triton X-100

1mM PMSF

1% Trasylol

3.2.9.3 GST Pull-down of [^{35}S]-labelled FMIP

GST-tagged proteins (GST-THOC7 and GST-THOC6) or GST alone were immobilized on Glutathion Sepharose beads 4B (Amersham) and incubated for overnight at 4°C with 25 µl of rabbit reticulocyte lysates containing the ^{35}S-labeled proteins ([^{35}S]-FMIP (wt), [^{35}S]-FMIP (1-289)) and 200µl of GST-binding buffer (section..). After incubation, Beads were washed 3 times with the binding buffer. The sampels were loaded onto 11% SDS-PAGE with 2µl [^{35}S]-methionine labelled protein imput for control. The polyacrylamide gel was then dried at 80°C for 2h under vacuum using

a gel dryer (BioRad model 543). Once dried, radio-labelled proteins were detected by autoradiography or Phosphorimager (Fujifilm BAS-1500).

3.2.9.4 GST pull-down using Xenopus Laevis extract

Twenty stage V/VI Xenopus oocytes were homogenized in lysis buffer H containing 1.2% Triton X-100 by pipetting up und down. The extracts were centrifuged at 13000 rpm at 4°C for 30 min. The supernatant was taken and centrifuged again at 13000rpm at 4°C for 30 min. The precleared supernatant was incubated overnight with GST-tagged THOC7 or GST alone at 4°C. Bound fractions were washed three times with the lysis buffer. The bound proteins were released by boiling in 4x SDS sample loading buffer and resolved by 11% SDS-PAGE and western blotting using monoclonal antibody raised against FMIP.

Lysis BufferH:

20mM Tris-HCl pH7.5

150mM NaCl

1.2% Triton X-100

1mM PMSF

1% Trasylol

3.2.10 Mammalian Tandem affinity purification of hFMIP

In TAP, almost the proteins that are in the same protein complex with the protein of interest will be pulled down after two-step affinity purification. The protein of interest is expressed as a fusion protein with TAP-tag being positioned either at the amino- or the carboxy-terminus. The TAP vector which contains Streptavidin Binding Peptide (SBP) and Calmodulin Binding Peptide (CBP) was applied in this work. The SBP tag allows for the protein of interest and its associated proteins to be captured through the tight binding of SBP to streptavidin resin and purified by eluting with excess biotin. The CBP tag allows for a second purification step in which the CBP tag binds to calmodulin resin and released by EGTA through calcium

(Ca^{2+}) chelation (Figure 2.1). The final eluate contains almost the proteins in the same complex with the protein of interest, and can be subsequently identified by massenspectroscopy (MS).

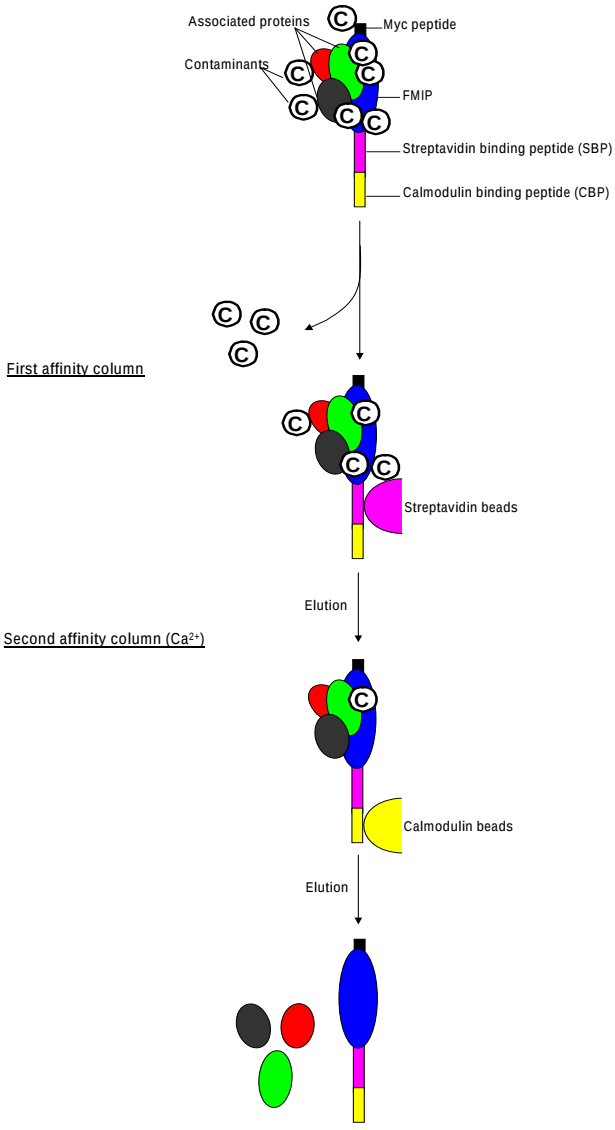

Fig. 3.1: **Overview of the mammalian tandem Affinity purification (TAP) procedure**. The high-affinity streptavidin-binding peptide (SBP) and calmodulin-binding peptide (CBP) tags allow for a two-step specific selection of the tagged protein and its associated proteins under mild conditions. Eluted proteins are subsequently identified by tandem mass spectrometry.

3.2.10.1 Cloning of TAP-tagged FMIP-Myc

The Myc-tagged FMIP fragment was amplified from pcDNA MycHis-Fmip vector using PCR techniques (EcoFMIPp, FMIPMycHis primesr section). The InterPlay™ N-terminal Mammalian TAP system pNTAP-vector (Stratagene) (Figure 2.2) was linearised using *EcoR I* and *Xho I*. The PRC product was then inserted in lienarised pNTAP vector. The resulting vectors, pNTAP-FMIP-Myc was verified by DNA sequencing.

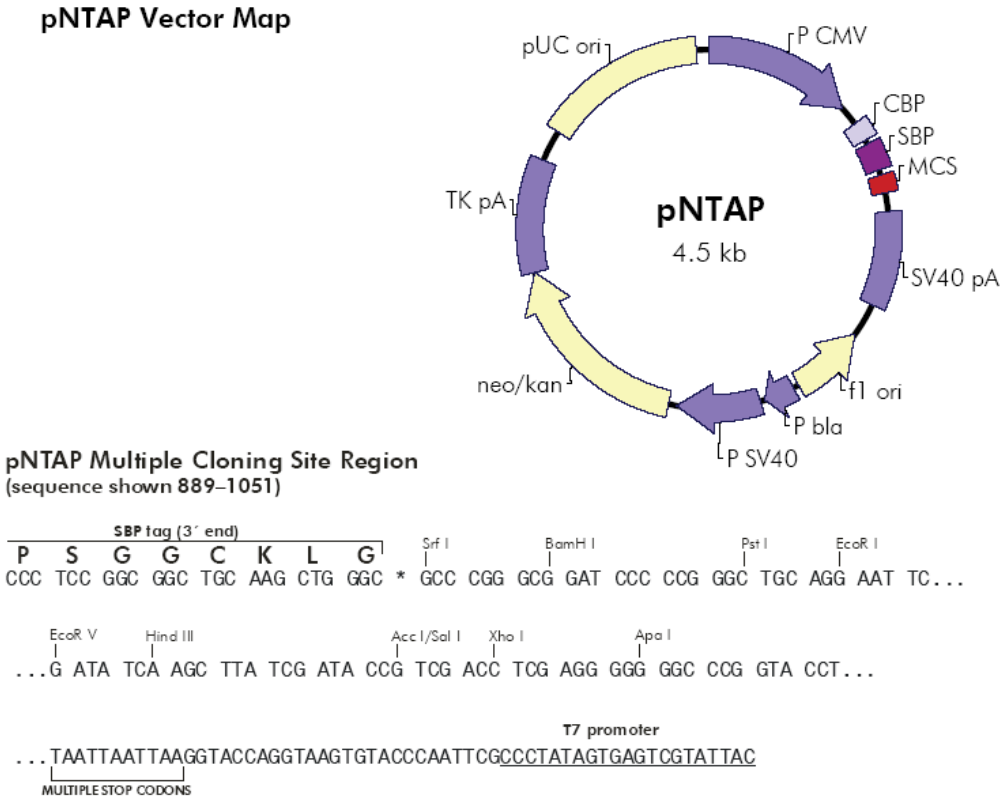

Fig. 3.2: Circular map of the pNTAP expression vectors used in this thesis. (Adapted from Interplay™ TAP Purification kit protocol)

3.2.10.2 Interplay™ TAP Purification kit

Purification of TAP-FMIP-Myc from HEK293 was first performed using the Interplay™ TAP Purification kit (Stratagene) following to the manufacturer's protocol.

3.2.10.3 Establishment of mammalian Tandem affinity purification

3.2.10.3.1 Cell lysates extraction

TAP-FMIP-Myc HEK293 transfected cells are washed 3 times in ice-cold PBS. The cells are lysed for 5 min in 3ml lysis buffer (3ml for each maxi-plate), and harvested by scraping. To improve protein recovery 2 freeze-thaw cycles were performed in liquid nitrogen and in 37°C water bath. The cell lysates were centrifuged for 15 min at maximum speed (13000rpm), and the supernatant was stored at -80°C.

3.2.10.3.2 Established mammalian TAP purification

The InterPlay™ Mammalian TAP system kit (Stratagene) protocol was followed with major modifications. To minimize protein degradation, all steps were performed on ice or in a 4ºC walk-in incubator for overnight steps. Because all agarose beads used for protein purification were preserved in buffers containing 20% ethanol, they were washed 3 times using their respective buffers and centrifuged at 2000rpm at 4°C. All spin steps involving beads are performed at very low speeds (2000rpm for 2min). Removel if the liquid is performed slowly. All spins and incubations are performed at 4°C, and all buffers are pre-chilled on ice. All eppendorf tubes were washed twice with 70% ethanol and 3 times with ultrapure water to remove residue contaminants that may interfere with MS analysis. In each step 20µl of aliquots was saved for analysis.

The cell lysates were incubated with 100µl of streptavidine sepharose beads (Amersham) in a volume of 8 ml- This slurry was incubated at 4°C, rotating overnight. After incubation, the beads were washed three times with 2ml

streptavidin binding buffer (SBB) buffer. The streptavidine sepharose were resuspended in 100µl SEB buffer and incubated overnight at 4°C. The beads supernatant was the retrieved and mixed with with 3 eluate volumes of calmodulin binding buffer (CBB), 1µl of 1M $CaCl_2$, and 80µl of calmodulin beads (Amersham). This mixture is incubated overnignht at 4°C, with rotating. After incubation, the beads were washed three times with 10 bead volumes of calmodulin buffer and eluatted two times with 150µl of calmodulin elution buffer (CEB). The eluates were then combined and the protein concentration was determined using the Bradford protein assay.

Lysis buffer

10mM	Tris-Cl pH8.0
150mM	NaCl
0.1%	NP40
1mM	PMSF
1%	Trasylol

Streptavidin binding buffer (SBB)

10mM	Tris-Cl pH8.0
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
10 mM	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 pH8.0
150mM	NaCl
1mM	Mg-acetate
10mM	β-Mercaptoethanol
1mM	imidazole
2mM	EGTA
0.1%	NP40

3.2.10.3.3 Purified protein separation and visualization

To analyze the results of the TAP procedure, protein aliquots taken from various steps were visualized on a 11% SDS-PAGE. The 0.5 to 4 µg of protein samples or 15 µL of beads were added to 2x SDS loading sample buffer. The samples were boiled for 12 minutes and loaded onto a 11% SDS gel. For the molecular weight markers, 1 µL of protein standards (Bio-Rad) with a range of 10 – 250 kDa were used. The

gel was then stained using the Silver Stain Plus Kit (Bio Rad) or stained for 2 hours using Coomassie stains (Sigma).

3.2.10.3.4 Mass spectroscopy and Maldi-TOFF

The identification of low level proteins from the SDS-PAGE was performed in the Laboratory of Prof. Tony Whethon at the University of Manchester (UK).
Mass spectroscopy is a powerful and versatile anayltical technique used extensively in proteomics. In order to identify the major proteins present in the SDS-PAGE gel. Gel spieces were cuted and sibjected toenzymatic digestion using trypsin, which typically generates peptides amenableto anylysis by mass spectroscopy. Separated peptides were eluated from the analytical column directlyinto the electrospry ion source of a QSTAR XL hybrid mass spectrometer. MS/MS datawas aquired using an established Information Dependent Acquisition (IDA) protocol enabling peptide sequence information to be obtained. The instrument was instructed to scan for potential peptide (multiply charged) ions. Fragement ionsin the spectrum could then be correlated to amino acids and the peptide sequence determined.

3.2.11 Protein localization

3.2.11.1 Immunoflorescence

Hela cells or MEF cells were transiently tansfected with plasmids containing FMIP-Myc, THOC7-Myc or NLS-THOC7-Myc cDNAs. Following transfection, medium was removed and cells were gently washed twice with PBS. PBS was aspirated and cells were fixed by addition of 500µl 3.7% formaldehyde directly to the cells, cells were incubated for 20 minutes at room temperature (RT), the washed 3 times with 500µl PBS for 5min at RT.Cells were permeabilised by the addition of 400µl 0.2% Triton X-100 for 5minutes at RT then washed 3X with 500µl PBS for 5min at RT. PBS was aspirated, 500µl of blocking solution containing 0.2% BSA was added an the cells were incubated for 20 min at RT then washed 3X with PBS. Primary antibody (MYC) was diluted in 1% BSA/PBS, 100µl was added drop-wise to each

well and the 24 well Platte was incubated for 2h or overnight at RT. After Incubation, the cells were washed 3X for 10 min with PBS. Bound primary antibody was detected using a secondary antibody FITC (Santa Cruz). FTIC was diluted in low light in 1%BSA/PBS, 100µl was added drop-wise to each well and the 24 well plates was incubated at RT for 1h shielded from light. After incubation, wells were washed 3X 10 min in PBS. Fluorescence microscopy analysis was carried out using an inverted Nikon fluorescence microscope (Nikon). Images were collected using the Metamorphe software (MetaMorph InVisitron System).

3.2.11.2 GFP-localization

GFP-tagged proteins were directly visualized after fixation with 3.7% formaldehyde in PBS for 20 min at room tempurature. Fluorescence microscopy analysis was carried out using an inverted Nikon fluorescence microscope (Nikon). Images were collected using the Metamorphe software (MetaMorph InVisitron System).

3.3 Cell culture

3.3.1 Cell culture and maintenance of cell lines

Human embryonic kidney (HEK) 293 cells, pluripotential mesenschymal progenitor cells C2C12 cells and Hela cells were routinely cultured at 37°C /5%CO_2 in Dulbecco´s modified Eagle medium (DMEM) (GIBCO) containing 10% foetal calf serum (FCS), 100µg/ml penicillin, 100µg/ml streptomycine and 2mM L-Glutamine in PBS. Medium was replaced every 2-3 days and cells were passaged at a dilution 1:5 or at 1:10 when cells are 90-95% confluent.

3.3.2 Passaging by trypsinisation

C2C12 and Hela cells were passaged every 2-7 days depending on the growth rate. After two washes with PBS, 0.5 ml trypsin solution was added to the cell layer. The cells were incubated at 37◦C for 5-10 min, then singled by tapping to the dish and

resuspended in medium. To concentrate, wash or harvest cells, centrifugation was always done at 1000 rpm, RT for 5 min.

3.3.3 Freezing cells for stock maintenance

Usually, cells from a confluent 10 cm flask were frozen as 2-4 vials. Cells were pelleted after trypsinisation, resuspended in 1 ml freezing medium per vial, immediately placed on ice and stored at -80 °C overnight. The vials were transferred to the liquid nitrogen container the following day.

Freezing Medium

90% FCS

10% DMSO

3.3.4 Thawing of cultured cell lines

The cell line aliquots were stored in 1 ml freezing medium at –80oC or in liquid nitrogen.

To grow up a cell line, the freeze down was thawed quickly in a 37 °C water-bath then transferred into a 75 cm2 cell culture flask containing 10 ml medium. After 4 h incubation under standard conditions (5% CO, 37°C) the medium was changed and the cells were further cultivated.

3.3.5 Cell counting

To determine the number of cells and the number of vital cells in culture, cell suspension in

DMEM is diluted 1:1 Trypan blue (Sigma) solution. This colored substance can enter and stain only dead cells. The cell suspension in Trypan blue is then transferred into two hemocytometer chambers. By using a 10x objective and light microscopy, it is possible to visualize the grid lines in the chamber, having each field an area of 1 mm^2. Then the cells are counted in 8 fields and the average

number of cells per volume (ml) is calculated (excluding cells stained in blue which are dead).

3.3.6 Transient transfection of HEK293, C2C12, MEF and Hela cells

Cells were grown to 70% confluence in 10cm Petri dishes. Prior to transfection, media was removed and replaced with 7ml fresh supplemented DMEM excluding antibiotics. Polyfect™ reagent was used as a method of DNA transfer following manufacturer's instructions. Briefly, 5µg DNA of interest was mixed in a total volume of 300µl DMEM without antibiotics. Subsequently, 30µl Polyfect was added to the mixture and incubated at room temperature for 5-10 min to allow the formation of liposomes. Following this incubation, 1ml of DMEM was mixed with reaction mixture and transferred to the cells, which were incubated at 37°C for a further 24-48hs. Transiently transfected cells were analyzed by western blotting for expression of the desired construct.

3.3.7 Establishment of FMIP and THOC7 stable cell lines

Myc-tagged FMIP, Myc-tagged THOC7, Flag-tagged FMIP plasmids or empty vector were expressed separately in HEK293, C2C12 or Hela cells. Transfections were performed with Polyfect™ as described above. Clones were selected in the presence of neomycin (G418) (1:200 in DMEM). After selection, cell extracts from selected clones were subjected to immunoblots using monoclonal anti-Myc or monoclonal anti- FLAG antibodies.

3.3.8 Adipocyte cell differentiation of C2C12 cells

Myc-tagged THOC7 or empty vector was expressed in C2C12 cells. After neomycine (G418) selection, colonies were obtained. Positive clones were incubated for 8 days in normal medium (DMEM). After incubation, cells were fixed with 3.7% paraformaldehyd (PFA) and stained by Oil Red (ORO).

3.3.9 Nuclear/cytoplasmic cell fractionation of HEK293 cells

Myc-tagged FMIP, Myc-tagged THOC7 plasmids or empty vector were expressed in HEK293. After transfection, cells were subjected to nuclear/cytoplasmic cell fractionation. Cytoplasmic fractions were obtained by lysis of cells in buffer **A**. After 5 min centrifugation, the supernatant (cytoplasmic fraction) was collected in a new eppendorf tubes. The nuclear fraction was obtained by resuspending the pellet in buffer **B**. Both fractions are obtained in equal volumes. Extracted nuclear and cytoplasmic fractions were used for immunoprecipitation using anti- THOC1 or anti-Myc antibody. Precipitates were analyzed by FMIP or THOC1 immunoblot.

Buffer A

10mM	HEPES pH7.9
1.4mM	$MgCl_2$
10mM	KCl
0.5mM	1.4-Dithiothreitol
0.5mM	PMSF
0.5%	NP40
1%	Trasylol

Buffer B

10mM	HEPES pH7.9
1.4mM	$MgCl_2$
375mM	KCl
0.5mM	1.4-Dithiothreitol
0.5mM	PMSF
0.5%	NP40
1%	Trasylol
10%	Gylcerol

3.4 *Xenopus* oocytes methods

3.4.1 Preparation of *Xenopus* Laevis oocytes extracts

Stage VI oocytes were prepared from ovaries of *Xenopus laevis* females, defolliculated as described by Gurdon and Wickens (1983) and incubated in 1x modified Barth's solution/HEPES (1xMBSH), 10 *Xenopus laevis* oocytes in stage V/VI were homogenized in lysis buffer (NET-2) containing 0.1% Nonidat (NP40). After removal of the yolk by centrifugation, Aliquots from the cleared extract was boiled in 4x SDS sample buffer. Extracted Proteins were resolved by 11% SDS/PAGE, followed by Western blot analysis by using monoclonal anti-FMIP (F6dII) antibody.

1xMBSH (modified Barth's solution/HEPES):
20 mM HEPES (pH 7.5)
80 mM NaCl
1 mM KCl
2.4 mM NaHCO3
0.82 mM MgSO4
0.33 mM Ca(NO3)2
0.41 mM CaCl2

NET-2 buffer
50 mM Tris-HCl, pH 7.4
150 mM NaCl
0.1% NP-40
0.21 mM PMSF
1µg/ml pepstatin

3.4.2 Cytoplasmic microinjection of GST-THOC7, GST-THOC7(1-66) and GST

Bacterially expressed and affinity-purified GST-THOC7, GST-THOC7 (1-66) or GST alone were released from glutathione beads and dialysed against PBS. Approximately 40-50nl of GST-THOC7, GST-THOC7 (1-66) or GST were injected separately into the cytoplasm of *Xenopus* oocytes. Microinjections were

performed in 1 X MBSH at 18°C. Nuclear (N) and cytoplasmic (C) fractions were manually dissected immediately after injection (0h) or after incubation (3h) at 18°C. From each injection 10-20 nuclei and cytoplasm were prepared. The nuclear and cytoplasmic fractions were homogenized in NET-2 buffer. Proteins were extracted from both fractions, and precipitated with TCA/Aceton (1:8). The dried protein pellets were dissolved in 2x SDS sample buffer and resolved by 11% SDS/PAGE, followed by Western blot analysis using monoclonal anti-GST antibody.

3.4.3 Nuclear Microinjection of radiolabeled AdML, U6snRNA and tRNA in oocytes

^{32}P-radiolabelled AdML pre-mRNA, U6snRNA and tRNA were generated using *in vitro* transcription. AdML pre-mRNA and U6 snRNA were synthesized with m7GpppG and γ–mpppG caps, respectively. *Xenopus* oocytes nuclei were injected with 10 to 20nl solution containing *in vitro* synthesised ^{32}P-radiolabelled RNAs (AdML pre-mRNA, U6nRNA and tRNA) and purified GST-hTHOC7 or GST alone. To control nuclear injection, samples were mixed (1:1) with a 20mg/ml solution of dextran blue. Nuclear (N) and cytoplasmic (C) fractions were manually dissected immediately after injection (0h) or after incubation (5h) at 18°C. After dissection only oocytes with blue nuclei were used. The distribution of radioactivity in the nucleus and cytoplasm was determined for each oocyte by Cerenkov counting. Injected RNA was from total oocytes, nuclear (N) and cytoplasmic (C) fractions were purified by homogenizing 20 oocytes/ml in RNase free NET-2 medium, followed by extraction with phenol chloroform. Approximatively 0.5 oocyte equivalents of extracted RNA were loaded per lane on 8% polyacrylamide gel containing 7 M urea.

4 RESULTS

4.1 Isolation of interacting proteins of FMIP by mammalian tandem affinity purification (TAP) methode

To further examine the function of FMIP, it is important to identify proteins that interact with FMIP. For this, FMIP complexes should be isolated and components of the complex identified by mass spectroscopy. As a first approach, tandem affinity purification (TAP) was chosen. TAP is a relatively new method for discovery of proteins involved in a protein complex. TAP was first developed in yeast (Rigaut et al., 1999). Several constructs have been developed by for use in mammalian systems (Burchstummer et al., 2006).

4.1.1 Establishment of TAP-FMIP purification methode

To isolate novel FMIP binding partners pNTAP plasmid from Stratagene was applied. The full-length human FMIP gene was inserted in-frame into downstream of the TAP sequence. Since we were not able to produce a big amount of FMIP monoclonal antibody, inserted FMIP gene was C-terminally tagged with Myc-epitope (**Figure 4.1A**). The Total gene cassette was placed under the control of the cytomegalovirus (CMV) to drive its constitutive expression.

