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Nuclear localization of the pre-mRNA associating protein THOC7 depends upon its direct interaction with Fms tyrosine kinase interacting protein (FMIP)

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ABSTRACT

THOC7 and Fms-interacting protein (FMIP) are members of the THO complex that associate with the mRNA export apparatus. FMIP is a nucleocytoplasmic shuttling protein with a nuclear localization signal (NLS), whereas THOC7 does not contain a typical NLS motif. We show here that THOC7 (50–137, amino acid numbers) binds to the N-terminal portion (1–199) of FMIP directly. FMIP is detected mainly in the nucleus. In the absence of exogenous FMIP, THOC7 resides mainly in the cytoplasm, while in the presence of FMIP, THOC7 is transported into the nucleus with FMIP. Furthermore, THOC7 lacking the FMIP binding site does not co-localize with FMIP, indicating that THOC7/FMIP interaction is required for nuclear localization of THOC7.

Structured summary:
MINT-6799962, MINT-6799973, MINT-6800005: *THOC7* (uniprotkb:Q6I9Y2) *physically interacts* (MI:0218) with *THOC5* (uniprotkb:Q13769) by *pull down* (MI:0096)
MINT-6800108: *FMIP* (uniprotkb:Q13769) and *THOC7* (uniprotkb: Q6I9Y2) *co-localize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-6800052: *FMIP* (uniprotkb:Q13769) *physically interacts* (MI:0218) with *THOC1* (uniprotkb: Q96FV9) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-6800022: *THOC7* (uniprotkb:Q6I9Y2) *physically interacts* (MI:0218) with *FMIP* (uniprotkb:Q6DFL5) by *pull down* (MI:0096)
MINT-6799989: *THOC7* (uniprotkb:Q6I9Y2) *binds* (MI:0407) to *FMIP* (uniprotkb:Q13769) by *pull down* (MI:0096)
MINT-6800071, MINT-6800089: *FMIP* (uniprotkb:Q13769) *physically interacts* (MI:0218) with *THOC7* (uniprotkb:Q6I9Y2) and *THOC1* (uniprotkb:Q96FV9) by *anti tag coimmunoprecipitation* (MI:0007)

1. Introduction

THOC7 was originally identified as a binding partner of a putative transcriptional repressor, Ngg1 interacting factor like 1 (NIF3L1) [1]. THOC7 contains 204 amino acids with a putative leucince zipper (LZ). Although THOC7 does not contain a nuclear localization signals (NLS) motif, THOC7 was detected in the nucleus and cytoplasm [1]. Fms-interacting protein (FMIP) was originally identified as a binding partner and substrate for several tyrosine kinases such as, Fms [2], Bcr-Abl, c-Kit (D816V), the Npm-Alk and Tel-PDGFβ receptor [3]. FMIP has also been identified as a downstream signaling molecule by insulin stimulation in pre-adipocytes [4] or by protein kinase C activation [5]. FMIP is a ubiquitous protein with a NLS, two LxxLL motifs, a putative PEST domain, and a putative LZ and is a nucleocytoplasmic shuttling protein [5].

Both THOC7 and FMIP have been found to be members of the THO complex, involved in mRNA processing [6,7]. The THO complex was first identified in *Saccharomyces cerevisiae* as a five-protein complex (THO2p, Hpr1p, Mft1p, Thp2p and Tex1p) [8–12] that plays a role in transcriptional elongations, nuclear RNA export and genome stability. In higher eukaryotes such as Drosophila melanogaster [6] or the human [13] system, three proteins (THOC1/hHpr1/p84, THOC2/hRlr1 and THOC3/fTex1) and three additional unique proteins were identified, namely FMIP/THOC5/fSAP79,

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THOC6/fSAP35 and THOC7/fSAP24/NIF3L1BP1, as members of the THO complex. It has been suggested recently that the THO complex binds to the 5′ end of mRNA via cap binding protein 80 and Aly/THOC4 [14]. However, by affinity purification using spliced human mRNPs under physiological conditions, FMIP and THOC7 were co-purified with Adenovirus pre-mRNA, but not with beta globin pre-mRNA [15]. Interestingly, another member of THOC, THOC6, was not detected in both cases. These data indicate that an individual member of the THO complex seems to bind to only a certain population of pre-mRNA as well as that all members of the THO complex may not function as a single subunit. Furthermore, it is not clear that the direct interaction partners of the whole six components in the complex. In this report we show that THOC7 interacts with FMIP but THOC6 does not. In addition, we have mapped the interaction domain of THOC7 to FMIP and also mapped the interaction domain of FMIP to THOC7. Finally, we show that interaction of THOC7 with FMIP is required for nuclear localization of THOC7.

