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## Characterization of hampin/MSL1 as a node in the nuclear interactome

Ruslan I. Dmitriev<sup>a</sup>, Tatyana V. Korneenko<sup>a,b</sup>, Alexander A. Bessonov<sup>a</sup>, Mikhail I. Shakhparonov<sup>a</sup>, Nikolai N. Modyanov<sup>b</sup>, and Nikolay B. Pestov<sup>a,b,\*</sup>

<sup>a</sup> *Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow 117997, Russia*

<sup>b</sup> *Department of Physiology, Pharmacology, Metabolism, and Cardiovascular Sciences, University of Toledo College of Medicine, Toledo, OH43614, USA*

### Abstract

Hampin, homolog of drosophila MSL1, is a partner of histone acetyltransferase MYST1/MOF. Functions of these proteins remain poorly understood beyond their participation in chromatin remodeling complex MSL. In order to identify new proteins interacting with hampin, we screened a mouse cDNA library in yeast two-hybrid system with mouse hampin as bait and found five high-confidence interactors: MYST1, TPR proteins TTC4 and KIAA0103, NOP17 (homolog of a yeast nucleolar protein) and transcription factor GC BP. Subsequently, all these proteins were used as baits in library screenings and more new interactions were found: tumor suppressor RASSF1C and spliceosome component PRP3 for KIAA0103, ring finger RNF10 for RASSF1C, and RNA polymerase II regulator NELF-C for MYST1. The majority of the observed interactions was confirmed in vitro by pull-down of bacterially expressed proteins. Reconstruction of a fragment of mammalian interactome suggests that hampin may be linked to diverse regulatory processes in the nucleus.

### Keywords

Hampin; MSL1; MYST1; interactome; TTC4; NOP17; NELF; RASSF1C; protein interactions

## INTRODUCTION

Hampin has been known for a long time as MSL1 protein of *Drosophila melanogaster* where its mutations are associated with Male-Specific Lethal phenotype [1]. We proposed a different name for its orthologs (*hampin* [2]) in order to emphasize the poor sequence conservation: even two drosophilid species show only 37% identity (*D. melanogaster* vs. *D. pseudoobscura*) and homology between mammalian hampin and *D. melanogaster* MSL1 is rather weak: only 26% identity is maintained, overwhelmingly in its C-terminal domain called IVpehe [3]. Primary structure of mammalian hampin was determined for the first time as a short partial sequence (Genbank Acc. Number **BM945758**) and full-length clones were identified later (Genbank Acc. Number **BC043039** etc).

\*Corresponding author. E-mail: korn@mail.ibch.ru.

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Some of the MSL1 and hampin functions accomplished via the IVpehe domain are identical: both human and *Drosophila* MSL orthologs are involved in multiprotein complexes (MSL) with histone acetyltransferase (HAT) activity [1,4], that is executed by MYST1/MOF. On the other hand, the *drosophila* MSL complex, called compensasome, seems to be highly specialized for one peculiar function, X-chromosome dosage compensation in males [1]. It should be noted that the use of compensasome for dosage compensation is known only in *drosophila* [1,5]. Also, mammalian MSL complex associates with all chromosomes [4] and its HAT activity is important for maintaining genome integrity, ATM-dependent DNA repair and tumor suppression [6–8].

It is interesting that murine hampin has at least five alternative splice variants [2]. These variants (A–E) contain 616 (GenBank acc. Number **NP\_082998**), 600 (**XP\_999185**), 463 (**XP\_999167**), 370 (**BAB29868**) and 233 (**ABD46887**) amino acid residues in its sequence and this diversity is a consequence of use of different exon sets. The longest variant A contains four domains: Pro-rich low complexity and highly variable domain I (absent in hampin D and E variants), short coiled-coil domain IIcc, modestly conserved domain III and highly conservative domain IVpehe (absent in C and E). All the variants were recently shown to be localized in nuclei [9].