To assess whether the tandem affinity tag constructs was in a correct fusion format and suitable for mammalian cell expression, HEK293 cells were transiently transfected with pNTAP-FMIP-Myc, pNTAP-FMIP and Myc/His tagged FMIP cDNA (as a control). The expression of the recombinant TAP-FMIP-Myc, TAP-FMIP and FMIP-Myc was analyzed by Western blotting anti-Myc or anti-FMIP antibody.

As shown in **Figure 4.1-B** all constructs were expressed. Inded, three specific bands of about 83 kDa (FMIP: 79 kDa + Myc epitope: 4 kDa); 85kDa (FMIP: 79 + TAP

epitope: 6 kDa) and 89 kDa (TAP: 6 kDa + FMIP-Myc: 83 kDa) were visualized by western blotting probed with anti-FMIP. To test whether the Myc epitope was inserted in TAP tagged FMIP construct (TAP-FMIP-Myc), the same aliquots were immunobloted using anti-Myc antibody. As expected, the FMIP-TAP was not detected by anti-Myc antibody, whereas recombinant TAP-FMIP-Myc was clearly detected from total lysate (**Fig. 4.1-B**). TAP-tagged FMIP-Myc construct was used for large-scale expression and purification FMIP binding protein complexes in HEK293 cells.

The mammalian TAP-tag purification was first performed using Interplay^{TM} TAP Purification (Stratagene) kit buffers. Cell extracts were prepared from TAP-FMIP-Myc transfected HEK293 cells. The efficiency of each purification step was monitored by analyzing aliquots on Western blots using anti-Myc antibody (Fig 2-C). As shown in **Fig 4.1-D**., the binding of fusion protein to streptavidin-sepharose and calmodulin-sepharose was highly efficient. However, the elution efficiency in the first and second purification steps was not complete (**Fig. 4.1-D**). This demonstrates that TAP-FMIP-Myc associated proteins were lost during the purification, indicating that the efficiency of TAP purification by the Interplay^{TM} TAP Purification kit was too low to allow isolation of large-scale of FMIP interacting proteins.

Since the elution in both affinity columns using Interplay^{TM} TAP Purification (Stratagene) kit was not complete, optimal Buffer conditions for the TAP-tag purification were established (**section 3**). Aliquots from each purification step were analyzed. The comparison between Interplay^{TM} TAP Purification kit steps using Kit buffers and established TAP using optimized buffers indicates that the elutions in both steps were efficient (**Fig.4.1**).

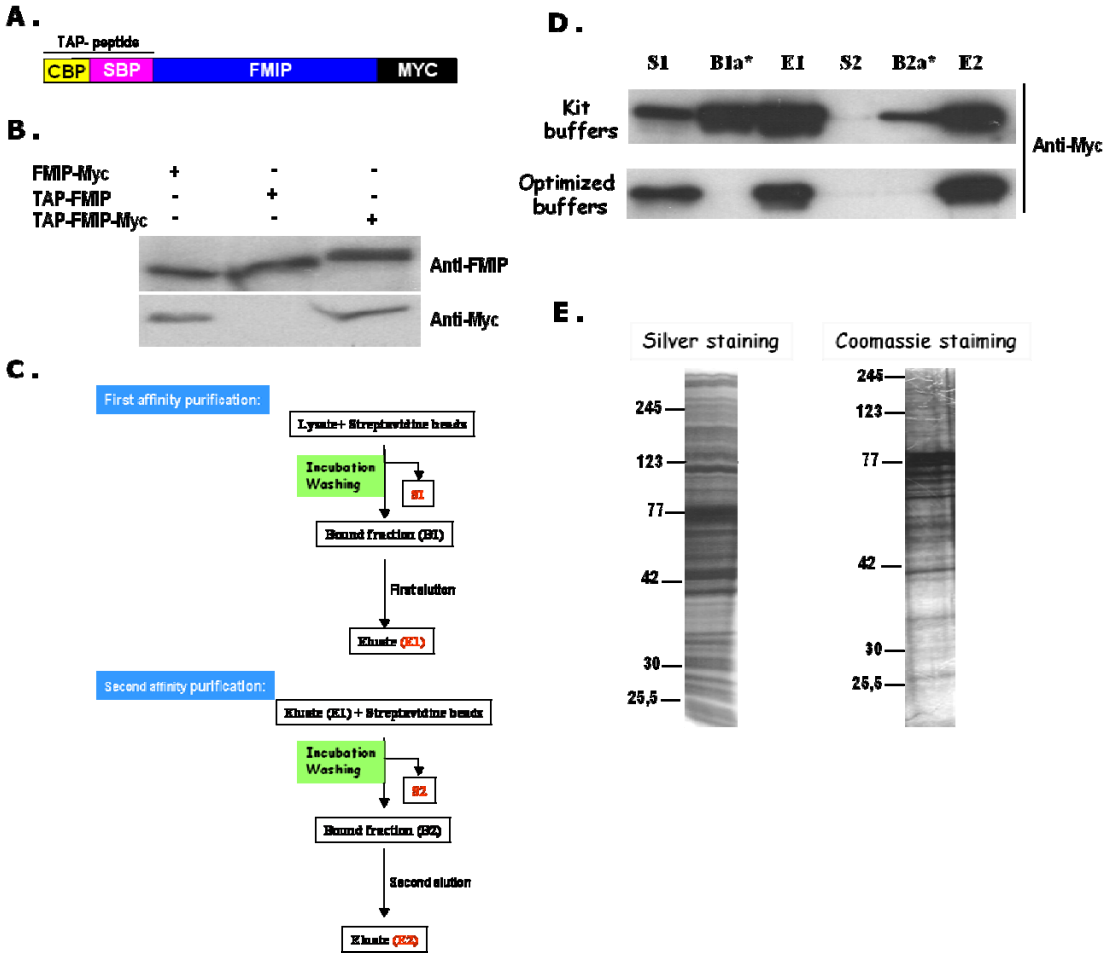

Fig. 4.1: Mammalian TAP-tag purification of hFMIP. A) Scheme of generated TAP-tagged FMIP-Myc construct. CBP: calmodulin binding peptide, SBP: streptavidine binding peptide, FMIP: fms interacting prtotein, Myc: Myc epitope. **B)** Expression of TAP-taged FMIP-Myc construct in HEK293 cells. N-terminal TAP- tagged FMIP-Myc, C-Terminal TAP-tagged FMIP and Myc tagged FMIP were separately expressed in HEK293 cells. After Transfection, cell lysates were separated by 11% SDS-PAGE and subjected to immunoblotting using a monoclonal anti-FMIP or anti-Myc **C)** Schematic illustration of different purification steps used for performing analysis. S1: supernatant 1, B1: bound fraction 1, E1: elute 1, S2: supernatant 2, B2: bound fractions 2, E2: elute 2. **D)** Comparison between Interplay™ TAP Purification (Stratagene) kit using Kit-buffers and established TAP-purification using optimized buffers. TAP-tagged FMIP-Myc was expressed in HEK293 cells. After transfection, TAP-tag purifications were performed using corresponding buffers of both Tap-purification. The efficiency of the different purification steps were controlled by Western Blot analysis. Approximately, 10% of the fractions from each purification steps were subjected to western blotting using anti-Myc antibody. **B1a*** and **B2a*** correspond to beads fractions B1 and B2 **a**fter elution, respectively. **E)** Siler and Coomassie staining of TAP-tag purified proteins. The eluate

from calmodulin beads were separated by SDS-PAGE and stained with silver staining or Coomassie blue brilliant. As a protein size marker prestained protein standard (Roth) was used.

4.1.2 FMIP is a member of THO complex

In collaboration with Prof. Antthony D. Whethon from the University of Manchester, large-scale of pooled TAP-FMIP protein complexes were separated by SDS polyacrylamide gel electrophoresis and stained with Coomassie Blue brillant G-250. Discrete gel slices were dissected from the top of the gel lane to the bottom and all regions were subjected to trypsin digestion. Digested materials were then analysed by mass spectrometry and database searching.

About 123 Proteins were identified in this large scale analysis of the TAP-tagged FMIP (**Table3**, page 116). The identified proteins were grouped and classified according to their subcellular localisation (**Figure 4.2**). Interestingly, More than 40% of isolated proteins are nuclear proteins. About 30% are located cytoplasmic and 14% are shuttling proteins.

Knowing that FMIP is mainly localized in the nucleus, analysis of identified proteins was focused on the nuclear proteins. For the interpretation of these results, the remaining 50 nuclear were classified and grouped according to their functions. Interestingly, about 36 percent of identified proteins were involved in mRNA processing and splicing (e.g. hnRNPs and processing factors) (**Figure 4.3**). Remarkably, Aly/REF1, UAP56 and members of THO complex (THOC1, THOC2 and THOC3), proteins that are known to be components of the TREX complex, were also identified in our TAP-purification.

By analysing the sequences of all THO complex members in The GenBank/EMBL and the Uniprot/Swissprot databases, we found surprisingly that the sequence of THO complex subunit 5, THOC5, shows a 100% homology to FMIP at the nucleotide and the amino acid level, suggesting that Fms interacting protein

(FMIP) is a member of the THO complex. This finding strongly demonstrates that FMIP may play a key role in the connection between receptor tyrosine kinases mediated extracellular signals and mRNA processing and /or export. The remaining proteins include proteins with a known function in replication or transcription (**Table 1**).

Additionally, 18% of isolated nuclear proteins are involved in cell cycle. Moreover, six proteins with potential regulatory roles were identified in apoptosis, DNA-damage or DNA-repair (**Table1**). This data indicates that FMIP may somehow be involved in the regulation of cell cycle, cell survival or apoptosis.

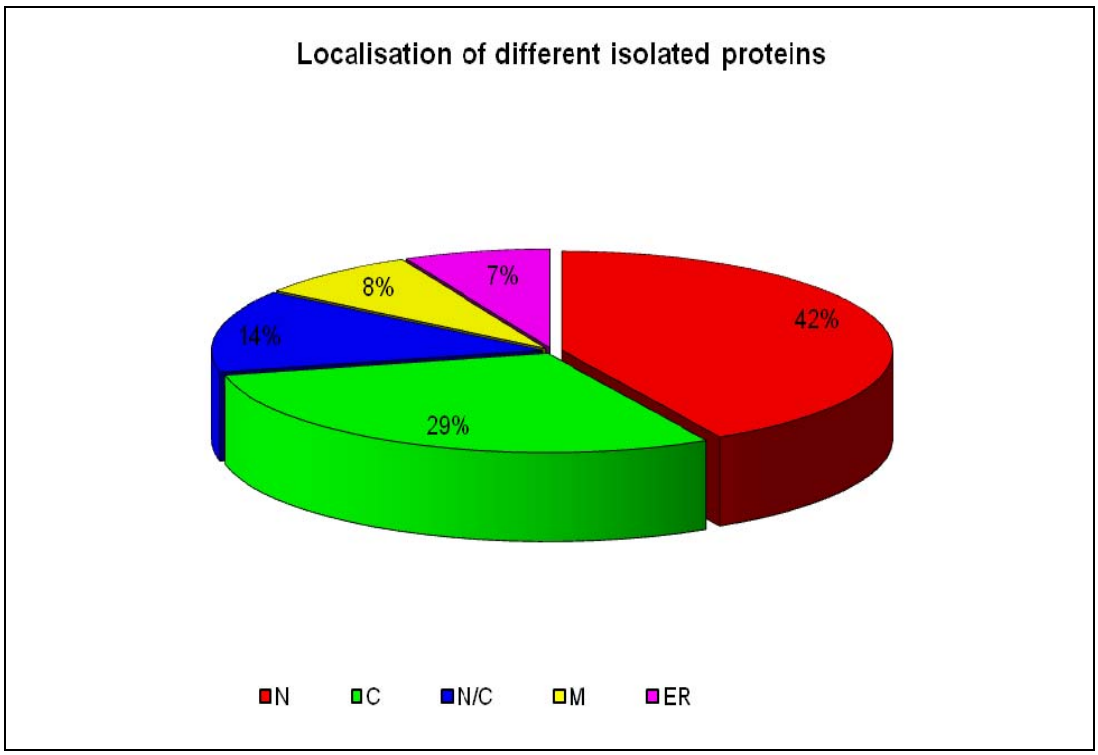

Fig. 4.2: Classification of identified proteins according to their localisation. N: Nuclear proteins, **C**: Cytoplasmic proteins, **N/C**: nuclear/cytoplasmic proteins, **M**: Mitochondria proteins, **ER**: Endoplasmatic reticulum proteins.

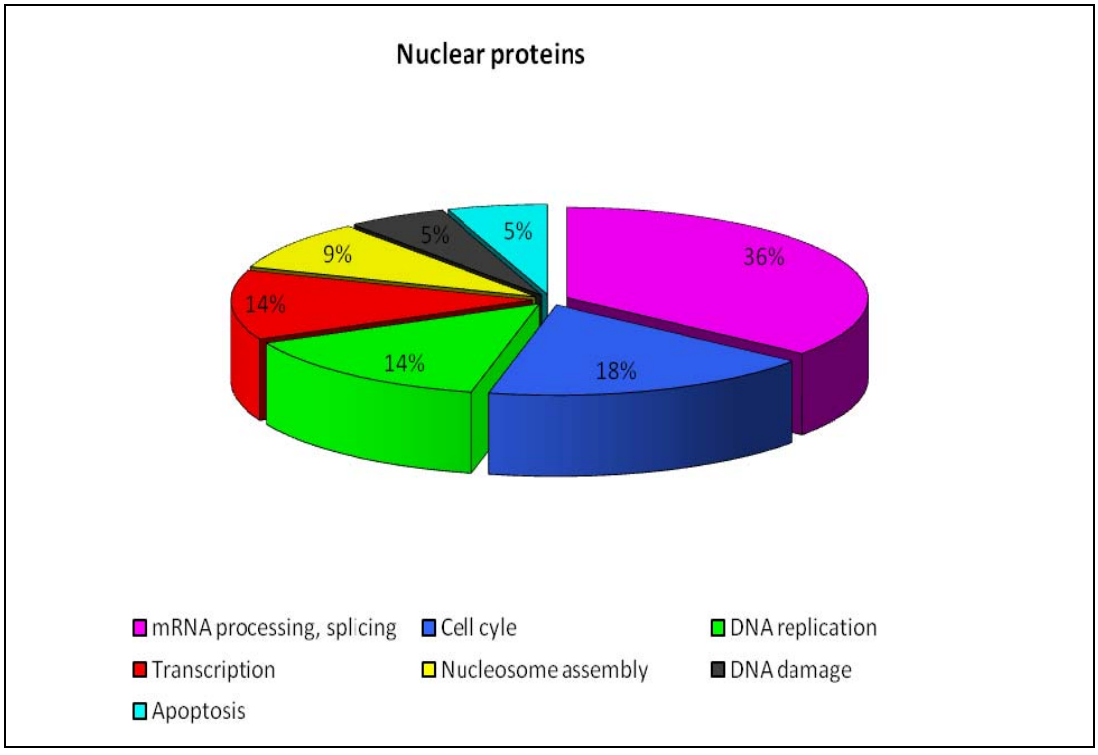

Fig. 4.3: Classification of identified nuclear proteins into seven functional classes.

Table1: Isolated nuclear proteins according to their function

Gene Name	Protein name	
	Short name	recommended name
mRNA processing and splicing		
DIMT1L	18S rRNA dimethylase	Probable dimethyladenosine transferase
NOL5A	Nop56	Nucleolar protein 5A
ASF/SF2	ASF-1	Splicing factor, arginine/serine-rich 1
HNRNPA2B1	hnRNP B1	Heterogeneous nuclear ribonucleoproteins A2/B1
HNRNPC	hnRNP C2	Heterogeneous nuclear ribonucleoproteins C1/C2
HNRNPK	hnRNP K	Heterogeneous nuclear ribonucleoprotein K
HNRNPM	hnRNP M	Heterogeneous nuclear ribonucleoprotein M
YBX1	EFI-A	Nuclease-sensitive element-binding protein 1
PCBP1	hnRNP-E1	Poly(rC)-binding protein 1
PCBP2*	hnRNP-E2	Poly(rC)-binding protein 2
PRPF6	U5-102 kDa protein	Pre-mRNA-processing factor 6
PRPF8	Splicing factor Prp8	Pre-mRNA-processing-splicing factor 8
SNRPB	snRNP-B	Small nuclear ribonucleoprotein-associated proteins B and B'
TARDBP	TDP-43	TAR DNA-binding protein 43
THOC1	Tho1	THO complex subunit 1
THOC2	Tho2	THO complex subunit 2
THOC3	Tho3	THO complex subunit 3
THOC4	Aly/REF1	THO complex subunit 4
UAP56	UAP56	Spliceosome RNA helicase BAT1
Cell cycle		-
CDC2	CDK1	Cyclin-dependent kinase 1
CDC23*	APC8	Cell division cycle protein 23 homolog
CDK9	C-2K	Cyclin-dependent kinase 9
CTBP1*	CtBP1	C-terminal-binding protein 1
DPH1*	DPH2-like 1	Diphthamide biosynthesis protein 1
MAD2L1	HsMAD2	Mitotic spindle assembly checkpoint protein MAD2A
RUVBL1*	TIP49a	RuvB-like 1
RUVBL2*	TIP49b	RuvB-like 2
SEPT2*	NEDD-5	NEDD-5
SMC1A	SMC1alpha	Structural maintenance of chromosomes protein 1A
Replication		
MCM2	MCM2	DNA replication licensing factor MCM7
ING5	p28ING5	Inhibitor of growth protein 5
ORC5L	-----	Origin recognition complex subunit 5
PCNA	Cyclin	Proliferating cell nuclear antigen
RFC2	RFC40	Replication factor C subunit 2
RFC3	RFC38	Replication factor C subunit 3

RFC4	RFC37	Replication factor C subunit 4
RFC5	RFC36	Replication factor C subunit 5
Transcription		
ILF2	-----	Interleukin enhancer-binding factor 2
GTF2H2	BTF2-p44	General transcription factor IIH subunit 2
GTF3C3	TF3C-gamma	Transcription factor IIIC subunit gamma
HDAC2	HD2	Histone deacetylase 2
HNRPDL	hnHNRP-DL	Heterogeneous nuclear ribonucleoprotein D-like
HSF4	HSF 4	Heat shock factor protein 4
POLR3D	RPC53	DNA-directed RNA polymerase III subunit RPC4
DNA damage, DNA repair		
SFN*	Stratifin	14-3-3 protein sigma
PRKDC	DNA-PKcs	DNA-dependent protein kinase catalytic subunit
MSH6	GTMBP	DNA mismatch repair protein Msh6
Apoptosis		
ING4	p29ING4	Inhibitor of growth protein 4
P53*	p53	Tumor suppressor p53
RPS3	-----	40S ribosomal protein S3

* Nuclear/cytoplasmic proteins which are mainly localized in the nucleus

4.2 Identification of THOC7 as a new binding partner of FMIP/THOC5

As shown above FMIP/THOC5 is a member of THO complex. Moreover, THOC1, THOC2, THOC3, Aly/REF1 and ASF/SF2 were detected as a binding partner using TAP purification in mammalian cells. Due to the reason that the interactions between THO complex members is still unknown, it was of interest to analyse potential protein-protein interactions between FMIP/THOC5 and detected members of human THO complex.

To address this question, GST-pulldown assays were performed to examine whether FMIP/THOC5 binds THOC3, SF2/ASF and the two other THO complex subunits (THOC6 and THOC7) found in Drosophila and human, but not in yeast. For this, THOC3, THOC6, THOC7 and SF2 were amplified from cDNAs using PCR and cloned into the pGEX-5X-1 vector. The resultant pGEX-5X-1 constructs contain GST coding sequence upstream of the cloned THOC3, THOC6, THOC7 and SF2/ASF

allowing production of GST fusion proteins. Sequence verified clones were transformed into a protease-deficient *Escherichia. Coli (E. coli)* DH5-α, and clones that showed induction of proteins of the predicted sizes were selected for large scale purification. Lysates were prepared and GST-fusion proteins were immobilised on glutathione sepharose. The purified proteins were separated on an SDS-PAGE gel and their concentrations estimated using Coomassie blue staining (**Fig. 4.4A**).

4.2.1 FMIP/THOC5 binds to THOC7, but not to THOC3, THOC6 and SF2

To perform GST-pulldown assays, eight µg of each purified GST fusion proteins (GST, GST-THOC3, GST-THOC6, GST-THOC7 and GST-SF2) were incubated with lysates from HEK293 cells overexpressing Myc-tagged FMIP. For expression of Myc-tagged FMIP in HEK293 cells, full length FMIP cDNA cloned in pcDNA Myc/His vector was introduced by transient transfection using Polyfect^{TM} (Qiagen). After transfection, the cleared cell lysates were incubated with purified GST fusion proteins at 4°C. GST was used as a negative control. After incubation and subsequent intensive beads washing, bound protein fractions were separated by SDS-PAGE and analyzed by immonoblotting using monoclonal anti-Myc antibody.

As illustrated in figure 5, a new interaction between FMIP/THOC5 and THOC7 *in vitro* was detected using GST- pull down approach. However, no interactions were observed between FMIP/THOC5 and THOC3, THOC6 or SF2/ASF using this method (**Fig. 4.4-B**).

The THOC7-FMIP interaction was confirmed by GST-pulldown using cell extracts of C2C12- or Hela cells overexpressing Myc-tagged FMIP and GST-THOC7 **(Fig. 4.4C&D**). This finding indicates that THOC7 is new binding partner of FMIP.