2. Materials and methods

2.1. Cell culture, plasmid construction and DNA transfection

HEK293, mouse embryo fibroblasts (MEF) and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS. Generation of green fluorescent protein (GFP) and Myc/His tagged FMIP was described previously [2,5]. BamHI–SalI or BamHI–HindIII fragments obtained by PCR using THOC6 (IRATp970A1277D, RZPD, Germany) and THOC7 (IRATp970D0486D) as templates, were subcloned into pEGFP (Clontech Laboratories, CA, USA), pcDNA3.1Myc His (Invitrogen, Carlsbad, CA, USA), or pGEX5X-1 (Amersham Pharmacia Biotech Europe GmbH, Freiburg,

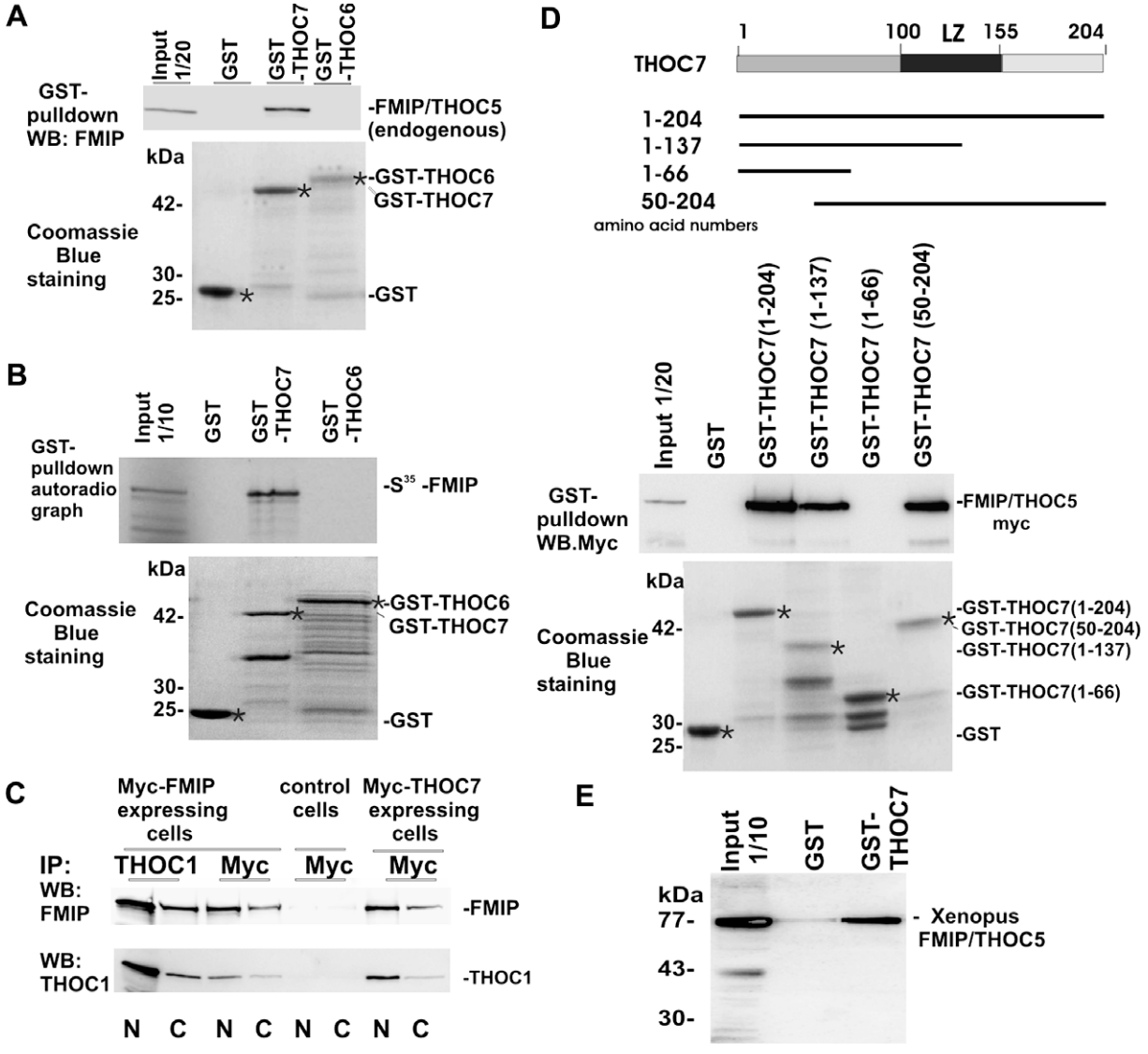

Fig. 1. FMIP forms a complex with THOC7. (A) Purified GST–THOC6, GST–THOC7 or GST (*: 2 μg each) were bound to GT-sepharose beads and incubated with HEK293 cell lysates. Bound protein was analyzed by SDS–PAGE followed by FMIP specific immunoblot. As a control, aliquots were analyzed by SDS–PAGE and staining with Coomassie brilliant blue (Coomassie blue staining). (B) ^{35}S-methionine labelled in vitro transcribed/translated FMIP (wt) and FMIP (1–289) were incubated with GST, GST–THOC7 and GST–THOC6 (*). Bound materials were analyzed by SDS–PAGE. (C) Myc-tagged FMIP (Myc-FMIP expressing cells) or THOC7 (Myc–THOC7 expressing cells) was expressed in HEK293 cells transiently and cell extracts obtained from nucleus (N) or cytoplasm were precipitated using anti THOC1 or anti-Myc antibody. Precipitates were analyzed by FMIP or THOC1 specific immunoblot. (D) HEK293 cells were transfected with Myc-tagged FMIP and cell lysates were incubated with GST, GST wild-type and mutants THOC7 (the number represents the amino acid number). Bound materials