RNA interference of either MYST1 or hampin A results in a decrease of histone H4 K16 acetylation [4]. Recombinant MYST1 is specific to H4 K16 *in vivo* but, *in vitro*, acetylates also H3 [6] indicating that its specificity may be attuned by other proteins. Apparently, the hampin/MSL1-MYST1/MOF association is conserved from arthropods to vertebrates. *Drosophila* MSL1 also interacts with MSL3 and MOF proteins via domain IVpehe and with MSL2 via domain IIcc [10]. It is known also that mammalian MYST1 participates in at least two protein complexes that possess histone acetylase activity, - one including proteins hampin A, MSL2, MSL3 and second complex including so-called “NSL”-proteins [11]. Both these complexes are evolutionary conserved, but interactions between their individual components are not well studied.

Therefore, it can be concluded that function both of hampin-MYST1 pair is poorly understood in the global interactome of mammals. To address this question, we searched for new protein-protein interactions of murine hampin and MYST1.

## MATERIALS AND METHODS

### Two-hybrid system

To construct the bait plasmids, the cDNA fragments corresponding to E266-K616 fragment of mouse hampin A was cloned by PCR into the GAL4 DNA binding domain fusion pGBKT7 vector at *Nde I/Sma I* sites. Bait-transformed yeast strain AH109 were mated with Y187 cells pretransformed with BD Matchmaker 17day mouse embryo cDNA library in pGADrec (pGAD-T7rec) vector (BD Biosciences) and grown on high stringency selection medium deficient for histidine, adenine, leucine, and tryptophan. Primary colonies were regrown several times on the selection medium with  $\alpha$ -X-Gal to select for induction of all three available reporter genes (HIS, ADE and MEL1). Plasmids were rescued from the positive clones by transformation of *E. coli* and retested to exclude false positives by cotransformation of AH109 cells with either hampin bait or empty pGBK plasmid. Plasmids clones passed all these tests were subjected to sequence determination.

### Dual-tag system and protein expression

Plasmids pHPMLQ, pCBDQ and pD1HTHQ were prepared by cloning fragments encoding T166-S371 (HPML), K1057-V1205 (CBD) fragments of human Plasma Membrane Calcium

ATPase (hPMCA4b) and M1-K394 of *E. coli* nicotinamide nucleotide transhydrogenase (NNT) in pQE42 vector (Qiagen) at *Bam*H I/ *Bgl* II sites (instead of DHFR coding sequence). These coding DNA sequences were amplified using PCR from pO plasmid (for hPMCA4b fragments) or from *E. coli* genomic DNA as template (for D1HTH). All other full-length ORFs were amplified by RT-PCR from various mouse cDNAs using specific primers and standard PCR conditions with the exception of hampin A and C: they required melting temperature to be set at 98 C. Murine ORFs for hampin A, C and RASSF1C were cloned into pD1HTHQ at *Bgl* II and *Hind* III sites, mouse KIAA0103 protein was cloned in pHPMLQ vector at *Bgl* II and *Sal* I sites. MYST1 - in pCBDQ at *Bgl* II / *Sma* I sites, NELF-C - at *Bgl* II and *Sph* I sites of pD1HTHQ. Full-length TTC4 ORF as well as a fragment corresponding to TPR-containing M1-R310 fragment (termed 51HIN) and mouse NOP17 full-length ORF were cloned into pQE30 vector (Qiagen) at *Bgl* II-*Bam* HI and *Hind* III sites. His<sub>6</sub>-tagged NOP17, 51HIN, D1HTH, CBD-MYST1, and CBD were expressed at +37 C for 30 min in presence of 1mM IPTG. Hampin fusions with D1HTH, His<sub>6</sub>-tagged TTC4, D1HTH-RASSF1C and D1HTH-NELFC were expressed for one hour at +19 C to avoid extensive degradation of the proteins. HMPL-tagged mouse KIAA0103 protein was expressed for 4 hours at +37 C.