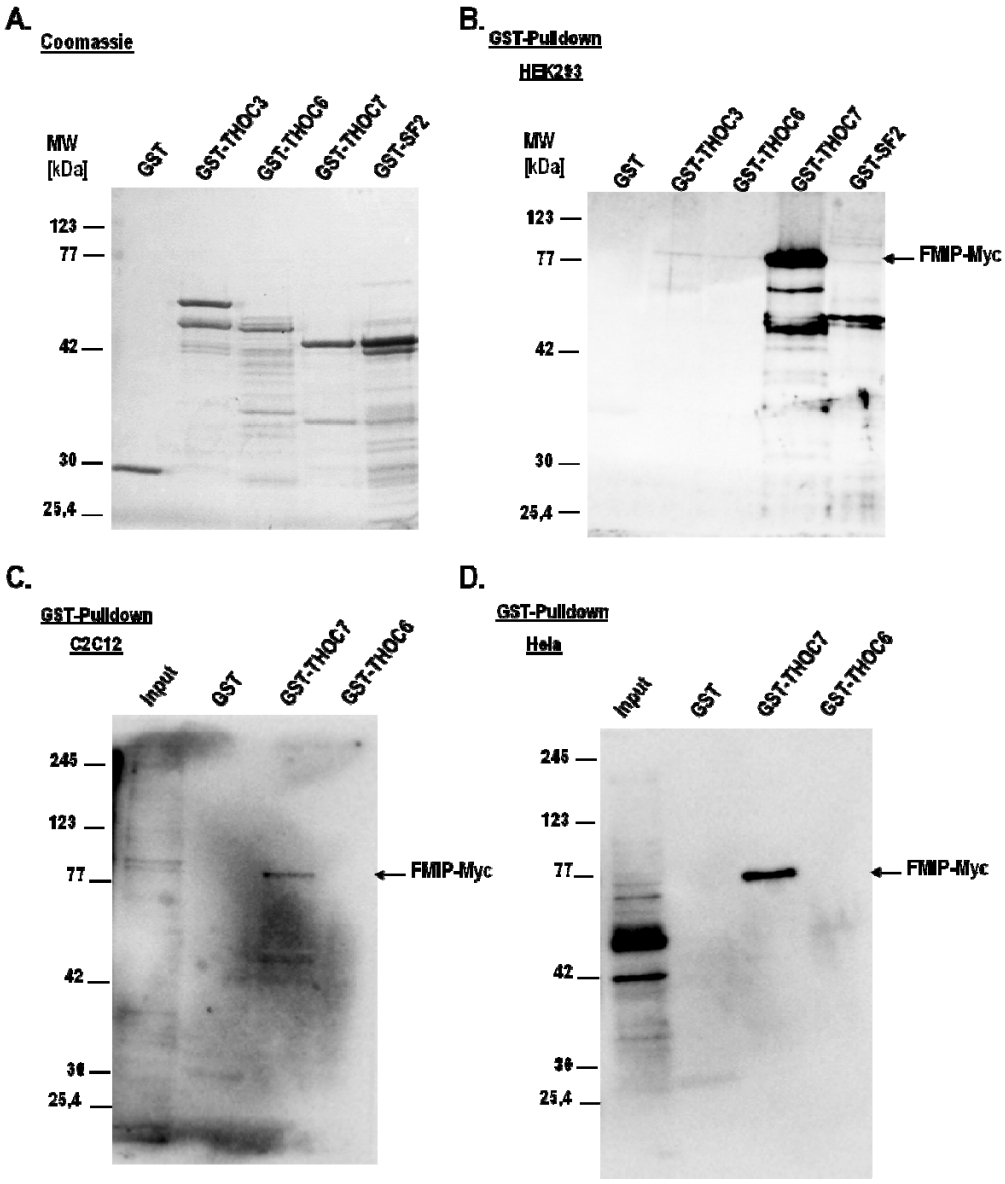

Fig. 4.4: THOC7 interacts *in vitro* with Myc-tagged FMIP/THOC5 in different cell lines. **A**. GST and GST fusion proteins (GST-THOC3 GST-THOC6, GST-THOC7 and GST-SF2) were expressed in *E. Coli* strain DH5-α, extracted and immobilised onto glutathione-sepharose beads. After extensive washing, 8µg of purified proteins were denaturated and separated by 11%SDS-PAGE. Recombinant proteins were then stained with Coomassie brilliant blue. **B**. To perform the GST-pull-down HEK293 were transiently transfected with Myc-tagged FMIP. 8µg of each purified fusion protein GST, GST-THOC3 GST-THOC6, GST-THOC7 and GST-SF2 were incubated with a detergent-solubilized (0.1% Triton X-100) cell lysate containing FMIP-Myc. After extensive washing, bound proteins were separated by SDS-PAGE. 20µl of cell lysate was FMIP-Myc was detected by immunoblotting using a monoclonal mouse anti-Myc antibody. **C**. & **D**. GST pull-down assays were performed as described

in (B) using Cell lysates of Myc-tagged FMIP transfected C2C12 cells, and cell lysates of Myc-tagged transfected Hela cells, respectively.

4.2.2 SR protein ASF/SF2 binds Aly/REF1

To further examine the association between isolated members of THO complex, ASF/SF2 and Aly/REF1; HEK293 cells extracts were incubated with purified GST-THOC3, GST-THOC6, GST-THOC7, GST-SF2 or GST alone. After incubation, bound fractions were separated by SDS-PAGE and subjected to Western blotting analysis using monoclonal antibody against endogenous Aly/REF1.

Surprisingly, a new interaction between SR protein ASF/SF2 and Aly/REF1 was detected *in vitro* (**Fig. 4.5**). This finding shows for the first time a new protein-protein interaction between TREX complex subunits and spliceosome complex members.

By analyzing aliquots of the same GST-pulldown by immunoblotting using monoclonal anti THOC1 antibody, no potential interactions between THOC1 and THOC3, THOC6, THOC7 and/or ASF/SF2 were detected (data not shown).

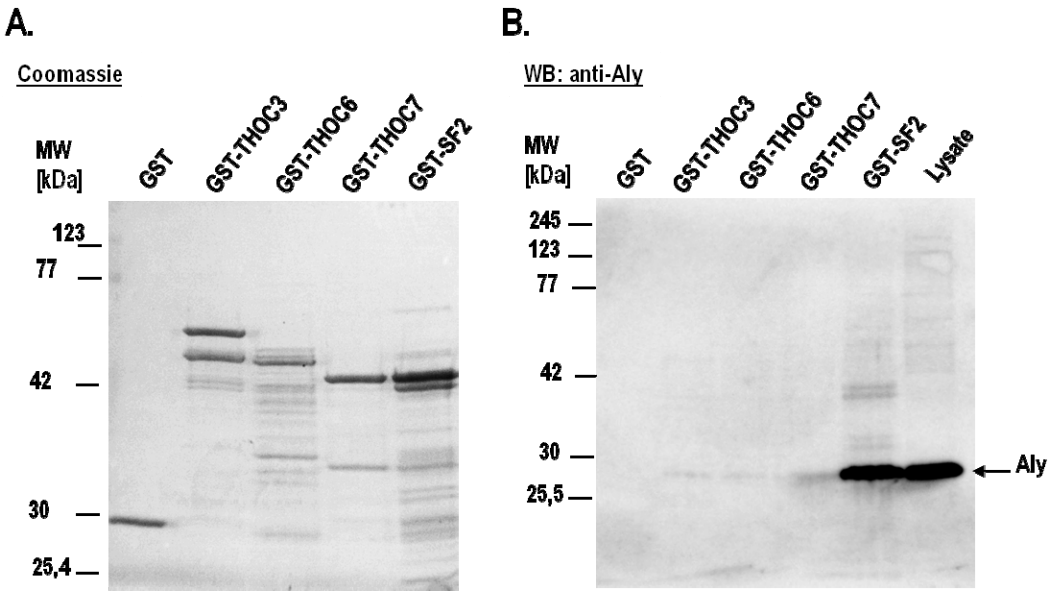

Fig. 4.5: ASF/SF2 associates with Aly/REF1 *in vitro*. **A**. GST and GST fusion proteins (GST-THOC3 GST-THOC6, GST-THOC7 and GST-SF2) were expressed in *E.Coli* strain DH5-α, extracted and

immobilised onto glutathione-sepharose beads. After extensive washing, 8µg of purified proteins were denaturated and separated by SDS-PAGE. Recombinant proteins were stained with Coomassie brilliant blue. **B**. To perform the GST-pull-down, 8µg of purified protein GST, GST-THOC3 GST-THOC6, GST-THOC7 and GST-SF2 were incubated with a detergent-solubilized (0.1% Triton X-100) HEK293 cell lysate. After extensive washing, bound proteins were separated by SDS-PAGE. Endogenous Aly/REF was detected by immunoblotting using a monoclonal mouse anti-Aly antibody (Santa Cruz).

4.3 Characterisation of THOC7-FMIP interaction

4.3.1 THOC7 associates directly with endogenous FMIP/THOC5

Given that ectopic FMIP/THOC5 associates with THOC7 *in vitro*, the interaction of THOC7 with FMIP/THOC5 was examined

To address this question, GST pull-down assays were performed using purified GST, GST-THOC6 and GST-THOC7. GST fusion proteins were incubated with HEK293 cell extract. After incubation, the beads were extensively washed and bound Proteins were separated by SDS-PAGE and subjected to Western blot using a monoclonal antibody against FMIP.

The immunoblot analysis revealed a significant interaction between endogenous FMIP/THOC5 and GST-THOC7. However, no association between FMIP/THOC5 and GST-THOC6 or GST alone was observed (**Fig.4.6-A**). This result confirmed the detected interaction between ectopic FMIP/THOC5 and THOC7 *in vitro*.

Because the interaction shown above in these GST pull-down assays can be mediated through other proteins in the HEK293 cells lysates, it was of interest to determine, whether THOC7 or THOC6 directly interact with FMIP/THOC5. Therefore, we performed *in vitro* pulldown analysis using bacterially expressed GST-THOC7, GST-THOC6 or GST alone and *in vitro* translated [^{35}S]-Methionine labelled FMIP (wild type). *In vitro* transcription/translation reaction was performed with the plasmid encoding full-length FMIP/THOC5. After translation, equal

amounts of recombinant GST or GST-THOC7 fusion protein were incubated with translated proteins. After incubation, beads were washed extensively. Bound proteins were separated by SDS-PAGE. The radio-labelled proteins were visualized by autoradiography.

As illustrated in figure 7, the [^{35}S]-Methionine labelled full-length FMIP (wt) was retained on beads that contains GST-THOC7 but not on beads with GST-THOC6 or GST alone (**Fig. 4.6-B**). These data further confirms a physical and direct association between FMIP and THOC7.

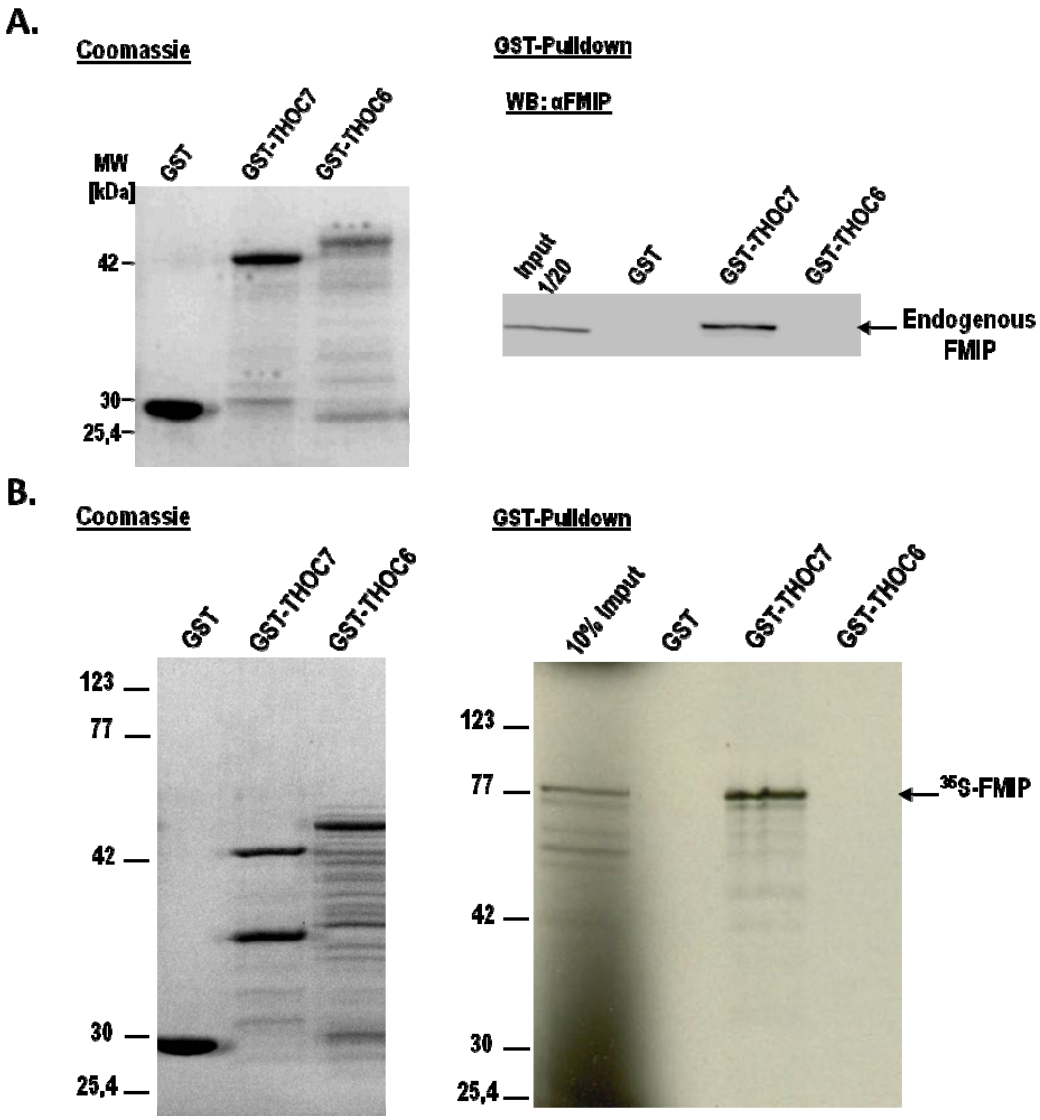

Fig. 4.6: THOC7 binds directly to FMIP/THOC5. A) GST, GST-THOC6 and GST-THOC7 were immobilized onto glutathione-Sepharose beads and incubated with a detergent-solubilized (0.1% Triton X-100) HEK293 cell lysate. After extensive washing, bound proteins were separated by 11% SDS-PAGE and subjected to western blotting using a monoclonal antibody raised against endogenous FMIP. 1/20 eluted HEK293 cell lysate was loaded as a control. **B)** The recombinant protein FMIP was labelled with [^{35}S]-Methionine using the *in vitro* transcription/translation system (Promega). The labelled protein was incubated with 8µg of purified protein GST, GST-THOC6 and GST-THOC7 for 2h. All unbound labelled proteins were removed by subsequent washing. Bound proteins were separated by 11% SDS-PAGE. The gel was dried, and radio-labelled proteins were visualized by autoradiography. (10% input corresponds to 10% of total labelled [^{35}S]-Methionine protein used in pull-down) (El Bounkari et al., 2008).

4.3.2 The binding domain of THOC7 to FMIP is located within the residues 50-137 of THOC7

To localize the site within THOC7 that contribute to FMIP/THOC5 interaction, GST-fusion proteins carrying splice variant of THOC7 (1-137), deletion mutants GST-THOC7 (1-66) and GST-THOC7 (50-204) were generated (**Fig. 4.7-A**).

The generated GST-fusions protein and the GST alone (control) were purified, prebound on glutathione-Sepharose beads and incubated with extract cell lysates of Myc-tagged FMIP transfected HEK293 cells. After incubation, bound fractions were subjected to immunoblot using anti-Myc antibody.

As shown in figure 8-B, THOC7 truncation construct (residues 1-66) failed to interact with FMIP/THOC5 in the GST Pull down assay, whereas splice variant GST-THOC7 (1-137) and deletion mutant GST-THOC7 (50-204) precipitated Myc-tagged FMIP (**Fig. 4.7-B**). These mapping studies indicate that the residue 50-137 within THOC7 amino acid sequence is required for THOC7-FMIP direct interaction.

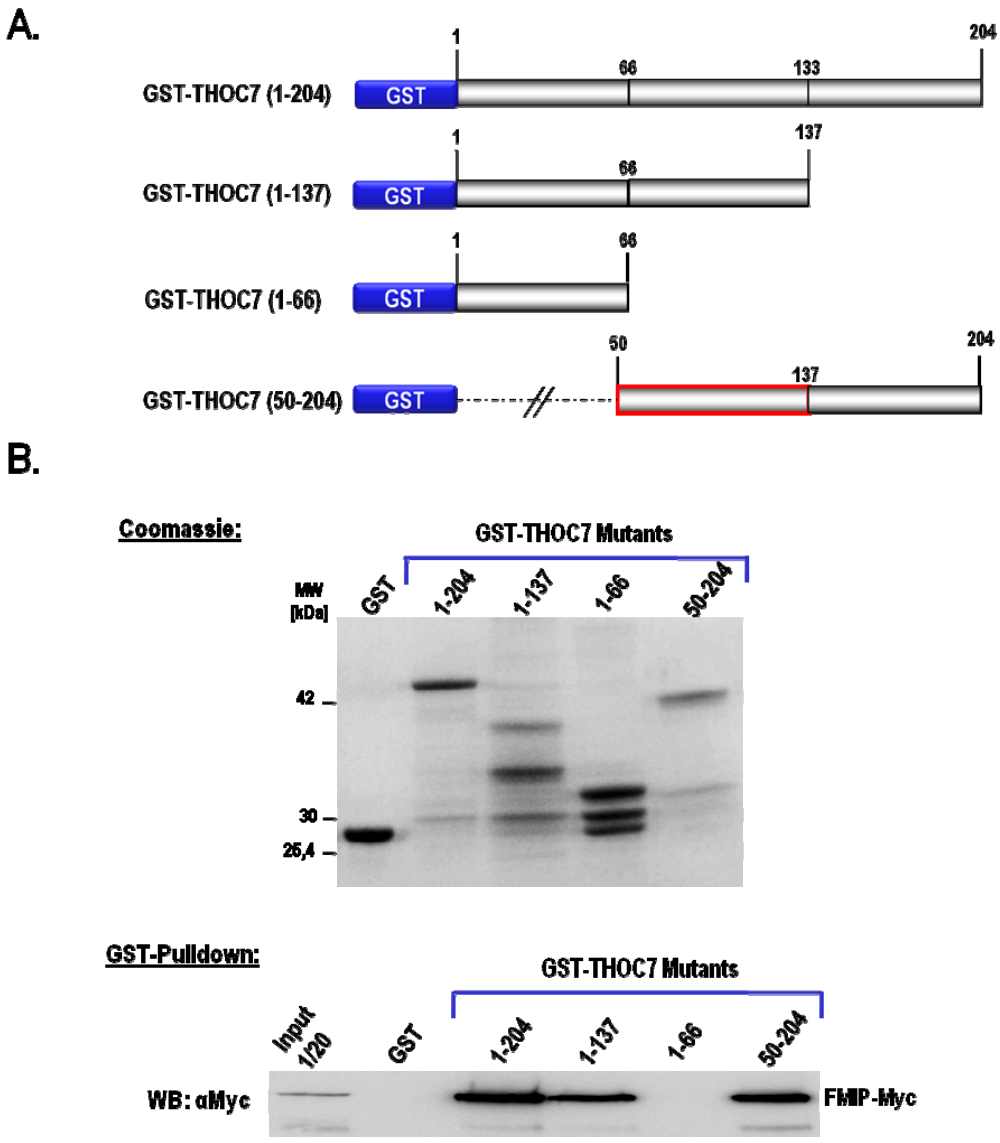

Fig. 4.7: The amino acide sequence (50-137) within THOC7 is responsible for the association to FMIP/THOC5. A) Scheme of different deletion mutants of THOC7, which were used to generate the pGEX5x-1-constructs, in approximate relation to the full length protein. The numbers indicate the beginning and ending amino acids of the residues. The motif in red corresponds to the binding domain responsible for the association with FMIP. **B)** GST and generated GST-THOC7 mutants were expressed, isolated from E.Coli and immobilized onto glutathione-Sepharose beads. Aliquots of recombinant proteins were stained with coomassie brilliant blue. 8µg of purified GST fusion proteins were incubated with the HEK293 cell lysate containing Myc-tagged FMIP. After extensive washing, bound proteins were separated by SDS-PAGE and subjected to Western blot using monoclonal anti-Myc antibody. Aliquot of the cell lysate of Myc-Tagged FMIP transfected HEK293 was diluted (1/20) and used as an input for the Western blotting analysis (El Bounkari et al., 2008).

4.3.3 N-terminal domain (residues 1-199) of FMIP/THOC5 is required for THOC7 association

FMIP binds to THOC7 directly, and the binding domain to FMIP is located within the centre portion of THOC7 sequence (50-137). The next question was which domain within FMIP/THOC5 is responsible for this interaction.

To answer this question, six C-terminal Myc-tagged deletion mutants Myc-tagged FMIP (1-559), FMIP (1-499), FMIP (1-409), FMIP (1-289), FMIP (1-199) and FMIP (1-100) were generated (**Figure 4.8-A**). Protein–protein interaction between THOC7 and FMIP/THOC5 or its deletion mutants were examined by GST-Pulldown assays.

Myc tagged wild-type (wt) FMIP or Myc-tagged FMIP deletion mutants were expressed in HEK293 cells, and the cell extracts were incubated with GST or GST-THOC7 for potential interactions. The binding proteins were analysed by immunoblot, using anti-Myc specific antibody. As expected, none of these proteins interacted with GST alone. When incubated with GST-THOC7, only FMIP (1-100) failed to bind to THOC7. In contrast FMIP(1-199) and all other proteins still binds to THOC7 (**Figure 4.8-B**). These results clearly showed that the interaction between FMIP and THOC7 is mediated by the N-terminal residues (1-199) of FMIP. Interestingly, this same region of FMIP was identified previously as the binding site for Fms tyrosine Kinase (Tamura et al. 1999).

GST-Pulldown assays using bacterially expressed GST-THOC7 or GST alone and *in vitro* translated [^{35}S]-Methionine labelled FMIP (wt) or [^{35}S]-Methionine labelled FMIP (1-289) show similar results obtained above, confirming a direct binding of THOC7 to FMIP/THOC5 and supporting the conclusion that the 1-199 amino acid sequence of FMIP/THOC5 is required for FMIP-THOC7 association (**Figure 4.8-C**).

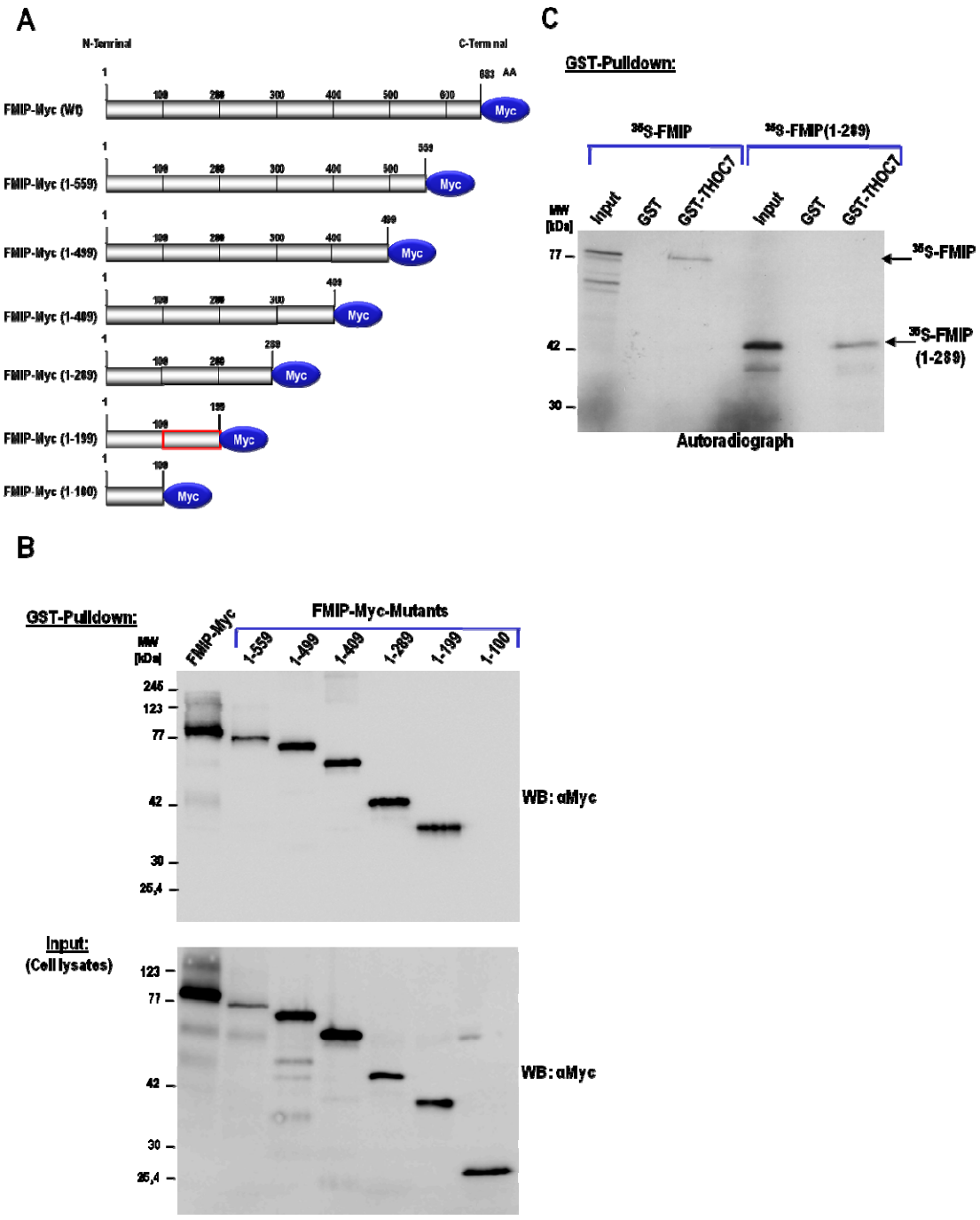

Fig. 4.8: The amino acuide sequence (1-199) within FMIP is responsible for the association to THOC7. A) Scheme of different deletion mutants of FMIP, which were used to generate the pcDNAMyc/His-constructs, in approximate relation to the full length protein. The numbers indicate the beginning and ending amino acids of the residues. The motif in red corresponds to the binding domain responsible for the association with THOC7. **B)** HEK293 cells were transiently transfected with generated Myc-tagged FMIP mutants (see A). 8 µg of purified GST-THOC7 was incubated with different HEK293 cell lysates containing Myc-tagged FMIP-mutants. After extensive washing, bound

proteins were separated by SDS-PAGE and subjected to Western blot using monoclonal mouse anti-Myc antibody. Aliquots of corresponding cell lysates used for the pull-down were analysd by Western blotting using anti-Myc antibody. **C)** The recombinant protein FMIP and deletion mutant FMIP (1-289) were labelled with [^{35}S]-Methionine using the *in vitro* transcription/translation system (Promega). Labelled proteins were incubated with 8µg of purified protein GST or GST-THOC7 for 2h. All unbound labelled proteins were removed by subsequent washing. Bound proteins were separated by 11% SDS-PAGE. Radiolabelled proteins were visualized by autoradiography. (10% input corresponds to 10% of total labelled [^{35}S]-Methionine protein used in pull-down) (EL Bounkari et al., 2008).