were analyzed by Myc-specific immunoblot. As a control, aliquots were investigated by SDS–PAGE and staining with Coomassie brilliant blue (Coomassie staining). *: GST and GST-fusion proteins. LZ: putative leucin zipper domain. E: *Xenopus* oocyte extracts were incubated with GST and GST–THOC7 and bound materials were analyzed by FMIP specific immunoblot.

Germany). DNA transfection was performed with the Polyfect™ reagent as described by the manufacturer (Qiagen, Hilden, Germany).

2.2. Antibodies

Monoclonal antibody against FMIP (F6d) was described previously [16]. Monoclonal antibody against Myc (9E10) was from Santa Cruz Biotechnology (Santa Cruz, USA). TRITC or FITC labelled anti-mouse or rabbit IgG were from Sigma–Aldrich GmbH. Monoclonal antibody against THOC1 (p84 N5) was from Gene Tex. Inc (San Antonio, TX, USA).

2.3. In vitro transcription/translation

In vitro transcription/translation in the presence of ^{35}S-methionine was performed using the TNT quick coupled transcription/translation system (Promega, Madison, USA) as described by the manufacturer.

2.4. Cell extract preparation

For the glutathione-*S*-transferase (GST)-pulldown assay, cells were extracted with lysis buffer containing 10 mM Tris–HCl, pH 7.6, 50 mM sodium fluoride (NaF), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA, 1% Triton X-100, 1% Trasylol (Bayer Vital, Leverkusen, Germany). For immunoprecipitation, the cytoplasmic fraction was prepared by buffer A (10 mM HEPES, pH 7.9, 1.4 mM $MgCl_2$ 10 mM KCl, 0.5 mM 1,4-dithiothreitol, 0.5 mM PMSF, 0.5% NP40 and 1% Trasylol). After centrifugation, the nuclear fraction was prepared with buffer A containing 375 mM KCl and 10% glycerol.