### In vitro binding assays

To prepare soluble cell extracts, *E. coli* cells were lysed in TE buffer (10mM Tris, 1 mM EDTA, pH 8) containing 1 mg/ml lysozyme, protease inhibitor cocktail (Sigma P2714), 1 mM PMSF for 30 min on ice, then sonified and centrifuged for 15000 rpm 15 min (Beckman JA-20 rotor). For co-immunoprecipitation, *E. coli* extracts were mixed with 1  $\mu$ l of ascites fluid and incubated for one hour at room temperature. Then 3  $\mu$ l Protein G-sepharose was added and incubated for one hour on rotary shaker, washed three times with "wash" buffer (25mM Hepes pH 7.6, 0.5% NP40, 400 mM NaCl, 1mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>) and eluted by 50  $\mu$ l of electrophoresis sample loading buffer (2xSLB). For co-immunoprecipitation experiments with HPML-tagged KIAA0103 protein, the protein was purified in denaturing conditions and dialyzed against PBS. For pull-down experiments on calmodulin-sepharose, protein extracts were mixed in the same way except that 20  $\mu$ l calmodulin-sepharose instead of Protein G-sepharose was used and 2 mM CaCl<sub>2</sub> was included in all buffers. For D1HTH-NELFC pull-down on CBD-MYST1, 10 mM MgCl<sub>2</sub> was also added. For pull-down from myeloma cells, nuclear extract from myeloma cell line X63AG8.653 was prepared as described [4] except that proteins were extracted using PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS (RIPA). The nuclear extracts were incubated overnight with either 51HIN or D1HTH-hampin immobilized on 20  $\mu$ l Ni-NTA-agarose (Sigma N3158) at +4°C, washed three times and eluted using 2xSLB buffer. Western blotting was performed as described earlier [12] using chemiluminescent substrates ECL+ (Amersham Biosciences) or Immun-Star (Bio-Rad).

### Antibodies and immunocytochemistry

His<sub>6</sub>-tagged and HPML-tagged KIAA0103 proteins were purified as described [13]. Rabbit polyclonal antibodies against hampin were obtained using His<sub>6</sub>-tagged fragment K259-A463 of hampin C as described in [2]. His<sub>6</sub>-tagged 51HIN was used for immunization of rabbits and mouse, whereas His<sub>6</sub>-tagged NOP17 was used only for immunization of mouse. Monoclonal and polyclonal antibodies to D1HTH domain (2G10) and monoclonal antibodies 2D8 against first cytoplasmic loop and 3C12 against calmodulin-binding domain of hPMCA4b were described earlier [14] (N.B.P., unpublished data). Mouse 3T3 fibroblasts and the myeloma cells were grown in DMEM containing 10% fetal bovine serum. For immunofluorescence, 3T3 fibroblasts grown onto glass tissue slides were fixed in methanol at -20°C, blocked for one hour in 5% normal bovine serum (Difco), incubated with the antibodies (1:1000 dilution for rabbit and 1:200 for mouse antibodies, 50 mins at RT) washed with TBST (tris buffered saline with 0.1% tween-20), then incubated with anti-rabbit (Alexa Fluor 555 nm) or anti-mouse (Alexa Fluor 594 nm) secondary antibodies (1:1000 dilution), washed with TBST,

counterstained with DAPI (Molecular Probes), mounted in Anti-Fade medium (Molecular Probes) and images collected on a fluorescent microscopy equipped with a digital camera.

## Results

### Search of hampin protein interactions in two-hybrid system

To find new protein partners of hampin, we screened a 17 day mouse whole embryo cDNA library using yeast two-hybrid method with E266-K616 fragment of mouse hampin A as bait. Using high stringency selection conditions and verification of the clones in order to exclude obvious false positives, we identified several clones encoding five different proteins: histone acetyl transferase MYST1, two tetratricopeptide repeat proteins – mouse KIAA0103 protein and TTC4, C2H2 Zn finger protein GC BP, and a novel protein homologous to yeast NOP17 (Table 1). To widen the interactome map centered on hampin, we screened the same library for novel interactors of these five proteins. No positive clones were identified in case of TTC4 and NOP17. Screening for interactors of KIAA0103 gave RASSF1C and PRP3. RASSF1C attracted our attention because of its role in oncogenesis: we chose it for a further screen and obtained one new clone, identified as RNF10 (RING-finger protein 10). Screening of interactions for MYST1 gave NELF-C/TH1L and also one clone encoding hampin A itself (coordinates Y437-K616).