4.3.4 The association between THOC5 and THOC7 leads to nuclear translocation of THOC7

THOC7 was originally identified as a binding partner of NIF3L1, NIF3L1 BP1, and the expression of green fluorescent protein (GFP) fusion protein NIF3L1 BP1-GFP in LNCaP and NIH3T3 cells revealed a subcellular localisation of NIF3L1 BP1/THOC7 in the cytoplasma as well as in the nucleus (Tascou et al., 2003). Furthermore previous studies in our group have shown that subcellular localisation of GFP-FMIP/THOC5 in mesenschymal progenitor cells C2C12 was mainly detected in the nucleus (Mancini et al., 2004).

To further study the effect of the direct interaction between FMIP/THOC5 and THOC7/ NIF3L1 BP1 on their subcellular distribution, changes in their subcellular localisation upon coexpression were examined.

In the first set of experiments, expression vectors GFP-THOC7 and GFP-THOC6 were constructed by fusing GFP to the N-terminus of full length of THOC7 and/or THOC6 in pEGFP vector. C2C12 cells were transiently transfected with GFP-FMIP/THOC5, GFP-THOC7 and GFP-THOC6. The green fluorescence was detected by confocal microscopy.

In agreement with previous data (Tascou et al., 2003), GFP-THOC7 is detected in the cytoplasm as well as in the nucleus, while GFP-FMIP/THOC5 is expressed in

nucleus exclusively. Furthermore, GFP-THOC6 was detected as spots in cytoplasm (**Figure 4.9-A**).

We next coexpressed GFP-THOC7, GFP-THOC6 or GFP alone with Myc-tagged FMIP/THOC5 in C2C12 cells. After transfection, the cells were briefly fixed, permeabilized and stained with anti-Myc antibody followed by TRITC conjugated anti-mouse IgG.

As illustrated in **Figure 4.9-B**, the subcellular localisation of THOC7 is exclusively dtetected in the nucleus upon co-expression with Myc-tagged FMIP/THOC5. By contrast, the co-expression of GFP-THOC6 and Myc-tagged FMIP/THOC5 shows no effect on the THOC6 localisation, suggesting that the interaction between THOC7 and FMIP/THOC5 may contribute to the import of THOC7 into the nucleus.

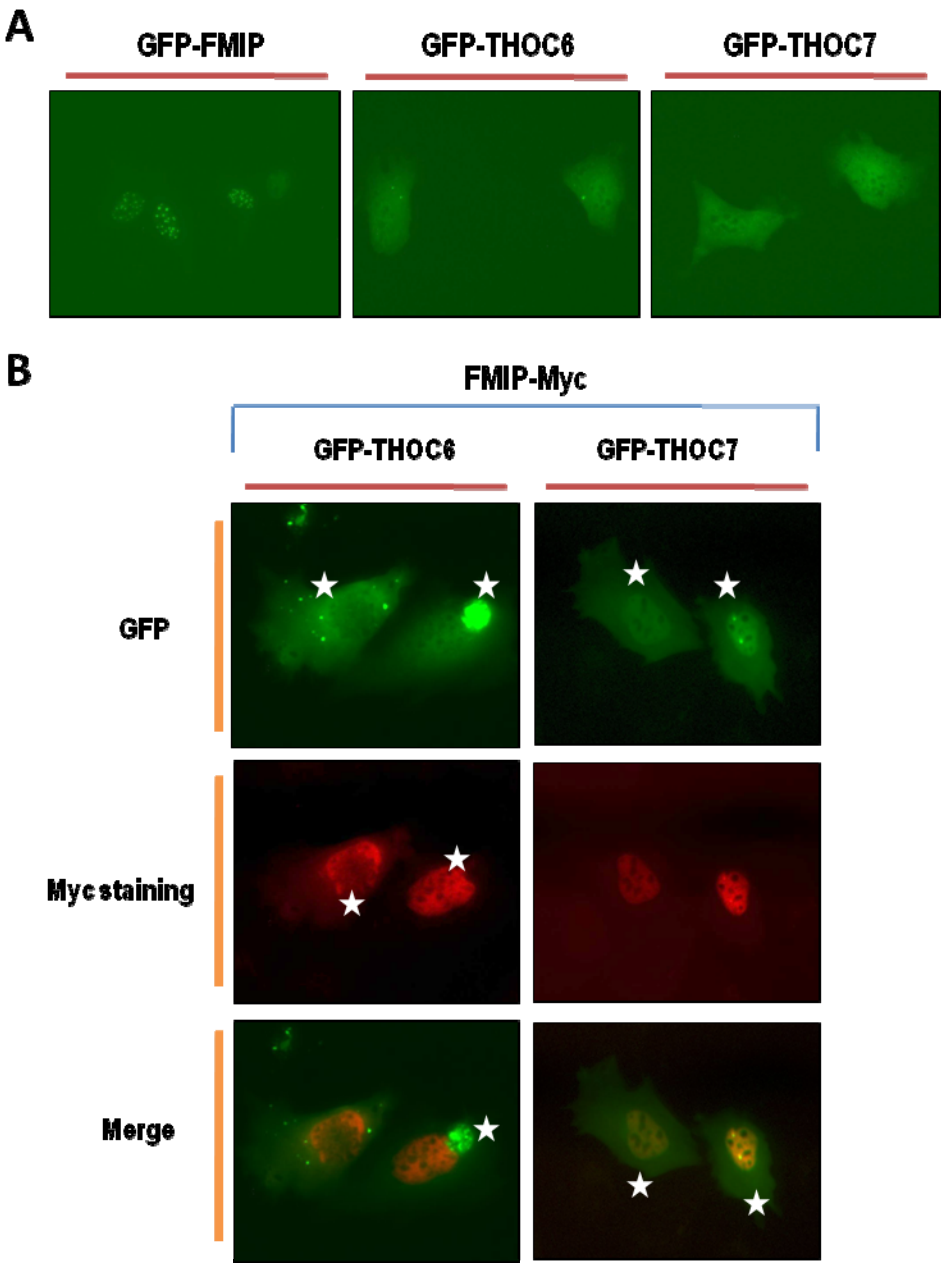

Fig. 4.9: FMIP/THOC5 affects the subcellular distribution of THOC7, but not of THOC6. **A)** C2C12 cells were transfected with pEGFP plasmids containing FMIP, THOC6 or THOC7 cDNAs. The subcelllular localisations of GFP-tagged proteins were directly visualized after fixation with 3.7% formaldehyde (PFA) using Fluorescence microscopy analysis. **B)** GFP-THOC6 and GFP-THOC7 plasmids were separately co-transfected with Myc-tagged FMIP in C2C12. After 28h, cells were fixed briefly with Formaldehyd (PFA), treated with 0.1 %Triton X-100 and were stained with anti-Myc antibody, followed by TRITC conjugated anti-mouse IgG (El Bounkari et al., 2008).

4.3.5 The binding domain of THOC7 (50-137) is required for the THOC5 dependent nuclear import

As mentioned previously, recent reports have demonstrated that FMIP/THOC5 is ubiquitous nuclear/cytoplasm shuttling protein and its translocation into the nucleus is abrogated upon the phosphorylation of both serines 5 and 6 by PKC (Mancini et al., 2004). As Shown above, THOC7 binds directly to FMIP/THOC5 and the cotransfection of both proteins in C2C12 cells leads to translocaion of THOC7 into the nucleus.

To examine whether the THOC7-FMIP/THOC5 interaction is required for the import of THOC7 into the nucleus, GFP-Fusion proteins carrying second splice variant of THOC7 (1-137), which contains the binding domain to FMIP/THOC5, and deletion mutant GFP-THOC7 (1-66), which failed to bind FMIP/THOC5 were generated. For subcellular localisation, generated constructs were transiently transfected separately in C2C12 cells. As shown in **Figure 4.10-A**, GFP-THOC7 (1-137) and GFP-THOC7 (1-66) show the same localisation as GFP-THOC7 (wt). All of them are detected in the cytpoplasm as well in the nucleus.

Next, GFP-THOC7(204), GFP-THOC7(1-137) or GFP-THOC7(1-66) were transiently co-expressed with Myc-tagged FMIP/THOC5 in C2C12 Cells. The cellular localisation of FMIP/THOC5 was visualised by immunofluorescence using anti-Myc antibody. Interestingly, both GFP-THOC7(204) and GFP-THOC7(1-137) were detected mainly in the nucleus with FMIP/THOC5. In contrast, the localisation of GFP-THOC7(1-66) did not change. Taken together, these data suggest that the binding domain of FMIP to THOC7 is responsible for the import of THOC7 into the nucleus, and thereby direct association between FMIP/THOC5 and THOC7 leads to its translocation/import into nucleus (**Fig. 4.10-B**).

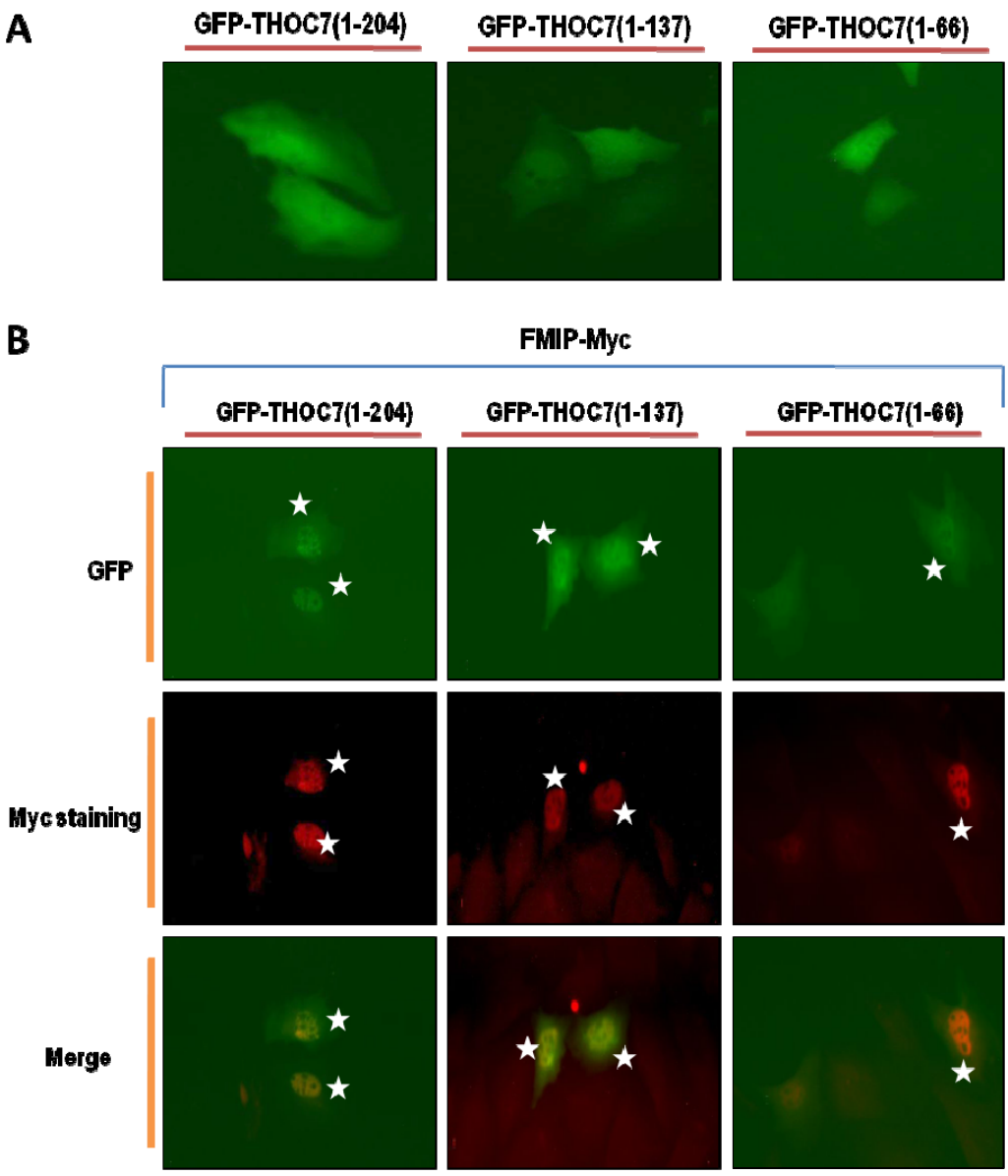

Fig. 4.10: the direct interaction between FMIP and THOC7 is required for THOC7 nuclear import in C2C12. **A)** C2C12 cells were transfected with pEGFP plasmids containing full lenght THOC7 (1-204) or THOC7 truncated mutants (THOC7(1-137), THOC7(1-66)) cDNAs. The subcelllular localisations of GFP-tagged proteins were directly visualized after fixation with 3.7% formaldehyde (PFA) using Fluorescence microscopy analysis. **B)** GFP-THOC7 (1-204), GFP-THOC7 (1-137) and GFP-THOC7 (1-66) plasmids were separately cotransfected with Myc-tagged FMIP in C2C12. After 28h, cells were fixed briefly with 3.7% Formaldehyd, treated with 0.1 %Triton X-100 and were stained with anti-Myc antibody, followed by TRITC conjugated anti-mouse IgG (EL Bounkari et al.,2008).

4.4 Role of THOC7/FMIP in cell growth, differentiation and apoptosis

4.4.1 Ectopic expression of THOC7 influences cell growth and differentiation of C2C12 cells

Previous studies in our group have shown that ectopic overexpression of FMIP/ THOC5 in mesenshymal progenitor C2C12 cells, which have the capacity to differentiate into myoblasts, osteoblasts, chondroblasts or adipocytes (Yamaguchi, 1995), enhanced cell growth and impairs the adipocyte differentiation induced by treatment with insulin, dextramethasone and 3-isobutyl-1-methyl-xanthine (IBMX). Furthermore, knockdown of endogenous FMIP by siRNA induced lipid droplets accumulation and adipocyte differentiation (Mancini et al., 2007).

The previous experiments showed that FMIP/THOC5 binds directly to THOC7 and leads to its import into the nucleus in C2C12 cells. To analyze the effect of THOC7 on cell growth and differentiation, THOC7 overexpressing C2C12 cell lines were established. C2C12 cells were trannsfected with plasmids that encode Myc/His tagged FMIP, THOC7 cDNA and/or empty vector alone. Transfected cells were selected in the presence of Neomycin (G418). Two different clones of each cell line expressing FMIP-Myc (clone#3 and clone#4) or THOC7-Myc (clone#3 and clone#5) were used for subsequent analysis. The level of the recombinant FMIP and THOC7 proteins was immunoprecipitated by anti-Myc antibody, and then monitored by Western Blotting using the same antibody. As shown in **Figure 4.11-A**, all selected clones express exclusively Myc-tagged FMIP and Myc-tagged THOC7.

Surprisingly, we obtained only 6 colonies from cells stably expressing THOC7, while more than 100 colonies were obtained from stable cell lines expressing FMIP under the same condition, we suggest that THOC7 may influences cell growth.
To test this hypothesis, aliquots of the same clones were isolated and cultured in DMEM medium containing 10% FCS for one, two or four days. The cells were stained with Trypan Blue, and living cells were counted.

As illustrated in **Figure.4.11-B** FMIP expressing cell lines grow 2-times faster than cells expressing THOC7. This indicates that overexpression of THOC7 slows down cell growth.

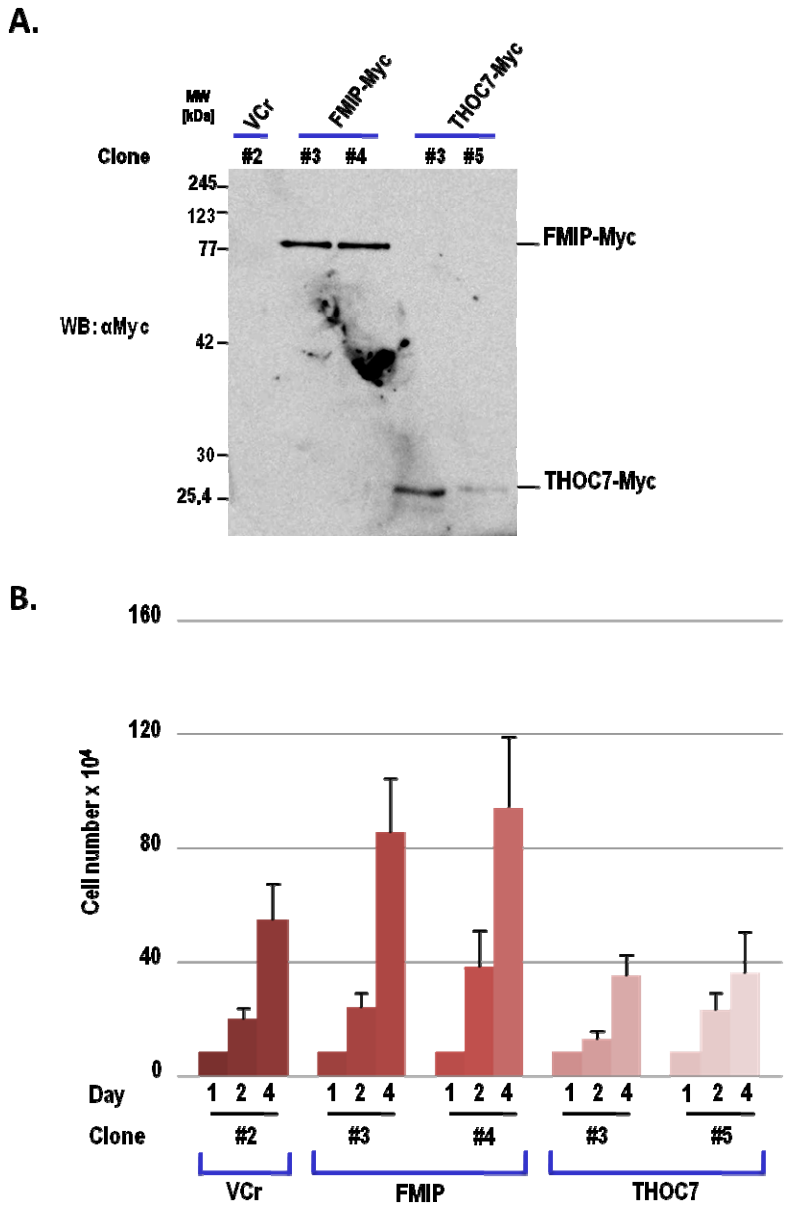

Fig. 4.11: Ectopic expression of THOC7 in C2C12 cells down regulates Cell-growth. Myc/His-taged FMIP and/or THOC7 cDNA were transfected into C2C12 cells. After Neomycin (G418) selection, colonies were isolated. **A)** Cell lysates of stable clones expressing FMIP (#3, #4), THOC7 (#3 and #5) or empty vector were prepared for western blot analysis with Myc specific antibody. **B)** Aliquots of same clones were grown in medium containing 10% FCS for 1, 2 and 4 days. Cells were stained with Trypan blue (sigma) and living cells were counted under microscope.

Given that ectopic overexpression of FMIP/THOC5 in C2C12 cells enhances cell growth and blocked adipocyte differentiation (Mancini et al., 2007), it was of interest to examine the effect of THOC7 overexpression on the C2C12 adipocyte diffrerentiation. For this, C2C12 cells expressing Myc tagged THOC7 (clon#3) or empty vector (clon#2) were incubated for 8 days in normal medium. The cells were fixed in paraformaldehyd and stained by Oil Red.

Interestingly, cells that stably overexpress THOC7 show accumulation of Oil Red-positive lipid droplets into an enlarged cytoplasm, while the empty vector-transfected cell lines did not show any phenotype (**Fig. 4.12**). Similar results were observed in our previous studies, showing that depletion of FMIP by siRNA induced lipid droplets accumulation and adipocyte differentiation (Mancini et al., 2007). This result suggests strongly that overexpression of THOC7 in C2C12 enhances adipocyte differentiation, and thereby may be a novel counteracting of FMIP/THOC5 by the regulation of cell growth and adipocyte differentiation.

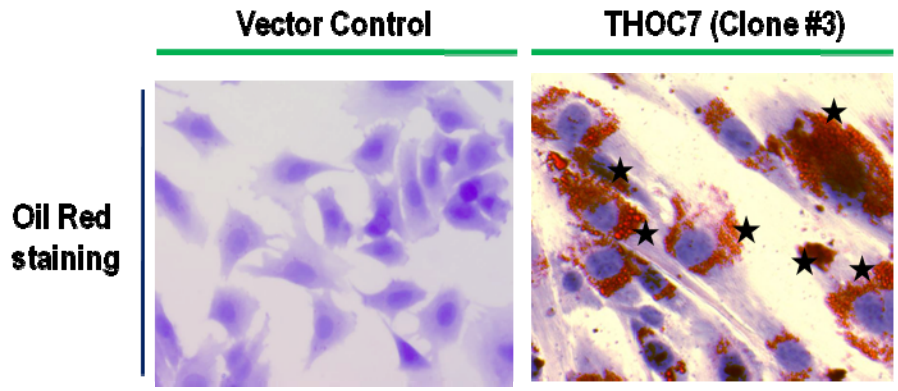

Fig. 4.12: Overexpression of THOC7 in C2C12 cells enhances adipocyte differentiation. Myc/His-taged THOC7 cDNA was transfected into C2C12 cells. After Gentamycine (G418) selection, colonies were isolated. Stable cell lines from clone #3 obtained in Figure.10 were incubated for 8 days in normal medium. After incubation, cells were fixed with 3.7% paraformaldehyde (PFA) and stained by Oil Red (ORO).

4.4.2 Functional analyses of THOC7 in Xenopus Oocytes

The direct association between THOC7 and FMIP/THOC5 leads to translocation of THOC7 into the nucleus, and the ectopic expression of both proteins shows opposite effects on the cell growth and cell differentiation. Hence, it is conceivable that THOC7 may antagonize FMIP/THOC5, and thereby may act as a negative regulator in mRNA-export. To test this hypothesis, microinjection experiments in *Xenopus oocytes* were performed. The enormous size of the *Xenopus* oocyte has made it one of the systems of choice for nuclear transport studies. Microinjection of radiolabeled or tagged transport cargo into either nuclear or cytoplasmic compartment, followed by manual oocyte dissection, allows rapid quantitation of nuclear import and export rates.