2.5. Western blot procedures

Details of immunoblotting have been described previously [17]. Results were documented on a LAS3000 imaging system (Fujifilm, Kanagawa, Japan).

2.6. GST-binding assay

Details of GST-binding assay have been described previously [18]. Binding materials were analyzed by SDS–PAGE and autoradiography or by immunoblotting.

2.7. Immunofluorescence

Cells were washed with ice-cold PBS, fixed in 3.7% formaldehyde in PBS for 20 min at room temperature, washed twice with PBS and permeabilized using 0.1% Triton X-100 in 5% FCS PBS for

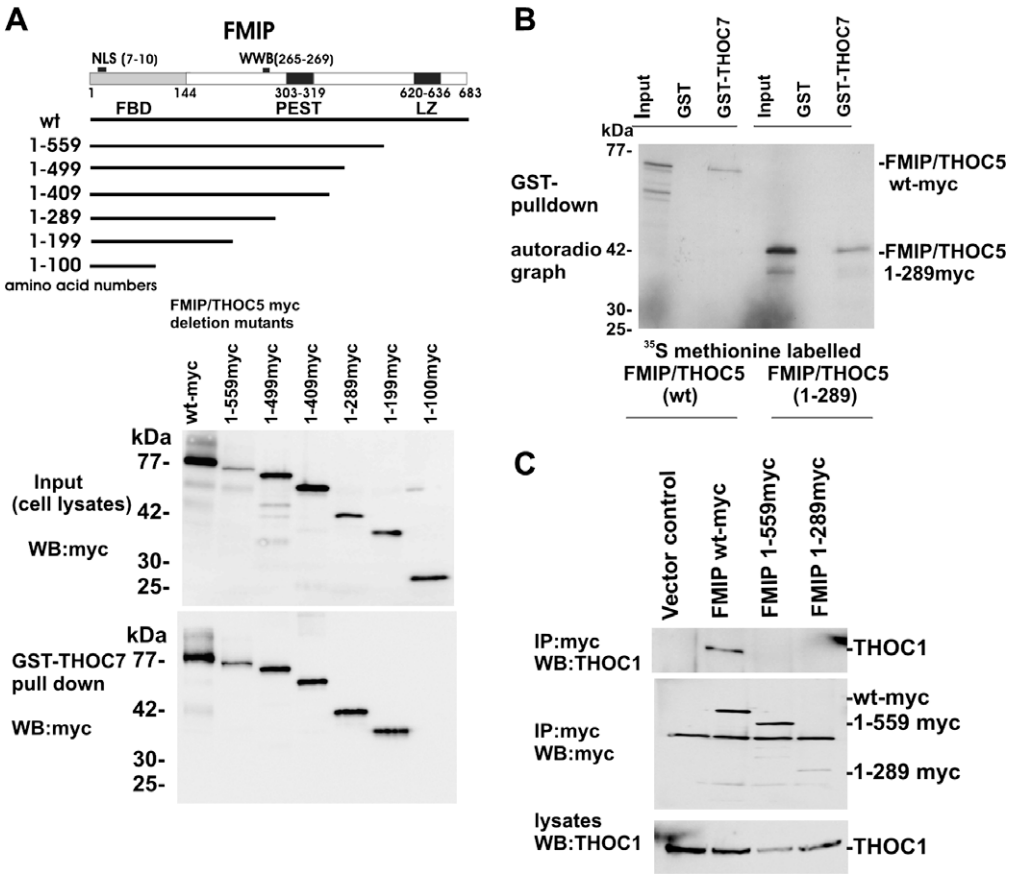

Fig. 2. N-terminal domain of FMIP (1–199) binds to THOC7 directly. (A) Wild-type and deletion mutants of FMIP (all numbers represent the amino acid numbers) were expressed in HEK293 cells transiently and cell extracts were incubated with GST or GST–THOC7 proteins. Bound materials were analyzed by Myc-specific immunoblot. As a control, inputs were analyzed by SDS–PAGE and staining with Myc-specific immunoblot (10% of input). NLS: nuclear localization signal; WWB: WW domain binding motif; PEST: PEST-like domain; LZ: a putative leucine zipper domain. (B) ^{35}S-methionine labelled in vitro transcribed/translated FMIP (wt) and FMIP (1–289) were incubated with GST and GST–THOC7. Bound materials were analyzed by SDS–PAGE. (C) Myc-tagged FMIP wt or its deletion mutants (1–559, 1–289) were expressed in HEK293 cells and extracts were then precipitated by anti-Myc IgG, followed by Myc or THOC1 specific immunoblotting.

1 min. Cells were incubated with anti-Myc antibody for 60 min at room temperature and stained using TRITC-anti-mouse IgG (Sigma–Aldrich GmbH, München, Germany) for 30 min.

3. Results and discussion

3.1. FMIP forms a complex with directly THOC7, but not with THOC6

Since unique members of the metazoa THO complex, THOC7, THOC6 and FMIP/THOC5 may not function as single subunit [15], we examined the binding partner among these three proteins. We first generated GST–THOC7 (52 kDa) and GST–THOC6 fusion proteins (63 kDa) (Fig. 1A, THOC7 antibodies are not available). These purified GST-fusion proteins were then incubated with HEK293 cell extract and bound proteins were first analyzed by FMIP/THOC5 specific immunoblot. GST–THOC7 precipitated endogenous FMIP, however, GST–THOC6 failed to do so (Fig. 1A). To examine whether THOC7 and FMIP interact directly, we utilized in vitro transcribed/translated FMIP for the GST–THOC7 and GST–THOC6 pull down assay. FMIP binds to THOC7, but not to THOC6, indicating that FMIP/THOC5 and THOC7 interact directly (Fig. 1B). To confirm that FMIP and THOC7 interact with each other in vivo, we transfected Myc-tagged FMIP, or Myc-tagged THOC7 into HEK293 cells, then nuclear and cytoplasmic extracts were precipitated with Myc antibodies. As expected, FMIP precipitates contain THOC1 (Fig. 1C, Myc–FMIP expressing cells), and THOC7 precipitates contain FMIP and THOC1 (Fig. 1C, Myc–THOC7 expressing cells). Furthermore, Myc-tagged THOC6 precipitates did not contain either FMIP or THOC1 in this condition (data not shown). We next mapped the binding domain of THOC7 to FMIP. For this purpose, we generated GST-fusion proteins carrying the second splice variant of THOC7 (1–137, all numbers represent the amino acid number) which is found in public databases (Accession No. NM_001013578), or deletion mutants THOC7 (1–66) and