4.4.2.1 FMIP/THOC5 and THOC7 are highly conserved between mammals and Xenopus laevis

The GenBank/EMBL and the Uniprot/Swissprot databases reveal, that the *Xenopus laevis* THOC5 exist in two isoforms, THOC5**A**_XENLA (Accession No: NM_001093029.1; MW 87.14 kDa; 679AA residues) and THOC5**B**_XENLA (Accession No: NM_001087199.1; MW 77.76 KDa; 678AA residues). Both *Xenopus Laevis* THOC5 isoforms show 93% homology at the amino acid level. Interestingly, the amino acide sequence alignment of the human and the two isoforms of *Xenopus Laevis* THOC5 (**A**,**B**) revealed 78% and 80% identity at the amino acid level, respectively (**Fig. 4.13-A**).

It is previously reported that mouse and human THOC7/NIF3L1 BP1 show 90 and 97% homology at the nucleotide level and an amino acid level, respectively (Tascou et al., 2003).

Remarkably, the comparison between human and *Xenopus laevis* THOC7 (Accession No: NM_001087182 MW 23.37 kDa; 201AA residues) reveals 87%

homology at the amino acid level (**Fig. 4.13-B**). This demonstrates strongly, that both FMIP and THOC7 are conserved in *Xenopus laevis*.

A)

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        10         20         30         40         50         60
MSSESSKKRK PKVIRSDGAP AEGKRNRSDT EQEGKYYSEE AEVDLRDPGR DYELYKYTCQ
MASDSLKKRK PKVNRN---- EDVKRGRHE- DQEGRYYSEE AEVDVRDPKE DYQLYKDTCL
MSSDSLKKRK PKVNRS---- EDGKRGRHD- EQEGRYYSEE AEVDVRDSRE DYQLYKDTCV

        70         80         90        100        110        120
ELQRLMAEIQ DLKSRGGKDV AIEIEERRIQ SCVHFMTLKK LNRLAHIRLK KGRDQTHEAK
DLQRLMSEIQ ELKSKGSRDS ALEIEEKKVQ SCVHFMTLKK LNRLAHIRLK KARDQTHEAK
ALQRLMSEIQ DLKSKGSKDS AMEIEEKKIQ SCVHFMTLKK LNRLANIRLK KARDQTHEAK

       130        140        150        160        170        180
QKVDAYHLQL QNLLYEVMHL QKEITKCLEF KSKHEEIDLV SLEEFYKEAP PDISKAEVTM
QKVDAYHLQL QNLLYEVMHL QKEITKCLEF KSKHEEIELV SVEEFYSKAP VAISKPEITS
QKVDAYNLQL QNLLYEVMHL QKEITKCLEF KSKHEEIELV SVEEFYSDAP AAISKPEITS

       190        200        210        220        230        240
GDPHQQTLAR LDWELEQRKR LAEKYRECLS NKEKILKEIE VKKEYLSSLQ PRLNSIMQAS
TDPHQQTLSR LDWELEQRKR LAEKYKECLA SKEKILKEIE IKKEYLNSLQ PQLNSIMQAS
TDPHQQTLSR LDWELEQRKR LAEKYKECLA SKEKILKEIE IKKEYLNTLQ PQLNSIMQAS

       250        260        270        280        290        300
LPVQEYLFMP FDQAHKQYET ARHLPPPLYV LFVQATAYGQ ACDKTLSVAI EGSVDEAKAL
LPVQEYLSMP FDCMHKQYET ARHLPAPLYV LFVQASAYSQ ACDRKLVVTI EGNVEEARAL
LPVQEYLSMP FDCMHKQYET ARHLPAPLYV LFVQASAYSQ ACDQKLVVAI EGNVEEARAL

       310        320        330        340        350        360
FKPPEDSQDD ESDSDAEEEQ TTKRRRPTLG VQLDDKRKEM LKRHPLSVML DLKCKDDSVL
FKPPEDSQDD ESDSDAEEEQ TTKRRRPTLG VQLDDKRKEM LKRHPLCVTI TLKCKEGSTL
FKPPEDSQDD ESDSDAEEEQ TTKRRRPTLG VQLDDKRKEM LKRHPLCVTL TLMCKEGSTL

       370        380        390        400        410        420
HLTFYYLMNL NIMTVKAKVT TAMELITPIS AGDLLSPDSV LSCLYPGDHG KKTPNPANQY
TLTFYFLMNL NILTVKVKIL PAFELSTAIS AGDLLNPDLM LSCLYQGDDG KTTPNPANQY
TLTFYFLMNL NILTVKVKIQ PAFELSTAIS AGDLLNPDLI LGCLYQGDDG KTTPNPANKY

       430        440        450        460        470        480
QFDKVGILTL SDYVLELGHP YLWVQKLGGL HFPKEQPQQT VIADHSLSAS HMETTMKLLK
QFDKIGILSL SDYISELGHP YIWVQAMGGL HFPTDQPQPA VVADNALSAN HMEKTIKLLR
QFDKIGILSL SDYISELGHP YVWAQTMGGL HFPTDQPQPE FVADNALSAS HMEKTIKLLK

       490        500        510        520        530        540
TRVQSRLALH KQFASLEHGI VPVTSDCQYL FPAKVVSRLV KWVTIAHEDY MELHFTKDIV
TRLLSRLSLH RQFASLEHGS IPVSLECQSF FPAKVISRLT KWNVITYEDY LALPYTKDVI
TRLLSRLSLH RQFASLEHGS IPVSLECQSL FPAKVISRLT KWNVIAYEDY LALPYTKDVV

       550        560        570        580        590        600
DAGLAGDTNL YYMALIERGT AKLQAAVVLN PGYSSIPPIF QLCLNWKGEK TNSNDDNIRA
ECGLAKETDQ YFNLLIERGT AKLNGVVVLN PGYCAVPPVF SLCLNWKGER LSSNDDNIRV
ECGLAKETDQ YFCLLIERGT AKLNGVVVLN PDYSSVPPVF SLCLNWKGER SSSNDDNIGV

       610        620        630        640        650        660
MEGEVNVCYK ELCGPWPSHQ LLTNQLQRLC VLLDVYLETE SHDDSVEGPK EFPQEKMCLR
MESEVNVYYK ELCGPPPGFQ LLTNQIQRLC MLLDVYLETE RHDNSVEGPH EFPPEKICLR
MESEVNVYYK ELCGPPPGFQ LLTNQIQRLC MLLDVYLETE RHDNSVEGPH EFPPEKICLR
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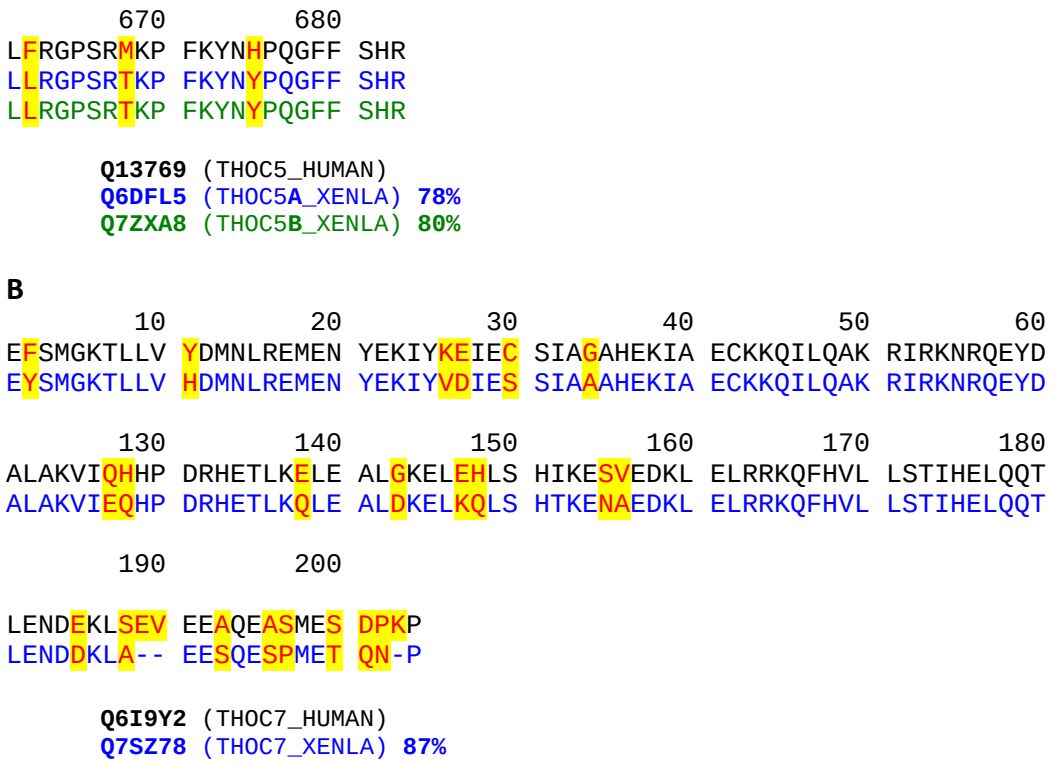

Fig. 4.13: FMIP/THOC5 and THOC7 are conserved in *Xenopus laevis*. A) Amino acide sequence comparison between human and *Xenopus* FMIP isoforms**. B)** Amino acide sequence comparison between human and *Xenopus* THOC7.

4.4.2.2 Xenopus laevis THOC5 /FMIP is mainly localized in the nucleus

Because of the homology of 76% of amino acid sequences between human and *Xenopus laevis* FMIP/THOC5, the detection of endogenous *Xenopus laevis* THOC5/FMIP with monoclonal antibody against human FMIP (hFMIP) was examined. For this, ten *Xenopus laevis* oocytes in stage V/VI were homogenized in 1x NET2 lysis buffer containing 0.1% NP-40. After removal of the yolk by centrifugation, the cleared extract was boiled in 2x SDS loading buffer. Proteins of this extract were separated by SDS-PAGE and subjected to immunoblotting using a specific antibody against human FMIP. Aliquot of HEK293 cell lysates was used as loading control.

As shown in **Figure 4.14-A** (line 1) endogenous levels of FMIP/THOC5 were readily detected in HEK293 cells by immunoblott analysis using a monoclonal anti-hFMIP antibody. Surprisingly, endogenous *Xenopus Laevis* THOC5 was detected with specific monoclonal antibody against hFMIP (Fig.3.14-A, line2).

To analyze the intracellular localisation of endogenous *X. laevis* THOC5, the *Xenopus* oocytes were manual dissected into nuclear and cytoplasmic fractions. The nuclear proteins were solubilised directly in 2x SDS loading buffer and the cytoplasms were homogenized in 1X NET2 lysis buffer containing 0.1% NP-40. After centrifugation the cleared cytoplasmic extract was mixed 1:1 with 2x SDS loading buffer. Proteins from the nuclear and cytoplasmic fractions were separated by SDS-PAGE. The distribution of THOC5 in both fractions was analyzed by immunoblotting using anti-hFMIP antibody. The data shown in **Figure 3.14-B** reveal that endogenous *X. laevis* THOC5 is exclusively detected in the nucleus, but also in the cytoplasm, suggesting that *X. laevis* THOC5 shuttles between nucleus and cytoplasm in almost the same manner as the human FMIP/THOC5.

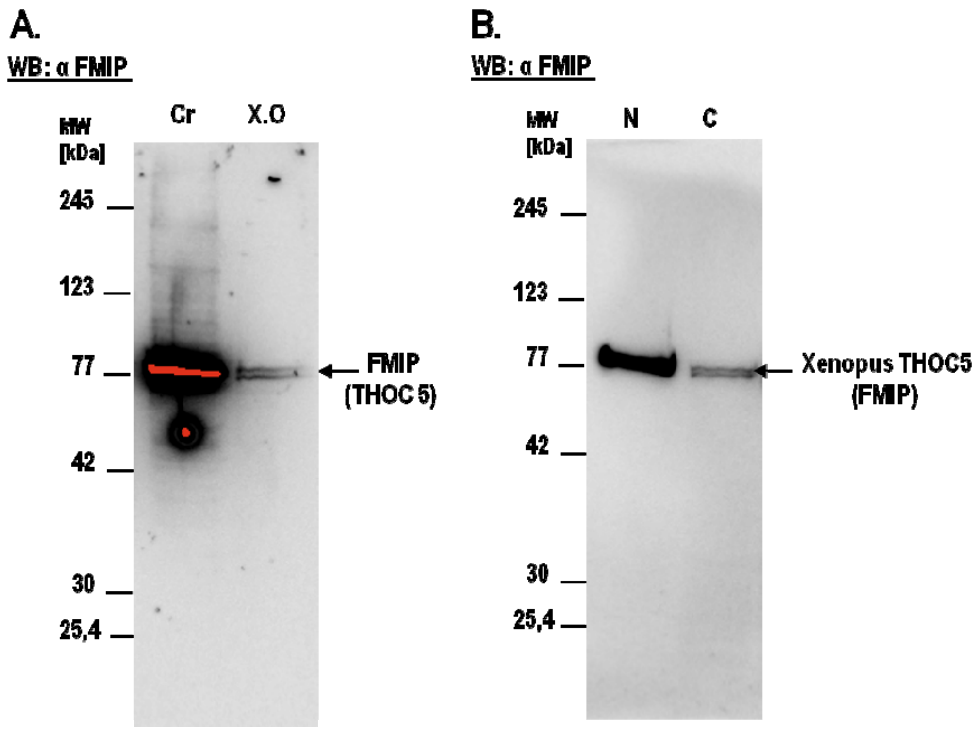

Fig. 4.14: *Xenopus* FMIP/THOC5 is mainly localised in the nucleus. A) 10 *Xenopus laevis* oocytes in stage V/VI were homogenized in lysis buffer containing 0.1% Nonidat (NP40). After removal of the yolk by centrifugation, Aliquots from the cleared extract (X.O) was boiled in 4x SDS sample buffer. Extracted Proteins were resolved by 11% SDS/PAGE, followed by Western blot analysis by using monoclonal anti-FMIP antibody. HEK293 cell lysate was used as control (Cr). **B)** Ncluar and cytoplasmic fractions of 12 xenopus oocyte were manual dissected. Nuclear fractions (N) were collected and boiled directly in 4x SDS sample buffer. Cytoplasimc fractions (C) were collected and homogenized in in lysis buffer containing 0.1% Nonidat (NP40). After removal of the yolk by centrifugation, the cleared extract was boiled in 4x SDS sample buffer. Extracted Proteins from both fractions were resolved by 11% SDS/PAGE, followed by Western blot analysis by using monoclonal anti-FMIP antibody.

4.4.2.3 Xenopus Laevis THOC5 binds to the human THOC7 in vitro

To examine whether *Xenopus laevis* FMIP/THOC5 binds to GST-human THOC7, GST pull-down assays were performed. GST fusion proteins of human THOC7 (GST-hTHOC7) and GST alone were purified and immobilized on glutathione-Sepharose as previously described (**Fig. 4.15-A**). Thirty stage V/VI *Xenopus oocytes* were homogenized in lysis buffer (50mM Tris/HCl pH 7.6, 150 mM NaCl, 0.1% Triton X-

100, 0.2mM PMSF and 3% Trasylol). After centrifugation, the cleared extract was removed in a new tube. Eight µg of immobilized GST-hTHOC7 or GST alone (control) were incubated overnight at 4°C with the cleared *Xenopus oocytes* extract. After extensive washing, bound fractions were resolved by SDS-PAGE, followed by Western Blot analysis using anti-FMIP antibody.

Using this assay, a new interaction between human THOC7 and *Xenopus laevis* THOC5 was identified. In support of this, *X. Laevis* THOC5 failed to bind in the same condition to GST alone (**Fig. 4.15-B**).

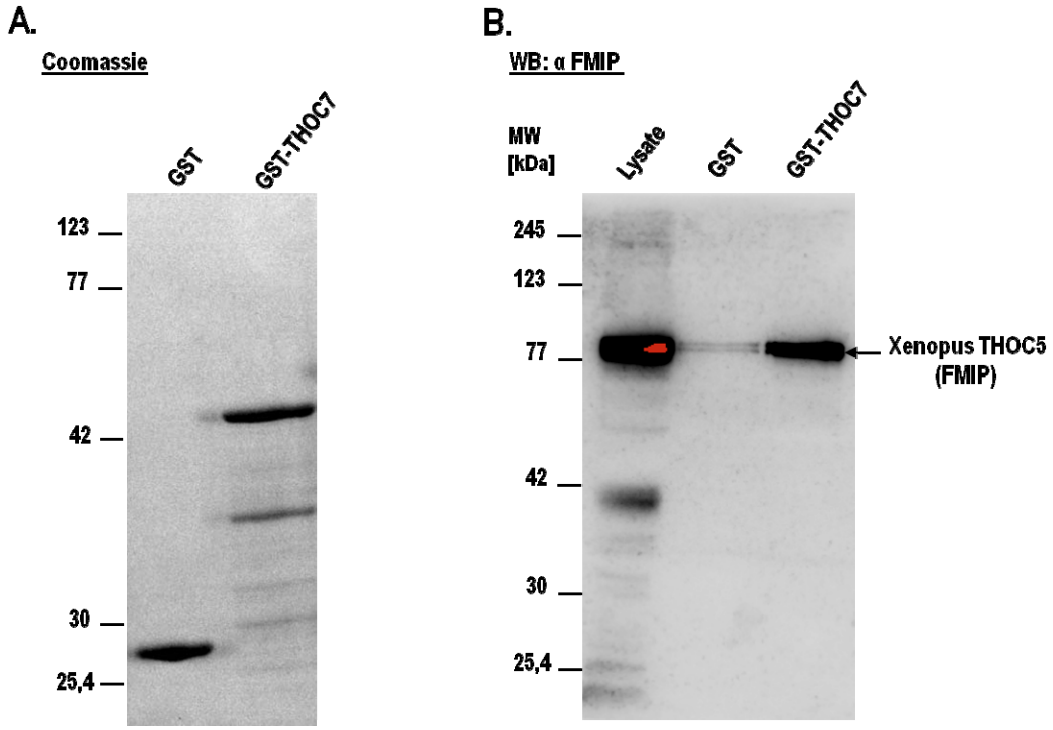

Fig. 4.15: Human THOC7 binds *Xenopus* FMIP/THOC5 *in vitro.* A) Bacterially expressed and affinity-purified GST-THOC7 or GST alone prebound on glutathione beads were resolved by 11%SDS-PAGE and stained with Coomassie brilliant blue. **B)** Thirty *Xenopus laevis* oocytes in stage V/VI were homogenized in lysis Buffer containing 0.1% Triton X-100. After removal of the yolk by centrifugation, the cleared extract was incubated with 8µg of purified GST and GST-THOC7. Bound fractions (Pellets) were resolved by 11% SDS/PAGE, followed by Western blot analysis by using anti-FMIP antibody. Aliquots from the *Xenopus* cell lysate were boiled in 4xSDS sample buffer, and used as a control (El Bounkari et al., 2008).

4.4.2.4 Human THOC7 is translocated into to the nucleus after cytoplasmic microinjection in Xenopus oocytes

To examine, whether *X. laevis* THOC5 affects the human THOC7 nuclear import in oocytes system, cytoplasmic microinjection of GST-hTHOC7 of stage V/VI oocytes was performed.

First, GST-hTHOC7(204) GST-hTHOC7(1-66) and GST were purified and immobilized on glutathione sepharose as described previously. Fusion proteins were released from the sepharose with reduced glutathione, passed a PD-10 desalting column (Microcon), and concentrated by size exclusion chromatography.

Ten nanogram of Purified full-length GST-hTHOC7 and GST-hTHOC7(1-66) were individually injected into the cytoplasm of stage V/VI oocytes, along with GST alone as a control. The nuclear and cytoplasmic fractions were then manually separated immediately after injection (0h) and after 3h of incubation at 18°C. The nuclear proteins were solubilised directly in 2x SDS loading buffer and the cytoplasms were homogenized in lysis buffer. The fractions were pooled and separated on SDS-PAGE, along with a total lysate derived from 10 cytoplasmically injected oocytes. Proteins of isolated fractions were analyzed by Western blotting using monoclonal anti-GST antibody.

As shown in **Figure 4.16**, both GST and GST-hTHOC7(1-66) remained in the cytoplasm 3h after injection, while full-length GST-hTHOC7 translocated to the nucleus. Remarkably, microinjected GST-THOC7(1-204) detected in nucleus was clearly degraded, indicating that nuclear localisation of THOC7 in *Xenopus* oocytes may affects its stability.

These data suggest that the nuclear import of hTHOC7 may depend on its association with *Xenopus* THOC5; raising the possibility that FMIP/THOC5 binding

domain is required for THOC7 nuclear import in *Xenopus* oocytes. These results are in accordance with the subcellular co-localisation studies of GFP-THOC7 and Myc-tagged FMIP/THOC5 cells showing that FMIP/THOC5 is required for the nuclear import of THOC7.

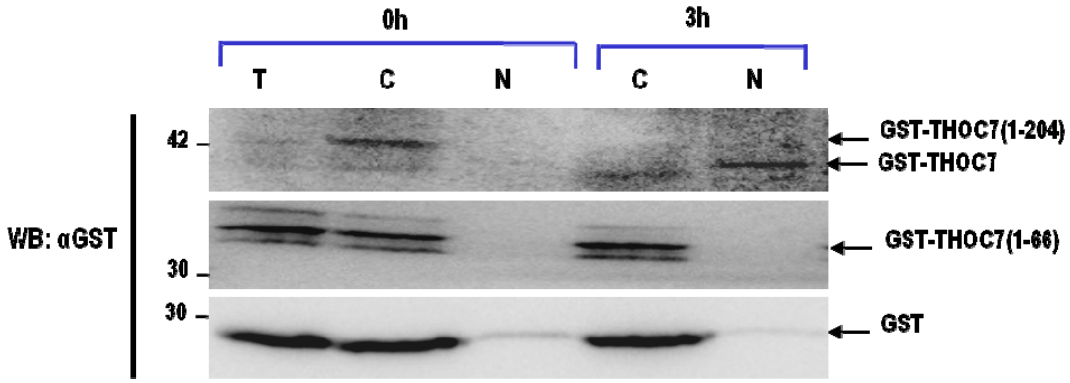

FIG. 4.16: GST-hTHOC7 is imported into the nucleus after cytoplasmic microinjection in the *Xenpous laevis* oocytes. A) Bacterially expressed and affinity-purified GST-THOC7(1-204), GST-THOC7(1-66) or GST alone were released from glutathione beads and dialysed in a PD-10 desalting column (Microcon) against PBS. Approximately 15nl of GST-THOC7(204), GST-THOC7(1-66) or GST were injected separately into the cytoplasm of oocytes. Nuclear (N) and cytoplasmic (C) fractions were manually dissected immediately after injection (0h) or after 3h incubation at 18°C. Proteins were extracted from both fractions, and precipitated with TCA/Aceton (1:8). The dried protein pellets were dissolved in 2x SDS sample buffer and resolved by 11% SDS/PAGE, followed by Western blot analysis using monoclonal anti-GST antibody. T: Total C: cytoplasmic fraction N: nuclear fraction.

4.4.2.5 Microinjection of human THOC7 in Xenopus oocytes causes AdML1 mRNA degradation

RNA export from the nucleus is an energy-dependent process that relies on recognisation of the RNA or ribonucleoprotein export substrates by saturable factors (Zasloff M., 1983; Bataillé et al., 1990; Jarmolowski et al., 1994). Microinjection experiments in *Xenopus* oocytes have revealed that different classes of RNA transport (Transfer RNA (tRNA), Uracil-rich small nuclear RNA

(UsnRNA), messenger RNA (mRNA) and ribosomal RNA (rRNA)) apparently occurs via different pathways (Jammolowski et al., 1994, Fischer at al., 1995).