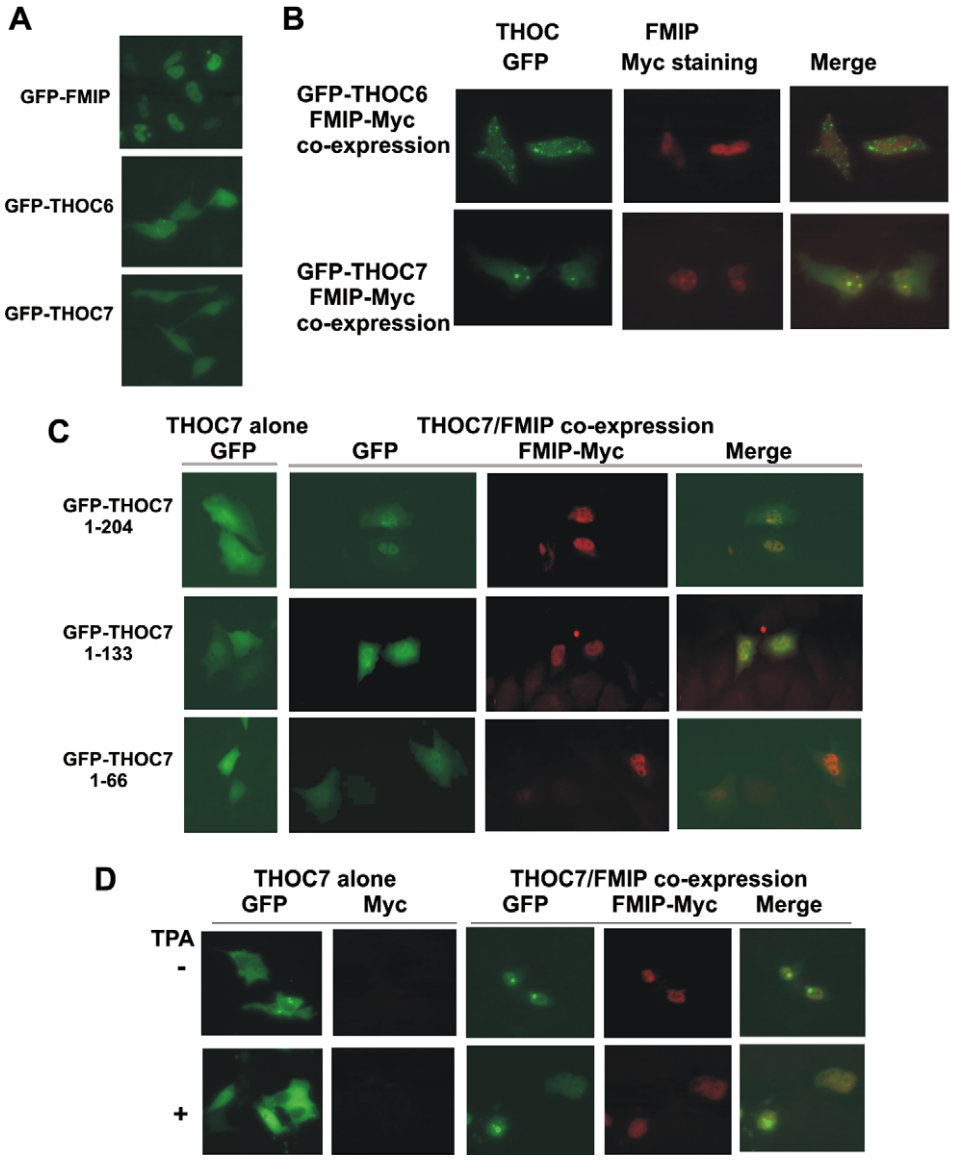

Fig. 3. FMIP recruits THOC7 in the nucleus. (A) Hela cells were transfected with pEGFP plasmids containing FMIP, THOC6 and THOC7 cDNA. (B) pEGFP–THOC6 (THOC6) and pEGFP–THOC7 (THOC7) were co-transfected with pcDNA3 containing Myc-tagged FMIP. Cells were stained with anti-Myc antibody followed by TRITC-conjugated anti-mouse IgG (Myc staining). (C) GFP–THOC7 (1–204), GFP–THOC7 (1–137) and GFP–THOC7 (1–66) were expressed in Hela cells with and without Myc-tagged FMIP. (D) GFP–THOC7 (1–204) with or without Myc-tagged FMIP were co-expressed in MEF and then treated with (+) and without (−) TPA for 2 h. Cells were stained with anti-Myc antibody followed by TRITC-conjugated anti-mouse IgG (FMIP–Myc).

THOC7 (50–204) (Fig. 1D). All GST–THOC7 deletion mutants except THOC7 (1–66) precipitated FMIP, suggesting that the FMIP binding domain of THOC7 is located within the center portion of THOC7 (50–137). This domain exhibits a part of the putative LZ domain (100–155, Fig. 1D) that may play a role in protein–protein interaction or protein–DNA interaction. Furthermore, the binding domain to FMIP differs from the NIF3L1 binding domain 1 (100–204) [1]. FMIP is conserved in all metazoa and *Xenopus* FMIP and THOC7 (public databases Accession Nos. NM_001087199, and NM_001087182) have 80% and 89% amino acid identity with human proteins, respectively. Since the mRNA export function of human Aly/THOC4 was measured using *Xenopus* oocyte system [14], we next examined whether *Xenopus* FMIP binds to GST–human THOC7. As shown in Fig. 1E, *Xenopus* FMIP binds to GST–THOC7. Furthermore, the monoclonal antibody raised against human FMIP [16] recognized *Xenopus* FMIP protein.