To examine whether THOC7 antagonizes FMIP/THOC5, and thereby acting as a negative regulator of mRNA-export in *Xenopus* Oocytes, we monitored the export of an intron-containing mRNA that was generated by splicing of injected ^{32}P-labelled adenovirus major late (AdML) pre-mRNA in oocyte nuclei (Hamm and Mattaj, 1990).

^{32}P-radiolabelled AdML pre-mRNA, U6snRNA and tRNA were generated using *in vitro* transcription. AdML pre-mRNA and U6 snRNA were synthesized with m7GpppG and γ–mpppG caps, respectively. *Xenopus* oocytes nuclei were injected with a mixture of *in vitro* synthesised ^{32}P-radiolabelled RNAs (AdML pre-mRNA, U6nRNA and tRNA) and purified GST-hTHOC7 or GST alone. U6 snRNA and tRNA serve as control RNAs. U6snRNA, which is not exported to the cytoplasm (Vankan et al., 1990; Terns et al., 1993a), is included as an internal control for nuclear envelope integrity and the accuracy of injections. Nuclear export of the initiator methionyl-tRNA is mediated by exportin-t, and it distinct from the export pathway of mRNAs (Mattaj and Englmeier, 1998). It therefore serves as a control for nuclear export. Nuclei (N) and cytoplasms (C) were isolated from injected oocytes by manual dissection. RNA samples from total oocytes, nuclear and cytoplasmic fractions were collected immediately (0h) or five hours (5h) after injections and analyzed by 8% denaturing polyacrylamid gel. The radiolabelled RNAs were visualized by autoradiography.

Even though repeating this experiment, no data could be detect indicating the export of AdML pre mRNA using this system (data not shown). It should be noticed that nuclear microinjection of GST-THOC7 was found to be toxic for *Xenopus* oocytes. To confirm this, we performed the nuclear microinjection of GST-hTHOC7 or GST alone in *X.* oocytes. After 5h incubation at 18°C, most of the oocytes were rapidly damaged through nuclear and cytoplasmic dissection in comparison with

the GST injected oocytes. Upon this observation, it could be possible that human THOC7 was rapidly degraded in oocytes nuclei, or its nuclear expression in *Xenopus* oocytes appears to be toxic. This indicates that its expression in oocytes leads probably to activation of signalling pathways involved in Apoptosis.

4.4.3 THOC7 overexpression induces Caspase-3 activation in mammalian cells

As shown above, by establishing cell lines that overexpress THOC7 only few colonies were obtained. Moreover, the overexpression of THOC7 in C2C12 cells slows down the cell growth. The nuclear microinjection of THOC7 appears to be toxic for Xenopus oocytes. Taken all these data together suggest that the overexpression of THOC7, which affect the stoichiometry of members of THO complex, may be involved in apoptosis. Therefore, the effect of THOC7 on the caspases activation in mammalian cells was examined.

4.4.3.1 Cytoplasmic THOC7 induces caspase-3 activation in mammalian cells

Caspase-3 has been found to be responsible for most of the morphological changes and intranucleosomal fragmentation in apoptosis. Furthermore, caspase-3 was found as the main executioner active caspase in Apoptosis (Hope J. kilic M. et al., 2001). Mouse embryo fibroblasts (MEF) cells were grown on coverslips and were transiently transfected with expression vectors containing Myc-tagged FMIP/THOC5 or Myc-tagged THOC7. After transfection, immunofluoerescence co-staining was performed using anti-Myc and anti-cleaved caspase-3, which recognizes p17 and p19 subunits of active caspase-3.

As shown in **Figure 4.17-A** cleaved caspase-3 was detected exclusively in cells transfected with THOC7. In contrast, hardly any activation of caspase-3 was detected in the cells overexpressing FMIP/THOC5.

The same experiment was performed using Hela cells. After transfection followed by immunostaining with anti-Myc and anti-caspase-3, the caspase-3 activation was also detected exclusively in THOC7 transfected cells, but not in FMIP transfected cells (**Fig. 4.17-Bb**).

4.4.3.2 Nuclear localisation of THOC7 leads to a reduction of caspase-3 activation

As mentioned above, the nuclear microinjection of THOC7 seems to be toxic for *Xenopus* oocytes. Therefore, the involvement of nuclear expression of THOC7 in mammalian cells in the activation of caspase-3 was examined.

To address this question, the 4-amino-acids of nuclear localisation signal (NLS) sequence (**KKRK**) derived from the FMIP/THOC5 protein were inserted in N-terminal of Myc-tagged THOC7 sequence to generate Myc-tagged NLS-THOC7 (NLS-THOC7-Myc) construct (**Fig. 4.17-Ba**).

Mcy-tagged FMIP/THOC5, Myc-tagged THOC7 or Myc-tagged NLS-THOC7 were transiently expressed in Hela cells. After transfection, the cells were fixed permeabilized and co-stained with anti-Myc and anti-cleaved caspase-3.

As shown in **Figure 4.17-Bb**, NLS-THOC7-Myc is exclusively expressed in nucleus unlike THOC7, which is mainly expressed in cytoplasm.
Similar to the overexpression of wild type THOC7, Myc-taged NLS-THOC7 contributes also to a lower the cleavage of caspase-3 in the same cells. Interestingly, this activation is highly reduced than that observed by overexpression of wild type THOC7. This indicates that the high level of cytoplasmic, rather than nuclear, THOC7 leads to activation of caspase-3 followed by apoptosis (**Fig. 4.17-Bb**). These data demonstrate strongly that the subcellular localisation of THOC7 influences the activation of caspase-3.

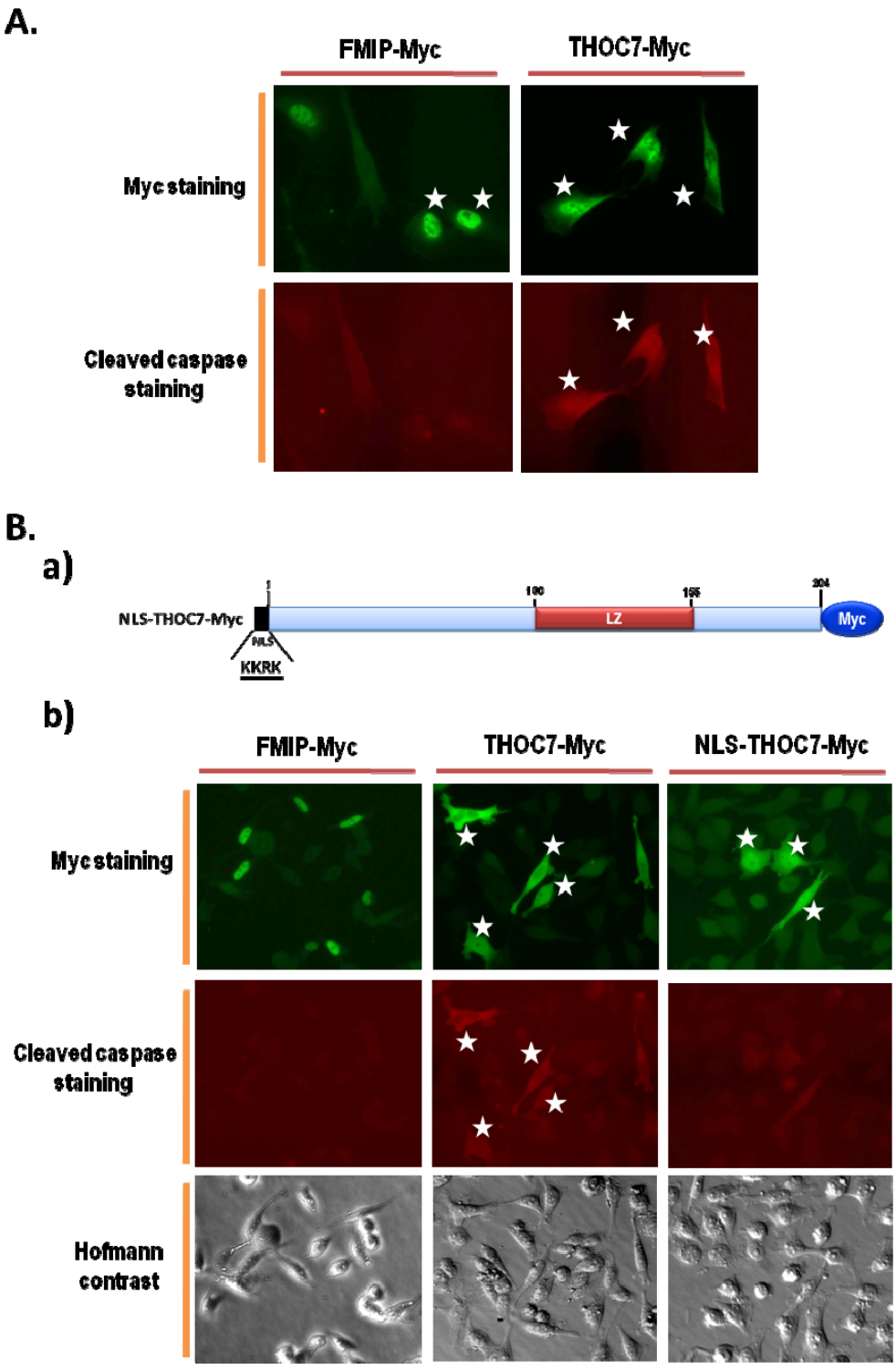

Fig. 4.17: Subcellular localisation of ectopic THOC7 influences the activation of caspase3 in mammalian cells. A) THOC7 overexpression induces Caspase-3 activation in MEF cells. MEF cells Hela cells were transfected with pcDNAMyc/His plasmids containing FMIP or THOC7 cDNAs. After

transfection, cells were fixed briefly with 3.7% Formaldehyd (PFA), treated with 0.1 %Triton X-100 and were stained with anti-Myc antibody, followed by cleaved caspase3 and finally by TRITC conjugated anti-mouse IgG. **B) a)** Schematic illustration of generated Myc-tagged NLS-THOC7. **b)** Nuclear localisation of THOC7 leads to a reduction of caspase-3 activation in Hela cells. Hela cells were transiently transfected with pcDNAMyc/His plasmids containing FMIP, THOC7 or NLS-THOC7 cDNAs. After transfection, cells fixed permeabilized and co-stained with anti-Myc and anti-cleaved caspase-3 (see A).

4.4.3.3 FMIP leads to reduction of THOC7-mediated caspase-3 activation

Having shown that nuclear, but not cytoplasmic, localisation of THOC7 leads to reduction of of caspase-3 activation in Hela cells and that FMIP/THOC5 overexpression leads to the translocation/import of THOC7 into nucleus, it was interesting to examine whether co-expression of THOC7 with FMIP/THOC5 can also lead to the reduction of THOC7 mediated caspase-3 activation.

To address this question, Flag-tagged FMIP overexpressing Hela cell lines were established. Hela cells were trannsfected with plasmids that encode Flag-tagged FMIP cDNA and/or empty vector alone. Transfected cells were selected in the Neomycin (G418) presence. Clones of each cell line expressing Flag-FMIP or empty vector were used for subsequent analysis. Expression of the recombinant FMIP protein was monitored by Western Blotting using anti-Flag antibody. As shown in **Figure 4.18-A**, the selected clone express exclusively Flag-tagged FMIP.

Myc-taged THOC7 was transfected into Hela cells expressing Flag-FMIP or empty vector. After transfection, immunofluorescence co-staining was performed using anti-Myc and anti-cleaved caspas-3. Remarkably, the coexpression of FMIP/THOC5 and THOC7 leads to less of caspase-3 activation in comparison with that observed when THOC7 is alone overexpressed; in contrast caspase-3 activation was clearly detected in Hela cells expressing emty vecotor (**Fig. 4.18-B**). These data are consistent with the results obtained upon overexpression of NLS-THOC7 in the same cell ine, demonstrating that the FMIP-mediated nuclear import of THOC7

leads to reduction of caspase-3 activation. This data suggests that FMIP/THOC5 appears to be a counteracting partner of THOC7 by activation of caspase-3 and probably by apoptosis induction. Further studies have to address the mechanism and functional implications of THOC7 on the caspase-3 activation.

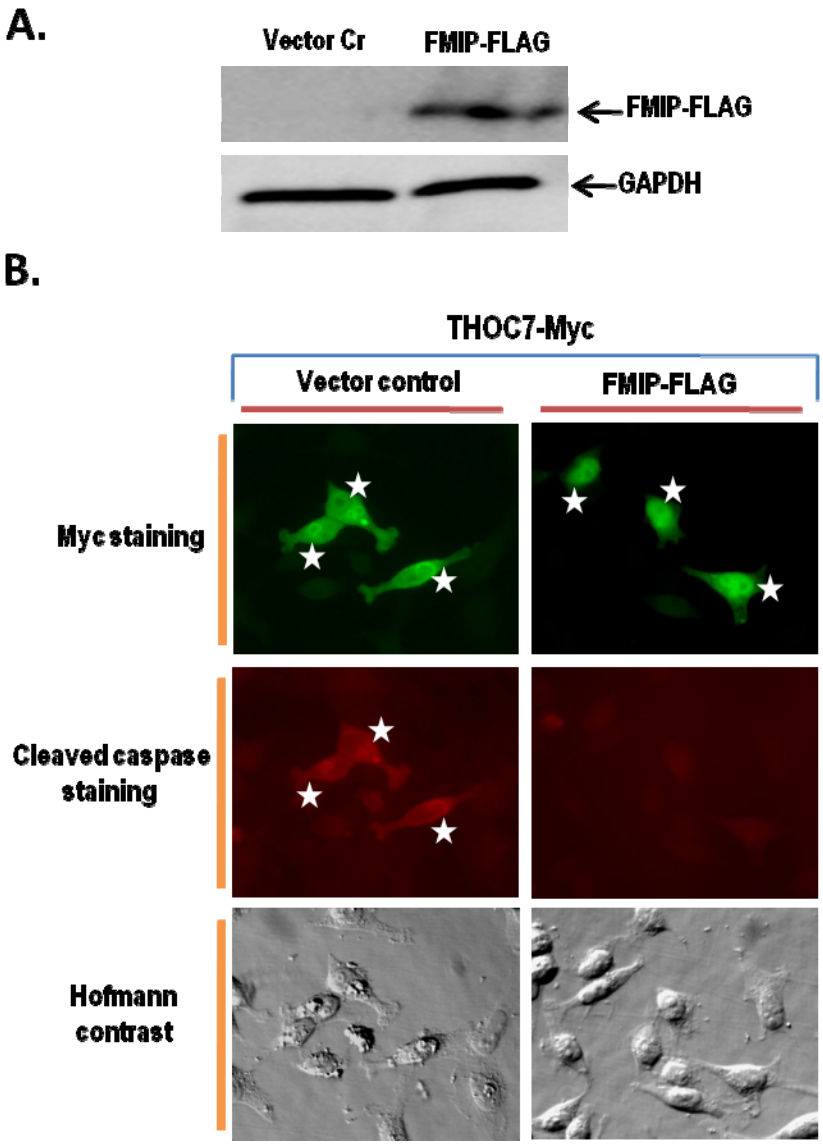

Fig. 4.18: THOC7-mediated caspase-3 activation is reduced upon FMIP coesxpression in Hela cells. **A)** Flag-taged FMIP or empty vector was transfected into Hela cells. After Neomycin (G418) selection, colonies were isolated. Cell lysates of selected clones expressing Flag-FMIP or empty vector were analysed by Western Blot using anti-Myc antibody. The protein among of used cell lysates was examined using anti-GAPDH antibody. **B)** The stable cell lines expressing Flag-FMIP or empty vector (see A) were transfected with Myc-tagged THOC7. Transfected cells were fixed briefly

with 3.7% Formaldehyd (PFA), treated with 0.1 %Triton X-100 and were stained with anti-Myc antibody, followed by cleaved caspase3 and finally by TRITC conjugated anti-mouse IgG.

4.5 FMIP/THOC5 forms a complex with THOC7/NIF3L1 BP and the death domain containing protein, p84N5/THOC1

The previous experiments have indicated that members of THO complex (THOC5 and THOC7) are somehow involved in the regulation of apoptosis. Interestingly, the overexpression of THOC1/p84N5, another member of THO complex, leads to loss of cell viability accompanied by changes in cellular morphology and internucleosomal DNA fragmentation inducing apoptosis (Doostzadeh-Cizeron et al., 1999). Furthermore, THOC1/p84N5 was originally identified as a binding partner of the tumor suppressor retinoblastoma (Rb) protein (Durfee etal., 1994). The association of THOC1/p84N5 and Rb leads to inhibition of THOC1/p84N5-induced apoptosis (Doostzadeh-Cizeron et al., 1999). In addition, higher level of THOC1/p84N5 in cancer cells induces apoptosis (Li et al., 2007). To examine whether FMIP/THOC5 is a common interacting partner for THOC7/NF3L1 BP and THOC1/p84N5 *in vivo* interaction between THOC5 and THOC1 was analysed by co-immunoprecipitaion experiments.

4.5.1 THOC1 as a new binding partner of FMIP/THOC5

Because the monoclonal antibody against FMIP/THOC5 poorly precipitates endogenous FMIP/THOC5 under non-denaturing conditions (data not shown), we performed the immunoprecipitation form HEK293 cell lysates using monoclonal anti-THOC1 antibody. THOC1-complex was pulled from the precleared Lysate with anti-THOC1 antibody coupled to protein A-Sepharose. After washing, immunoprecipitated proteins were released from the matrix addition and subsequent boiling in 2x SDS-PAGE sample buffer. Precipitated proteins were then analysed by immunblotting using monoclonal antibody against endogenous FMIP and against p84N5/THOC1.

As shown in **Figure 4.19-A** THOC1 co-immunoprecipitate endogenous FMIP. This finding identifies a new binding partner of FMIP, indicating that FMIP/THOC5 acts as a common binding partner of THOC7 and HOC1/p84N5.

4.5.2 C-terminal domain (559-683) of FMIP is required for THOC1 association

To further localize the binding site within FMIP/THOC5 that contributes to THOC1/p84N5 association, HEK293 cells were transiently transfected with plasmids expressing Myc-Tagged full-length FMIP(1-683), Myc-tagged truncated FMIP-mutants (FMIP(1-559), FMIP(1-289)) or empty vector alone. Cell extracts from transfected HEK293 cells were used to perform immunoprecipitations using anti-Myc antibody. Precipitated proteins were then analysed by immunoblotting using both antibodies (**Fig. 4.19-B**).

As shown in **Figure 4.19-B** the level of endogenous human THOC1 in HEK293 cells was sufficient to allow detection following immunoprecipitation of Myc-tagged FMIP (IP-Myc, WB: THOC1). However, no association between both FMIP-truncated mutants (FMIP(1-559), FMIP(1-289)) and THOC1 could be detected. This data suggest strongly that the C-terminal domain (559-683) is responsible for the interaction between FMIP/THOC5 and THOC1/p84N5.

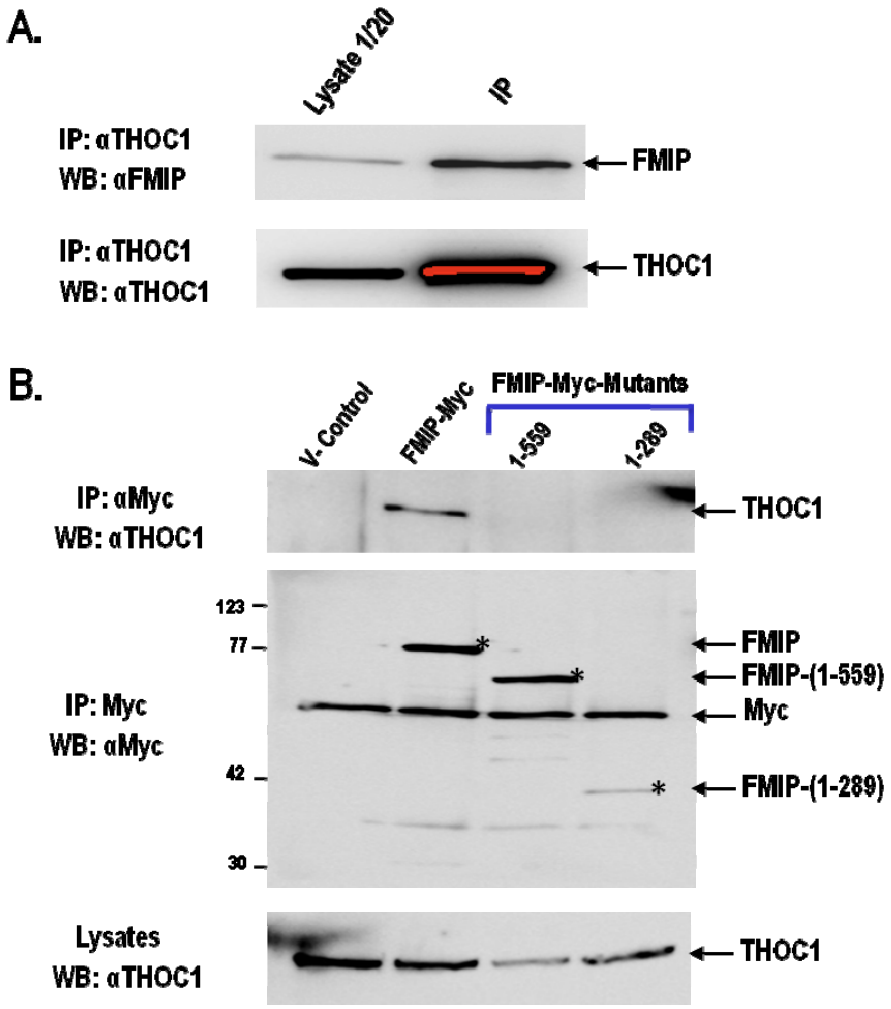

Fig. 4.19: THOC1 as a novel interacting partner of FMIP. A) Endogenous FMIP associates with THOC1 *in vivo*. Cell extract from HEK293 cells was incubated with 20µl A-Sepharose beads and monoclonal anti-THOC1 antibody. After incubation, Precipitated proteins were separated by SDS-PAGE and analysed by immunblotting using monoclonal anti- FMIP or anti-p84N5/THOC1. Aliquots from cell lysate was diluted and loaded on the gel as control. **B)** Amino acide sequence (559-683) within FMIP is responsible for THOC1 association. HEK293 cells were transfected with Myc-taged FMIP, Myc-tagged truncated FMIP-mutants (FMIP(1-559), FMIP(1-289)) or empty vector alone. Cell extract from transfected cells were subjected to co-immunoprecipitation using monoclonal anti-Myc antibody. After incubation, immunoprecipitated proteins were separated by SDS-PAGE and analysed by immunblotting using monoclonal anti- THOC1 or anti-Myc. Aliquots from transfected lysates were immunoblotted with anti-THOC1 as control.

4.5.3 THOC1-FMIP-THOC7 complex is located in the nucleus

As mentioned above, FMIP/THOC5 seems to be a common interacting partner for THOC7 and THOC1/p84N5. Although, these three THO complex proteins show different subcellular localisations, it was of interest to characterise which cellular compartment is required for the interactions found between FMIP/THOC5- THOC7 and/or -THOC1/p84N5.

To address this question, HEK293 cells were transfected with Myc-tagged FMIP/THOC5, Myc-tagged THOC7 or empty vector. After transfection, nuclear (N) and cytoplsamic (C) fractionations were performed. Both fractions were subjected separately to immunoprecipitation using monclonal anti-THOC1/p84N5 or monoclonal anti-Myc antibodies. Precipitated proteins were analyzed by Western blotting using anti-FMIP or anti-THOC1/p84N5.

As shown in **Figure 4.20**, precipitated FMIP associated with THOC1 mainly in nucleus, but less in cytoplasm. Similar data were obtained performing the immunoprecipitation with anti-THOC1 antibody, demonstrating that the interaction between FMIP and THOC1 occurs in nucleus, but less in the cytoplasm. It is important to mention that the splice version of THOC1/p84N5, which lacks the death domains, is mainly localized in the cytoplasm (Gasparri et al., 2004). This is consistent with the fact that the interaction between THOC5 and THOC1 is also detected in the cytoplasm (**Fig. 5.20**). Remarkably, similar results are observed concerning FMIP-THOC7 interaction. Surprisingly, immunoprecipitation of Myc-tagged THOC7 from nuclear fractions demonstrates that THOC1 form a complex with THOC5 and THOC7 in the nucleus, but not in the cytoplasm (**Fig. 4.20**).

This result indicates that FMIP recruits THOC7 into the cytoplasm to form a triple complex with THOC1 composed from THOC1-THOC5-THOC7 in the nucleus, which may be involved in the regulation of caspase-3 activation and apoptosis.

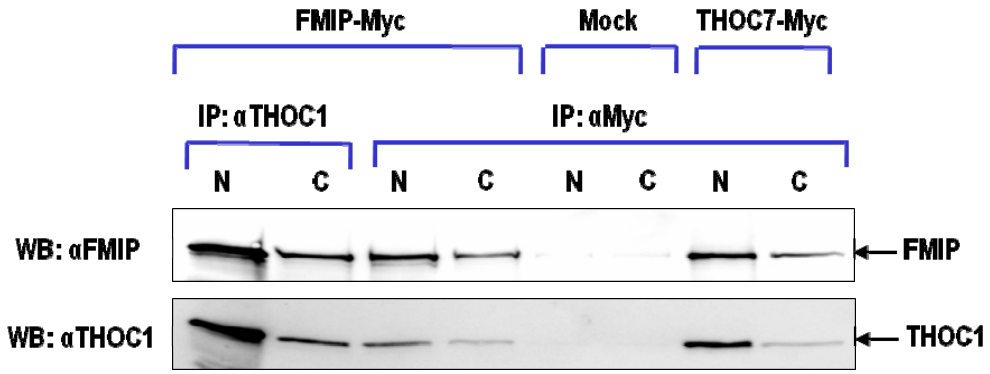

Fig. 4.20: THOC1, FMIP and THOC7 form a complex in the nucleus. HEK293 cells were transfected with Myc-taged FMIP, Myc-tagged THOC7 or empty vector alone. Transfected cells were subjected to cell fractionantion. Nuclear (N) and cytoplasmic (C) cell extracts were subjected to immunoprecipitations using monoclonal anti-Myc or anti-THOC1 antibodies. After incubation, immunoprecipitated proteins were separated by SDS-PAGE and analysed by immunblotting using monoclonal anti- FMIP or anti-THOC1.

5 DISCUSSION

Cell proliferation, differentiation and survival are processes that are tightly governed by soluble growth factors via their membrane bound receptors. One of these receptors is the macrophage colony-stimulating factor receptor (M-CSFR), which associates with the nuclear/cytoplasmic shuttling protein termed FMIP. In this study, putative new interaction partners of FMIP were identified, and characterised FMIP as a member of mRNA processing THO complex. Below, we discuss the key findings from these thesis, and approaches for further investigations.

5.1 FMIP (THOC5) as a member of THO complex

Identification of interaction partners is one of the most successful methods to improve the understanding of the role for a given protein. In the present work a large scale co-precipitation approach using TAP-tagged FMIP was applied to search for proteins that physically interact with FMIP *in vivo*.

The TAP-tag purification of hFMIP shows the identification of different proteins that are involved in processing and splicing of mRNA. In addition, proteins involved in DNA-Replication, cell cycle regulation and DNA damage/repair were also identified in the purified TAP-FMIP complex. Remarkably, members of THO/TREX complex were also identified in this purification. Three members of THO complex were isolated, namely THOC1, THOC2, and THOC3. Additionally, mRNA export factors Aly/REF1 and UAP56/BAT1 were identified. By analysing the sequences of THO complex members, we found surprisingly that the Sequence of THO complex subunit5, THOC5 (also termed fSAP79), shows a 100% homology to FMIP sequence, indicating that Fms interacting protein (FMIP) belongs to the THO complex. This finding demonstrates for the first time a kind of protein that interact with the trans-membrane receptor tyrosine kinase and at the same time a

member of a nuclear complex (THO complex), which is somehow involved in mRNA processing or export. This indicates that receptor tyrosine kinases may be somehow involved in the regulation of mRNA processing and/or mRNA export.

Masuda et al. (2005) characterized a human THO complex *in vitro* using a combination between GST-UAP56 and GST-THOC4 Pulldown from RNase treated Hela nuclear extract, indicating that THO complex is conserved in *human* and *drosophila* (Rehwinkel et al., 2004).

Indeed, with the exception of THOC6 (fSAP35) and THOC7 (fSAP24), we detected nearly all known components of the human TREX complex using the mammalian tandem affinity purification of hFMIP/THOC5.

Alternatively, there are several possible explanations for the loss of the THOC7 and THOC6 during the TAP-purification procedure applied in this work. The affinity of one protein for another can be altered by the presence or absence of an effector such as ATP. In addition, a change in physiological conditions such as temperature, pH, salt concentration or covalent modification including phosphorylation can all affect protein binding (Nooren and Thornton, 2003). Another possible explanation is that N-terminal TAP-tagged sequence leads to conformation changes and thereby impairs the binding domain of FMIP to THOC7 and/or that the interaction between THOC7 and other members of THO complex may be transient *in vivo*. Transient protein-protein interactions are regulated by physiological conditions that have the ability to change affinity between partners thereby allowing for the effective control of dynamic protein complexes (Nooren and Thornten, 2003). In addition, protein-protein interactions can also be classified as obligate and non obligate. In an obligate protein-protein complex, the proteins forming this complex are not found as stable structures on their own in vivo (Nooren and Thornton, 2003).

To date, little is known about dynamic interactions, assembly and stability of THO complex. This work shows and examines for the first time a possible assembly of THO complex. Multiple GST-pulldown assays and immunoprecipitation studies of THO complex members were unable to give a general view about the assembly of this complex. Anyhow, only two new interactions could be characterized, the direct association between THOC5 and THOC7 and the association between THOC5 and THOC1, showing that THOC5 is a common partner between THOC1 and THOC7. In order to characterize the assembly and the stability of THO complex, it would be interesting to further examine new interactions between THO complex members using alternative interaction approaches. Clearly, understanding the precise molecular function of the THO complex awaits further biochemical characterization of its subunits. Hence, it will be important to focus on the characterization of each component of THO complex. This would be helpful to illustrate the function of this complex.

Remarkably, most of isolated nuclear proteins are involved in mRNA processing and splicing. This is consistent with the previous data showing that THO complex is somehow involved in mRNA processing and splicing. Interestingly, previous *in vitro* spliceosome proteomics using pre-mRNAs derived from either the adenovirus major late (AdML) or ß-globin always yield some, but not all, members of THO complex (Rappsilber et al.2002; Zhou et al., 2002; Neubauer et al., 1998, Merz et al., 2007, Masuda et al., 2005) (**Table 2**).

Table 2 lists different THO complex members identified using different proteomics approaches. Most of These are performed *in vitro*. Remarkably, *in vivo* spliceosome proteomics performed by Chen et al. (2007) yield the same THO complex members identified in our TAP-tag purification (**Table 2**). However, THOC7 and THOC6 were not detected. This indicates that the assembly of THO complex may not at once contains all members of this complex *in vivo*.

Recently, *in vitro* studies indicated that components of the human TREX complex are associated with spliced mRNA (Masuda et al. 2005). Furthermore, Cheng et al. (2006) demonstrate that human TREX complex is associated with nuclear cap-binding complex (CBC). This association occurs via a protein-protein interaction between CBP80 and Aly/REF1, which bound on mRNA closest to the cap (Cheng et al., 2006). In the present study, we were able to detect proteins involved in pre-mRNA splicing. Unfortunately, no members of nuclear cap-binding complex (CBP80/CBP20) were characterized. However, nothing is known about the regulatory role of THO complex within the TREX complex.

Additionally, it is also unclear whether THO complex coupled just splicing or mRNA-export. The tandem affinity purification among the proteins involved in replication, four subunits of replication factor C (RFC) complex (RFC2, RFC3, RFC4 and RFC5) were isolated, except the subunit 1 (RFC1). This finding indicates that human THO complex may also be involved in coupling transcription-splicing and mRNA export.

GST-pulldown experiments performed in the present work demonstrated a new putative interaction between pre-mRNA splicing factor SF2/ASF and Aly/REF1. SF2/ASF belongs to the Serine and Arginine-rich (SR) protein familiy, which consists of eight conserved proteins. SR proteins have well-characterized roles in pre-mRNA splicing including exon definition and assembly of the mature spliceosome (Hastings et al., 2001; Ram et al., 2007). Furthermore, SF2/ASF is a nucleo-cytoplasmic shuttling RNA binding protein, which plays a role in mRNA translation (Sanford et al., 2005; Sanford et al., 2004). Recently, it was shown that SF2/ASF has the capacity to regulate the nuclear and cytoplasmic processing of specifi mRNAs (Sanford et al., 2008). The new interaction between SF2/ASF and ALy/REF1 shows for the first time a novel connection between members of the TREX complex and the spliceosome components. This would support the fact, that components of the human TREX complex are associated with spliced mRNA (Masuda et al., 2005). It is also possible that this interaction is indirect or caused by strong unspecific binding

of the GST-SF2 construct. Thus, further experiments should be performed to confirm this interaction.

Table 2: Isolated THO complex subunits by different proteomics approaches found in the literature

Bait	Isolated THO complex subunits	Methods and conditions	literatures
AdML pre-mRNA	THOC1, THOC2, THOC3, THOC5, THOC7	*in vitro* spliceosome proteomics	Merz et al., *Cell* 2005
β-globin pre-mRNA	THOC2	*in vitro* spliceosome proteomics	Merz et al., *Cell* 2005
AdML pre-mRNA	THOC1, THOC2, THOC3,THOC5, THOC6, THOC7	*in vitro* spliceosome proteomics	Zhou et al., *Nature* 2002
AdML pre-mRNA	THOC1	*in vitro* spliceosome proteomics	Rappsilber et al., *Genome Research* 2002
β-globin pre-mRNA	THOC1	*in vitro* spliceosome proteomics	Rappsilber et al., *Genome Research* 2002
GST-Aly	THOC1, THOC2, THOC3,THOC5, THOC6, THOC7	*in vitro* GST-pulldownRNAse treated Hela nuclear Extract	Masuda et al., *Cell* 2005
GST-UAP56	THOC1, THOC2, THOC3,THOC5, THOC6, THOC7	*in vitro* GST-pulldownRNAse treated Hela nuclear Extract	Masuda et al., *Cell* 2005
CBP80	THOC2, THOC3	Immunoprecipitation -RNAse treated Hela nuclear Extract	Cheng et al., *Cell* 2006
THOC2	THOC1, THOC3,THOC5, THOC6	Immunoprecipitation -RNAse treated Hela nuclear Extract	Cheng et al., *Cell* 2006
Human suprаspliceosome-associated polypeptides and snRNAs	THOC1, THOC2, THOC3	*in vivo* spliceosome Hela suprаspliceosome	Chen et al., *Nucleic acid research* 2007
TAP-THOC5	THOC1, THOC2, THOC3	TAP-purification HEK293 cells	This Thesis
THOC1	THOC5	Immunoprecipitation -HEK293 cell lysate	This Thesis
GST-THOC7	THOC5	Immunoprecipitation -HEK293 cell lysate	This Thesis

5.2 THOC7 is a putative interaction partner of FMIP/THOC5

In the present work the uncharacterized protein THOC7/NIF3L1 BP (fSAP24) was identified as a novel and direct putative interaction partner of FMIP/THOC5. However, no interaction between FMIP and THOC6 was detected. GST-pulldown assays using *Xenopus laevis* oocytes extracts indicates that THOC7-FMIP association is probably conserved in different species.

GST-pulldown assays using deletion mutants of FMIP revealed that approximately the first 200 amino acids of FMIP/THOC5 were required for this interaction. Remarkably, these 200 amino acids contain the macrophage colony stimulating factor (M-CSF) receptor (CSF1R) binding site. Therefore, further studies will be necessary to determine whether THOC7 acts as competitor or regulator on FMIP-CSF1R interaction, and probably affecting M-CSF receptor signalling. Additionally, mutation of the amino acid sequence residue (50-137AA) of THOC7 prevented the association with THOC5 using GST-pulldown approaches, further suggesting the role of this motif in mediating this interaction.

5.3 Nuclear import of THOC7 is mediated by FMIP/THOC5

It has been reported that THOC7, which binds NIF3L1, is mainly localized in the cytoplasm, however, THOC7 is also present in the nucleus (Tascou et.al, 2003). The THOC7 amino acid sequence does not show any nuclear localisation signal (NLS) and therefore, several possibilities exist by which the protein would be able to translocate into the nucleus. First, it has been reported that ions, small metabolites, and proteins below 40kDa are able to diffuse into the nucleus through the nuclear pore complex (NPC) (Mattaj et al., 1998). Another possibility is that THOC7 translocation into the nucleus is caused by co-transport with nuclear proteins. The finding that THOC7, but not THOC6, interacts directly with FMIP/THOC5 further supports the idea of distinct subcellular localisations of the

three THO complex family members, and thereby investigating the effect of FMIP/THOC5-THOC7 interaction on the subcellular localisation of both proteins.

Expression of different GFP-tagged constructs demonstrated an exclusively cytoplasmic localisation of THOC7 and a nuclear localisation of THOC5, which are consistent with the previous reported data (Tascou et al., 2003, Mancini et al., 2004). Furthermore, we show for the first time in this thesis that THOC6 is localizes as spots primarily in cytoplasm.

Interestingly, the co-expression of FMIP with THOC7 and/or THOC6 shows a significant localisation of THOC7, but not THOC6, in nucleus, suggesting that the association between both proteins is responsible for THOC7 nuclear import. The dependent nuclear translocation of THOC7 on FMIP was confirmed by co-expression of GFP-tagged THOC7 mutants with FMIP, indicating that the binding domain of FMIP within THOC7 sequence is required for THOC7 import into the nucleus. Furthermore, this data was supported by the cell fractionation of HEK293 cells expressing both proteins showing that the association FMIP/THOC5-THOC7 is mainly detected in the nucleus. All three proteins are nuclear/cytoplasmic shuttling proteins; however it is still unclear whether THOC7/THOC5 is exported from nucleus to the cytoplasm as a single molecule.

Additionally, we were able to confirm this data basing on the interaction between *Xenopus laevis* THOC5 and human THOC7. The cytoplasmic oocytes microinjection of human THOC7 demonstrates a significant import of microinjected THOC7 into the nucleus of *Xenopus* oocytes after nuclear/cytoplsamic dissection. However, the microinjected THOC7 mutant, which fails to bind *Xenopus laevis* THOC5, shows no translocation in the nucleus of *Xenopus* oocytes, suggesting that *Xenopus* system is one of suitable system to analyze the unclear function of THO complex.

These co-localization data of FMIP/THOC5 and THOC7 based on their direct association suggest that THOC5 recruits THOC7 into the nucleus to form a stable

complex in the nucleus. Interestingly, the same effect was observed between NIF3L1, which binds THOC7, and Trip15/CSN2 protein (Akiyama et al., 2003). Trip15/CSN2 encodes the second subunit of the COP9 signaleosome complex (CSN), which involved in various cellular and developmental processes. Trip15 bind directly NIF3L1 and caused its translocation to the nucleus (Akiyama et al., 2003). It would be interesting to analyze whether FMIP/THOC5 binds to NIF3L1 or Trip15/CSN2 to form a tetramer complex composed of THO5, THOC7, NIF3L1 and Trip15/CSN2. However, it remains to be determined whether FMIP/THOC5-THOC7 association rather THOC7 translocation has any significant effect on the linking between receptor tyrosine kinase signalling and mRNA-processing and/or export.

5.4 THOC7 and THOC5 show counteracting effects on cell growth and adipocyte differentiation

In previous studies it has been shown that FMIP is involved in cell proliferation and cell differentiation (Mancini et al., 2004). Moreover, FMIP overexpression impairs the induced adipocyte differentiation of multipotent C2C12 mesenchymal progenitor cells (Mancini et al., 2007). In the present work, we further elucidate the function of uncharacterised THOC7 protein in cell signalling, by analyzing the effect of the overexpression of THOC7 on cell proliferation and cell differentiation.

The overexpression of THOC7 in C2C12 shows a significant effect on cell growth. In addition, it should be mentioned that only a few clonies were obtained from cells that stably expressed THOC7. In contrast, more that 100 colonies were detected from cell lines expressing FMIP. This is consistent with the recent published data showing that THOC5 expression under M-CSF stimulation promotes cellular proliferation (Carney et al., 2008). This data demonstrate that the expression of THOC7 and/or of FMIP/THOC5 is required for the regulation of cell proliferation. However, we could not rule out whether overexpression of THOC7 slows down or causes cell cycle arrest.

Surprisingly, THOC7 overexpression in C2C12 cells, which were grown in normal medium, showed a similar phenotype observed by downregulation of endogenous FMIP using siRNA in the same cells (Mancini et al., 2007). In contrast ectopic expression of FMIP in the same cells under the same condition blocked adipocyte differentiation (Mancini et al., 2007). This data indicate that THOC7 acts as a negative regulator of FMIP/THOC5 by mediating adipocyte differentiation. It would be interesting to analyse the putative effect of downregulated endogenous THOC7 in the same cell line and under the same condition by using small interfering RNA (siRNA).

It has been reported that THOC5 downregulates the CCAAT-enhancer binding protein alpha (C/EBPα) that is a key regulator for adipocyte differentiation (Mancini et al., 2007) as well as myeloid differentiation (Carney et al., 2008). This indicates that THOC5 may act as a transcription regulator of some genes involved in a variety of different cell processes including hematopoesis, adipogenesis and/or morphogenesis.

Remarkably, the amino acid sequence of FMIP/THOC5 contains two Leu-Xaa-Xaa-Leu-Leu (LXXLL) motifs. LXXLL motifs, also called the nuclear receptor (NR) box, participate in many protein-protein interactions, specifically with nuclear receptors. This interaction is associated with different aspects of transcriptional regulation of many biological events such as cell proliferation, differentiation reproduction and development (McKenna et al., 1999; Mangelsdorf et al., 1995; Pelvin et al., 2005; Savkur et al., 2004).

A common LXXLL motif present in a number of co-activator molecules is necessary and sufficient to mediate the interactions between the co-activator and the nuclear receptor are known to act as co-activator or co repressor (Heery et al., 1997; McInerney et al., 1998). Based on this, it can be possible that THOC5 could act as co-activator of nuclear receptors. It would be interesting to further examine

this hypothesis. Thus, further studies may explore whether apart from its role in mRNA export, the THO complex functions in additional post-transcriptional steps of gene expression in higher eukaryotes. Taken all these data, THOC7 acts as a counteracting of FMIP/THOC5 on cell proliferation and cell differentiation.

Although THOC7 is a member of THO complex, the role of THOC7 in mRNA-export is largely unclear- mRNA-export assay using *Xenopus laevis* oocyte system was not successful, because the nuclear overexpression of THOC7 appears to be toxic for the oocytes. By means of THOC7 and AdML co-expression in mammalian cells, followed by northern blotting analysis, we were able to demonstrate that THOC7 overexpression shows no significant effect on AdML mRNA export, suggesting that THOC7 may not impair the mRNA export (data not shown).

5.5 Effect of THOC1-FMIP-THOC7 complex on apoptosis and cell survival

The counteracting effect of THOC7 against FMIP/THOC5 observed in cell proliferation and cell differentiation may be due to other function of THOC7 other than mRNA export.

Effectively, immunofluorescence studies revealed that the overexpression of THOC7 in mammalian cells induces caspase-3 activation. In contrast, the overexpression of FMIP/THOC5 shows no effect on the caspase-3 activation. Furthermore, the co-expression of FMIP/THOC5 and THOC7 leads to a moderate reduction of caspase-3 activation, suggesting that FMIP-mediated nuclear import of THOC7 leads to a reduction of caspase-3 activation. The overexpression of generated THOC7 mutant (NLS-THOC7-Myc), which is located in the nucleus, leads to similar reduction of caspase-3 observed by co-expression of FMIP/THOC5 and THOC7.

We conclude from these finding that the high level of cytoplasmic, rather than nuclear, THOC7 leads to activation of caspase-3. However, the mechanism of this activation is still unclear.

In addition to investigating the previously identified THOC5-THOC7 interaction, we were able to identify a novel interaction between THOC5 and THOC1/p84N5 in whole cell lysates using immunoprecipitation. Additionally, we could demonstrate that the C-terminal domain (559-683) is required for this interaction.

The association between THOC5 and THOC1 is mainly detected in the nucleus. Furthermore,
Myc-tagged THOC7 immunoprecipitation from nuclear fractions reveals that THOC1, THOC5 and THOC7 form a tight complex in the nucleus.

On the contrast to THOC7, nuclear localisation of THOC1 is required for induction of apoptosis through the death domain located in its C-terminus (Evans et al., 2002). THOC1 interacts with the nuclear retinoblastoma (Rb) tumor suppressor protein and this association inhibits THOC1-mediated apoptosis (Dootzadeh-Cizeron et al., 1999). Moreover, it is important to mention that THOC1 is identified as a unique death domain-containing protein that functions within the nucleus to trigger apoptosis. Since both THOC7 and THOC1 interact with FMIP/THOC5 and since the import of THOC7 into the nucleus is mediated by FMIP/THOC5 and leads to the reduction of caspase-3 activation, we hypothesize that the interaction FMIP/THOC5-THOC7 participates in THOC1 mediated apoptosis signalling initiated from within the nucleus that is regulated by Rb. Consistent with this hypothesis, FMIP/THOC5 was shown to be a substrate of ataxia telangiectasia mutated (ATM) kinase, one of the tumor suppressor protein p53 kinases shown to be by different types of DNA damage (Banin S.et al., 1998; Canman C. E. et al., 1998). Additionally, the amino sequence of THOC1 and THOC7 contain putative consensus sequences SQ, indicating that both proteins may be a substrate for ATM. This observation

indicates that the three proteins are probably involved in DNA damage/ repair. Further experiments should be performed to examine this.

The finding that FMIP/THOC5 interacts with both THOC7 and THOC1 allows postulating that the three proteins are implicated in the regulation of the cell proliferation and apoptosis and thereby acting differently by the regulation and the stability of the triple complex THOC1-THOC5-THOC7.

It seems probable that THOC7-FMIP-THOC1 network regulate multiple pathways, probably only a specific pathway required for mediating some of Rb's inhibitory effects on apoptotic responses triggered by DNA damage. Thus, additional experiments would be necessary to characterize the mechanism and the common pathway of this protein-network.

5.6 Conclusion

Results from the present work revealed that FMIP is a member of THO complex, termed THOC complex subunit 5 (THOC5), and thereby acting as a unique nuclear/cytoplasm shuttling protein that interact with trans-membrane CSF1R receptor tyrosine kinase and at the same time a member of a nuclear THO complex, indicating that FMIP may play a key role in the connection between receptor tyrosine kinase-mediated extracellular signals and mRNA processing. This finding indicates that receptor tyrosine kinases may be somehow involved in the regulation of mRNA processing and/or mRNA export (**Fig. 5.1**). To substantiate these results, it would be interesting to investigate the influence of the interaction between CSF1R and FMIP, with or without MCSF stimulation, on mRNA processing or mRNA export.

Furthermore, the present study identified two new binding partners of FMIP/THOC5 namely, THOC7 and THOC1. The direct interaction and binding sites responsible for the interaction between FMIP/THOC5 and THOC7 were

characterised in this study. Remarkably, the FMIP-THOC7 association leads to the translocation of THOC7 into the nucleus. Previous data in our group show that PKC mediated phosphorylation of FMIP/THOC5 at serines 5 and 6 affects the translocation of FMIP into the nucleus and the mutation of both serines leads to the accumulation of FMIP/THOC5 in the nucleus (Mancini et al., 2004). Taken all these data, it would be interesting to examine whether the PKC mediated phosphorylation of FMIP influences THOC5-THOC7 interaction or affects nuclear translocation of THOC7 (**Fig. 5.2**).