3.2. The N-terminal domain of FMIP (1–199) binds to THOC7 directly, while the C-terminal domain (559–683) is required for THOC1 association

We next examined the binding site of FMIP to THOC7. We generated six deletion mutants of Myc-tagged FMIP (1–559, all numbers represent the amino acid number), FMIP (1–499), FMIP (1–409), FMIP (1–289), FMIP (1–199) and FMIP (1–100) (Fig. 2A). FMIP (1–199) still binds to THOC7 but FMIP (1–100) does not, indicating that the FMIP binding site of GST–THOC7 is located at the N-terminal portion (1–199) (Fig. 2A). It is noteworthy that this domain was identified previously as the binding site for Fms tyrosine kinase (1–144) [2]. To confirm whether THOC7 and the N-terminal domain of FMIP interact directly, we utilized in vitro transcribed/translated FMIP (wt) and FMIP (1–289) for the GST–THOC7 pull down assay. Both FMIP (wt) and FMIP (1–289) bind to THOC7 (Fig. 2B). To examine whether the binding domain for THOC7 is different than the domain for THOC1, we once again utilized the FMIP deletion mutant. Myc-tagged FMIP (wt), FMIP (1–559) and FMIP (1–289) were expressed in HEK293 cells and cell lysates were precipitated with Myc antibody. THOC1 was detected only from precipitates with FMIP (wt), suggesting that the C-terminal domain (559–683) that contains a putative LZ domain (620–637) is required for THOC1 association with FMIP (Fig. 2C). The expression of C-terminal truncated FMIP reduces the total amount of THOC1 protein (Fig. 2C) and cells go into apoptosis (data not shown). It is not clear at present how mutant FMIP destabilizes THOC1. Interestingly, THOC1 is also nuclear–cytoplasm shuttling protein with the death domain [19]. It has been shown that overexpression as well as depletion of THOC1 cause apoptosis in several cells [20]. We are currently investigating the molecular mechanisms of the effect of FMIP mutants.

3.3. FMIP recruits THOC7 to the nucleus

It was shown that GFP–NIF3L1BP1/THOC7 fusion protein was located in the cytoplasm as well as in the nucleus [1], while we have shown previously that GFP–FMIP is mainly located in the nucleus [5]. Since THOC7 does not contain a typical NLS motif, we examined the nuclear import of THOC7. We first studied the subcellular localization of THOC7. As a negative control, we also examined GFP–THOC6 that does not bind to FMIP. In agreement with previous data, GFP–THOC7 is detected in the cytoplasm as well as in the nucleus, while GFP–FMIP is found exclusively in the nucleus (Fig. 3A). GFP–THOC6 was detected in the cytoplasm. Since the overexpression of THOC7 may cause impairment of stoichiometry of endogenous proteins, we next co-expressed GFP–THOC7 with Myc-tagged FMIP, all of which are directed by CMV promoter. To examine whether FMIP influences the subcellular distribution of THOC7, we co-expressed GFP–THOC7 with Myc-tagged FMIP. Interestingly, THOC7, but not THOC6 was detected mainly in the nucleus when FMIP was co-expressed (Fig. 3B), suggesting that THOC7 is imported into the nucleus together with FMIP. To confirm that the binding site of THOC7 to FMIP is important for co-localization, we co-transfected GFP–THOC7 (1–204), GFP–THOC7 (1–137) and GFP–THOC7 (1–66) with Myc-tagged FMIP. GFP–THOC7 (1–204) and (1–137) were detected mainly in the nucleus with FMIP, however, the location of THOC7 (1–66) did not change (Fig. 3C). We have shown previously that activation of protein kinase C (PKC) alpha induces the cytoplasmic translocation of FMIP [5]. Therefore, we next examined whether THOC7 is also translocated with FMIP upon stimulation with TPA, an activator of PKC-α. As shown in Fig. 3D, THOC7 was translocated with FMIP into the cytoplasm within 2 h, indicating that phosphorylation states of FMIP influence subcellular localization of THOC7. Furthermore, by affinity purification using spliced human mRNPs under physiological conditions, endogenous FMIP with endogenous THOC7 were co-purified in all cases, but not with THOC1, THOC2 and THOC6 [15], suggesting that the FMIP and THOC7 complex may act as one functional unit.

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