The positive THOC1-THOC5 co-immunoprecipitation and the relevance of the C-terminal amino acid sequence for this interaction is a further result of the present work. Nuclear and cytoplasmic fractionations revealed that FMIP/THOC5-THOC1 association occurs in the nucleus. This demonstrates that FMIP is a common interacting partner for THOC1 and THOC7. The trimer complex THOC1-FMIP-THOC7 is detected in the nucleus. Surprisingly, THOC7 overexpression influences cell growth and adipocyte differentiaton. Further more its ectopic expression leads to caspase3 activation and thereby to apoptosis. However, the mechanism responsible for this activation is still unclear. Remarkably, nuclear localisation of THOC7, induced by inserting an ectopic nuclear localisation signal, and co-expression of THOC5 and THOC7 lead to a reduction of caspase3 activation. Furthermore, the complex previous studies showed that nuclear localisation of THOC1 leads to apoptosis. It could be possible that THOC5 may acts as a negative regulator that inhibits the THOC7 induced caspase3 activation and THOC1 induced apoptosis, and thereby acting as a positive regulator on cell proliferation, differentiation and apoptosis (**Fig. 5.2**).

The finding described in this thesis provide for the first time new insights into the role played by the members of THO complex (THOC5, THOC7, THOC1) in the regulation of caspase-3 activation, cell growth, differentiation and apoptosis.

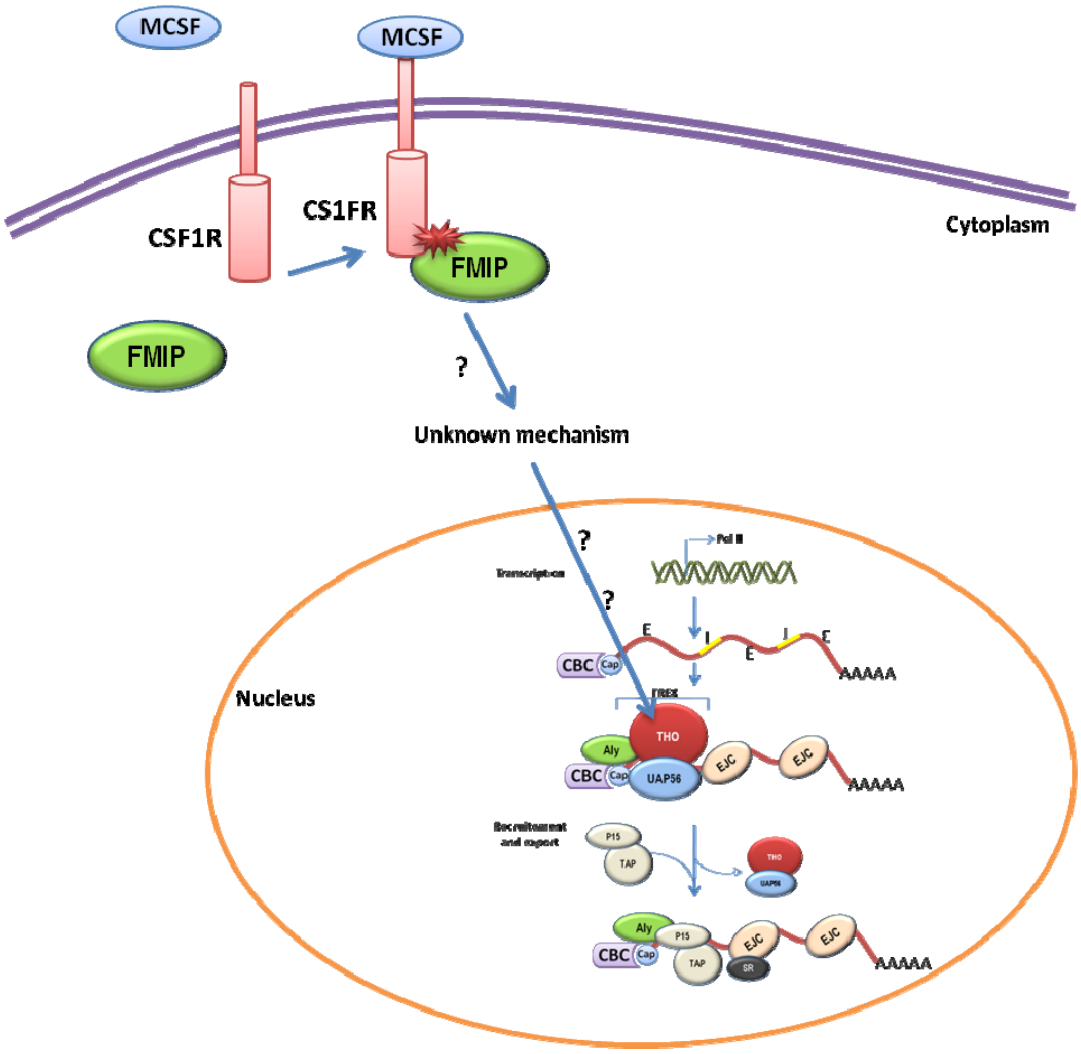

Fig. 5.1: Schematic illustration of the connection between extracellular signaling mediated by CSF1R rezeptortyrosine kinase and mRNA processing/splicing.

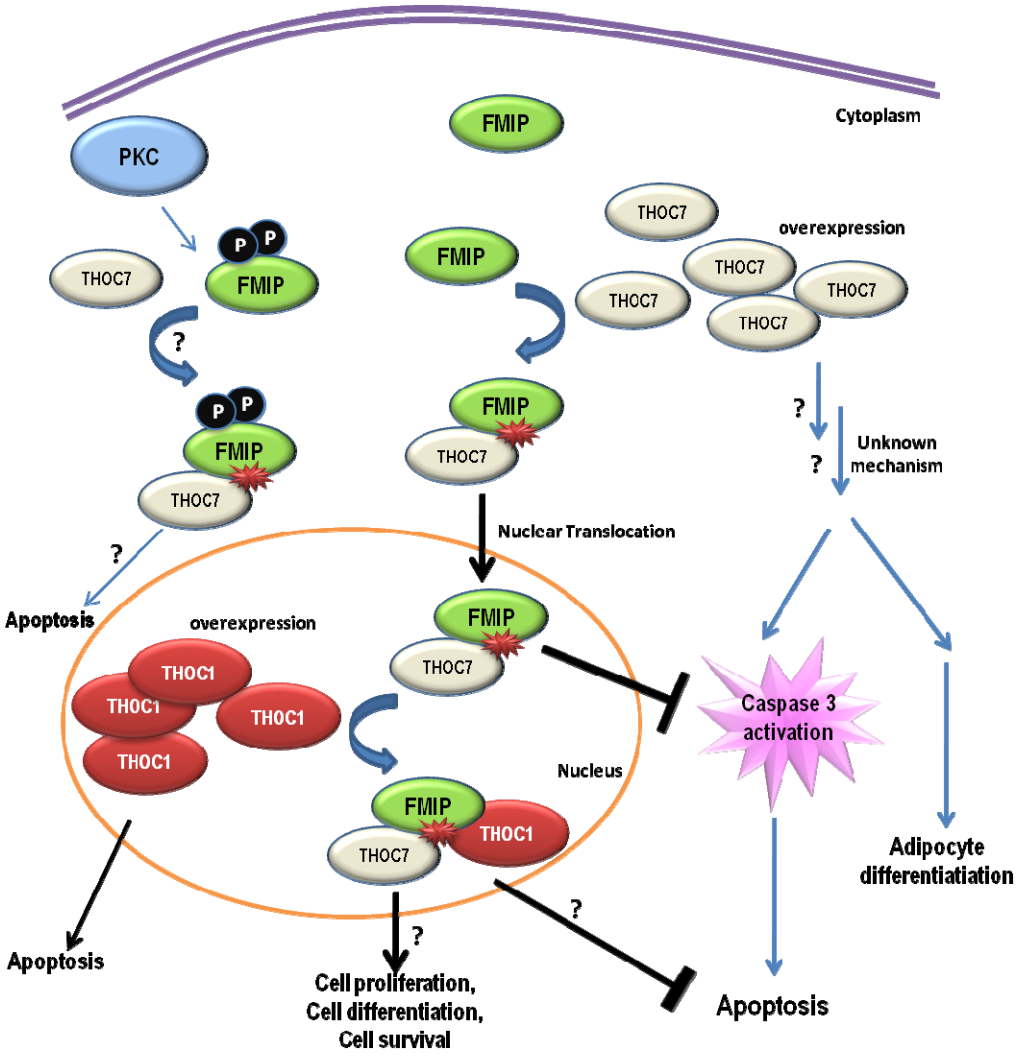

Fig. 5.2: A model for FMIP/THOC5 signaling function. FMIP/THOC5 binds directly THOC7 and leads to its nuclear translocation. Ectopic expression of THOC7 leads to caspase3 activation. This activation is reduced upon THOC5 mediated nuclear import of THOC7. THOC5-THOC7 association leads to the translocation of THOC7 into the nucleus. PKC mediated FMIP phosphorylation leads to accumulation of FMIP in the cytoplasm. This phosphorylation may enhance the THOC7 mediated caspase3 activation. The translocated THOC7-FMIP binds THOC1 in nucleus. This leads to formation of a trimmer complex THOC7-THOC5-THOC1, which may play a role in regulating ofcell proliferation, differentiation, survival or apoptosis.

Table3: All isolated proteins using TAP-tag purification of hFMIP-Myc

Gene name	Protein name	Function
Nuclear Proteins		
CDC2	Cyclin-dependent kinase 1	Cell cycle division control
CDC23*	Cell division cycle protein 23 homolog	Cell cycle, Cell division, Mitosis
CDK9	Cyclin-dependent kinase 9	Cell proliferation, RNA elongation from RNA polymeraseII promotor
CTBP1*	C-terminal-binding protein 1	negative regulation of cell proliferation
DPH1*	Diphthamide biosynthesis protein 1	Cell cycle, translation regulation
MAD2	Mitotic spindle assembly checkpoint protein MAD2A	Cell cycle, Cell division, Mitosis
RUVBL1*	RuvB-like 1	Cell cycle, cell division, growth regulation
RUVBL2*	RuvB-like 2	DNA rcombination growth regulation
SEP2*	NEDD-5	Cell cycle, cell division
SMC1A	Structural maintenance of chromosomes protein 1A	Cell cycle, Cell division, DNA damage, DNA repair, Meiosis, Mitosis
MCM2	DNA replication licensing factor MCM7	DNA replication and transkription regulation, Cell cycle,
ING5	Inhibitor of growth protein 5	Negative regulation of cell proliferation, Cell growth
ORC5L	Origin recognition complex subunit 5	DNA replication initiation
PCNA	Proliferating cell nuclear antigen	DNA replication, Cell proliferation
RFC2	Replication factor C subunit 2	DNA replication
RFC3	Replication factor C subunit 3	DNA replication
RFC4	Replication factor C subunit 4	DNA replication
RFC5	Replication factor C subunit 5	DNA replication
DIMT1L	Probable dimethyladenosine transferase	rRNA processing
NOL5A	Nucleolar protein 5A	rRNA processing
ASF/SF2	Splicing factor, arginine/serine-rich 1	mRNA processing, splicing
HNRNPA2B1	Heterogeneous nuclear ribonucleoproteins A2/B1	mRNA processing, splicing
HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2	mRNA processing, splicing
HNRNPK	Heterogeneous nuclear ribonucleoprotein K	mRNA processing, splicing

HNRNPM	Heterogeneous nuclear ribonucleoprotein M	mRNA processing, splicing
NSEP1	Nuclease-sensitive element-binding protein 1	mRNA processing, splicing
PCBP1	Poly(rC)-binding protein 1	nucleare mRNA splicing, via spliceosome
PCBP2	Poly(rC)-binding protein 2	nucleare mRNA splicing, via spliceosome
PCBP2*	Poly(rC)-binding protein 2	nucleare mRNA splicing, via spliceosome
PRPF6	Pre-mRNA-processing factor 6	mRNA processing, splicing
PRPF8	Pre-mRNA-processing-splicing factor 8	mRNA processing, splicing
SNRPB	Small nuclear ribonucleoprotein-associated proteins B and B'	mRNA processing, splicing
TARDBP	TAR DNA-binding protein 43	mRNA processing, splicing, transcription
THOC1	THO complex subunit 1	mRNA processing, splicing, transport
THOC2	THO complex subunit 2	mRNA processing, splicing, transport
THOC3	THO complex subunit 3	mRNA processing, splicing, transport
THOC4	THO complex subunit 4	mRNA processing, splicing, transport
UAP56	Spliceosome RNA helicase BAT1	mRNA processing, splicing
HIST1H2BC	Histone H2B	Nucleosome assembly
H2AFV	Histone H2A.V	Nucleosome assembly
HIST1H1D	Histone H1.3	Nucleosome assembly
HIST1H4H	Histone H4	Nucleosome assembly
ILF2	Interleukin enhancer-binding factor 2	positve reghulation of transcription
GTF2H2	General transcription factor IIH subunit 2	Transcription regulation, DNA damage, DNA repair
GTF3C3	Transcription factor IIIC subunit gamma	Transcription
HDAC2	Histone deacetylase 2	Transcription regulation, Cell cycle progression
HNRPDL	Heterogeneous nuclear ribonucleoprotein D-like	Transcription regulation, Stress response
HSF4	Heat shock factor protein 4	Transcription regulation, Stress response
POLR3D	DNA-directed RNA polymerase III subunit RPC4	Transcription
Cytoplasmic Proteins		
PC	Pyruvate carboxylase	Glyconeogenesis, lipid synthesis

PSMD3	26S proteasome regulatory subunit S3	proteasome
EEF1A1	Elongation factor 1-alpha 1	protein biosynthesis
EEF1G	Elongation factor 1-gamma	protein biosynthesis
CCT1	T-complex protein 1 subunit alpha	protein folding
EIF4A1	Eukaryotic initiation factor 4A-I	protein biosynthesis
EIF3E	Eukaryotic translation initiation factor 3 subunit E	protein biosynthesis
LDHB	L-lactate dehydrogenase B chain	Glycolysis
EIF3I	Eukaryotic translation initiation factor 3 subunit I	protein biosynthesis
PSMD1	26S proteasome non-ATPase regulatory subunit 1	proteasome
PFKL	Phosphofructo-1-kinase isozyme B	Glycolysis
RARS	Arginyl-tRNA synthetase	protein biosynthesis
PFKM	Phosphofructo-1-kinase isozyme A	Glycolysis
ACLY	ATP-citrate synthase	Lipide synthesis
EIF3F	Eukaryotic translation initiation factor 3 subunit F	
CASP8	Caspase-8	Apoptosis
EIF3B	Eukaryotic translation initiation factor 3 subunit B	protein biosynthesis
OTUB1	Ubiquitin thioesterase OTUB1	Ubiquitin dependent protein catabolic process
ADSS	Adenylosuccinate synthetase isozyme 2	Purine biosynthesis
EIF2B4	Translation initiation factor eIF-2B subunit delta	protein biosynthesis
GNB1	Transducin beta chain 1	Ras protein signal transduction
ASNS	Asparagine synthetase	amino-acid biosynthesis
LDHA	L-lactate dehydrogenase A chain	Glycolysis
AGGF1	Angiogenic factor with G patch and FHA domains 1	Angiogeneses, differentiation
EEF1B2	Elongation factor 1-beta	protein biosynthesis
PRDX1	Peroxiredoxin-1	cell proliferation, hydrogen peroxide catabolic process
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis
PKM2	Pyruvate kinase isozymes M1/M2	Glycolysis
EEF1D	Elongation factor 1-delta	protein biosynthesis
EIF2A	Eukaryotic translation initiation factor 2A	protein biosynthesis, Translation regulation

EIF3G	Eukaryotic translation initiation factor 3 subunit G	protein biosynthesis
MAP3K3	Mitogen-activated protein kinase kinase kinase 3	signal transduction,NF-kappa-B cascade
	c-AMP-Dependent Protein Kinase	
EIF4G2	Eukaryotic translation initiation factor 4 gamma 2	Cell cycle arrest, cell death, translation regulation
RAF1	RAF proto-oncogene serine/threonine-protein kinase	Apoptosis, cell proliferation
WASF2	WASP family protein member 2	G-protein signaling,
EIF3D	Eukaryotic translation initiation factor 3 subunit D	regulation of translational initiation
nucleear/cytoplasmic		
CSE1L	Exportin-2	protein transport
RUVBL2	RuvB-like 2	DNA rcombination growth regulation
SFN	14-3-3 protein sigma	DNA damage/ signal transduction resulting in apoptosis
RUVBL1	RuvB-like 1	Cell cycle, cell division, growth regulation
RANBP7	Importin-7	protein transport
DPM1	Dolichol-phosphate mannosyltransferase	
RANBP9	Importin-9	protein transport
KPNA2	Karyopherin alpha 2	protein import into the nucleus
RANBP5	Importin-5	prortein transport
P53	Tumor suppressor p53	Apoptosis, cell cycle
PSMA4	Proteasome subunit alpha type-4	regulation of Ubiquitin-protein ligase during mitotic cell cycle
PCBP2	Poly(rC)-binding protein 2	nucleare mRNA splicing, via spliceosome
CTBP1	C-terminal-binding protein 1	negative regulation of cell proliferation
RANBP11	Importin-11	protein transport
CCT2	T-complex protein 1 subunit beta	protein folding
Sep 02	NEDD-5	Cell cycle, cell division
	ER-protein	
DDOST	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	
	Tubulin alpha-1A chain	
	carbamoyl-phosphate synthetase 2,	
AP3D1	AP-3 complex subunit delta-1	
RPN2	Ribophorin II	
TRAPA	Translocon-associated protein subunit alpha	

PDHB	Pyruvate dehydrogenase E1 component subunit beta	
ERLIN1	Endoplasmic reticulum lipid raft-associated protein 1	
Mitochondrium proteins		
PHB	Prohibitin	transcription repressor activity
CLPX	ATP-dependent Clp protease ATP-binding subunit clpX-like	Protein folding
HK1	Hexokinase-1	Glycolysis
NDUFS2	NADH-ubiquinone oxidoreductase 49 kDa subunit	electron transport
Yme1l1	ATP-dependent metalloprotease YME1L1	Metalloprortease
SUCLA2	Succinyl-CoA synthetase, betaA chain	Succinyl CoA pathway
ACAT1	Acetyl-CoA acetyltransferase, mitochondrial	ketone body metabolism
ACAD9	Acyl-CoA dehydrogenase family member 9, mitochondrial	
NDUFA10	NADH-ubiquinone oxidoreductase 42 kDa subunit	
PDHA1	Pyruvate dehydrogenase E1 component subunit alpha	Glycolysis

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7 APENDIX

7.1 List of Figures

7.2 List of Tables

7.3 Abbreviations

2YT	modified Luria-bertani broth
AA	Amino acid
ADP	Adenosine diphosphate
Amp	Ampicilin
app.	Approximately
APS	Ammonium persulfate
ATP	Adenosine-5'-triphosphape
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumine
Ci	Curie
C-terminal	Carboxy-terminal
DMEM	Dulbecco´s modified Eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethlylemediaminetetraacetic acid
ER	endoplasmatic reticulum
FCS	Fetal calf serum
Fig.	Figure
FITC	Fluoresceine-isothiocyanate
FMIP	Fms interacting proteine
GAPDH	Glutamat dehydrogenase
GFP	Green fluorescent protein
GST	Glutathione S-transferase
GTP	Guaninetriphosphate
HEPES	N-2-hydroxyethylenepiperazine-N´-2-ethanesulfonic acid
hnRNP	Hetrogenous nuclear ribonucleoprotein
IgG	Immunoglobulin G
IP	immunoprecipitation
IPTG	Isopropyl ß-D-thiogalatopyranoside
kb	kilobasepairs
kDa	kiloDalton
M	molar
mM	millimolar
MOPS	3-(N-Morpholino) propanesulfonic acid
mRNA	Messenger RNA

NCBI	National Center of Biotechnology Information, USA
NEB	New England Biolabs
NLS	Nuclear localisation signal
NP-40	nonident P-40
nt	Nucleotide
N-terminal	Amino-terminal
OD	Optical density
OD600	opical density at 600 nm
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	-log10 c(H+)
Pre-mRNA	Precursor messenger RNA
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
rpm	Rounds per minute
rRNA	Ribosomal RNA
RT	Room temperature
RZPD	German Ressource Center for Genome Research
SDS	Sodium dodecylsulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SSC	Saline-sodium citrate buffer
TAP	tandem affinity purification
TBE	Tris-EDTA-borate buffer
TCA	trichloracetic acid
TEMED	N, N, N´, N´-tetramethyl-ethylene diamine
THOC	THO subunit complex
TREX	Transcription and export complex
Tris	Tris(hydroxymethyl)-amino-methane
Triton X-100	alkylpehnylpolyethylenglycol
TTP	Tymidinetriphosphate
Tween 20	Polyoxyethylene-sorbitan-monolaurate
Uniprot	universal protein data base
UTP	Uridine triphosphate
v/v	volume/volume
Vol	volume
w/v	weight/volume
wt	wild type
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
YPD	yeast complete medium

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Berufliche Aktivitäten

Okt 2007- Nov 2007	Betreuung der Studierenden Human- und Zahnmedizin im biochemischen Praktikum und Durchführung eines begleitenden Seminars
Nov 2006- Jan2007	Durchführung des Seminars “Onkogenese: Zelltransformationen und Rezeptortyrosinkinasen“ für Studierende der Biochemie (8.Semester)
Apr 2005- Feb 2006	Betreuung der Studierenden Human- und Zahnmedizin im biochemischen Praktikum
Jun 2000-Dez 2004	Betreuung der Studierenden der Human- und Zahnmedizin im chemischen Praktikum und Durchführung eines Praktikumsbegleitenden Tutorials

Publikationen:

Mancini A., Niemann-Seyde S.C., Pankow R., **El Bounkari O**., Klebba-Faerber S., Koch A., Jaworska E., Spoocer E., Gruber A. D., Whetton A. D., Koch A., Tamura T. **(2009)**. *M-CSF receptor interacting protein (FMIP)/THOC5, a member of the mRNA processing complex plays a role in maintenance of haematopoiesis in vivo*. ***Oncogene*** [Submitted]

El Bounkari O., Guria A., Klebba-Faerber S., Claußen M., Pieler T., Griffiths J.R., Whetton AD., Koch A., Tamura T. **(2008)**. *Nuclear localization of the pre-mRNA associating protein THOC7 depends upon its direct interaction with Fms tyrosine kinase interacting protein (FMIP)*. ***FEBS Lett.*** **583(1)**:13-8. Epub 2008 Dec 4.

Mancini A., **El Bounkari O**., Norrenbrock A.-F., Scherr M., Schaefer D., Eder M., Banham, A.H., PulfordK., Lyne L., Whetton A. D. and Tamura T. **(2007).** *FMIP controls the adipocyte lineage commitment of C2C12 cells by downmodulation of C/EBP alpha*. ***Oncogene*** **26**, 1020-7.

Koch A., Mancini A., **El Bounkari O**., and Tamura T. **(2005).** *The SH2-domain-containing inositol 5-phosphatase (SHIP)-2 binds to c-Met directly via tyrosine residue 1356 and involves hepatocyte growth factor (HGF)-induced lamellipodium formation, cell scattering and cell spreading*. ***Oncogene*** **24**, 3436-47

Erklärung zur Disseration:

Hiermit erkläre ich, Omar El Bounkari, dass ich diese Dissertation selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den

Omar El Bounkari