### Confirmation of interactions in vitro

To assess reliability of the interactions identified by the two-hybrid screenings, we tested them *in vitro*. These experiments were also aimed at investigating whether the interactions are direct and whether they require any modifications specific to eukaryotes. For this purpose, we expressed His<sub>6</sub>-tagged proteins NOP17 and TTC4 in *E. coli*. Expression of full-length hampin A and C was not successful either as His<sub>6</sub>-tagged proteins or as fusions with several other proteins, such as DHFR (results not shown). After these attempts, we decided to employ C-terminal fusions with nicotinamide nucleotide transhydrogenase domain I (D1HTH), and two domains of hPMCA4b (HPML or CBD). With these domains, we developed a new dual tag system useful for immunoprecipitation and pull-down. This system relies on available precipitating monoclonal antibodies against the tags (described in Methods). Finally, we successfully expressed in *E. coli* soluble full-length hampins A and C, RASSF1C and soluble NELF-C as C-terminal fusions with D1HTH, whereas MYST1 was expressed as C-terminal fusions with CBD. Expression of KIAA0103 was successful in the case of HMPL fusion.

Fig. 1 illustrates confirmation of identified interactions *in vitro*. Known interaction between hampin A and MYST1 was confirmed using our dual-tag system (see Supplementary Fig. S1.). Interactions of hampin with His-tagged TTC4 and HPML-tagged KIAA0103 proteins were tested by co-immunoprecipitation from *E. coli* extracts with D1HTH-tagged hampin A and C (Fig. 1A) or, in case of NOP17 protein, with D1HTH-tagged hampin A only (Fig. S2). It was shown that TTC4 co-precipitates with both hampin isoforms whereas KIAA0103 - only with hampin A. It would be also interesting if the observed interactions can be confirmed with endogenous proteins; D1HTH-tagged hampin A and C were used for pull-down of myeloma extract. NOP17 was detected in the eluate from immobilized hampin A, and was lacking in the eluate from hampin C (Fig. 1B). All these results indicate that domain IVpehe of hampin is required for binding NOP17 and KIAA0103 whereas domain III is necessary for interaction with TTC4.

Interaction MYST1-NELF-C was confirmed by pull-down from *E. coli* extracts using our dual-tag system (Fig. 1C) and interaction KIAA0103-RASSF1C was confirmed using co-immunoprecipitation from *E. coli* extracts (Fig. 1D).

In all cases when we were able to bacterially express protein pairs, we successfully detected the protein-protein interactions by pull-down/co-immunoprecipitation. This provides an estimate that interactions identified in this work by the yeast two-hybrid system have an excellent rate of true positives.

### Demonstration of nuclear localization of new hampin-interacting proteins

It is known that hampin is localized exclusively in nuclei [4,9]. However, TTC4 and NOP17 are almost unstudied; therefore we characterized them in more detail. We performed immunofluorescence experiments and showed that mouse proteins TTC4 and NOP17 are also localized predominantly in nuclear compartments (Fig. 2 and Fig. S3). Nuclear localization of NOP17 and TTC4 provides additional support for the existence of protein complexes with both TTC4 and NOP17 associated with hampin.

### Discussion

In this work, the interaction of mouse hampin/MSL1 with MYST1/MOF histone acetyltransferase has been found using yeast two-hybrid system, in accord with recent independent reports of Smith *et al* and [4] and Mendjan *et al* [11] where cultured human cells were used. This interaction requires domain IVpehe of hampin and is conserved from insects to mammals despite poor overall sequence conservation of hampin/MSL1. MYST1/MOF can be regarded as a catalytic subunit of the MSL complex, which function is acetylation of histone H4 at Lys16.

Functions of other identified hampin interactors are largely unknown. For example, the only available information about KIAA0103 is its presence in inner nuclear membrane [15]. Structurally, it belongs to TPR proteins; TPR is a repeat structure of typically 34 amino acids that is present in a large number of functionally diverse proteins [16]. Another TPR protein interacting with hampin is TTC4. Its gene was reported to be frequently damaged in breast cancer [17] and melanoma [18] (the latter is contradictory [19]). Its *Drosophila* homologue, Dpit47, has an interesting function as a nuclear co-chaperone of Hsp90 protein that interacts with DNA polymerase  $\alpha$  [20]. However, our results indicate that TTC4 binds both hampin A and C isoforms and, therefore, the hampin-TTC4 interaction apparently occurs through variable domain III.

No studies on mammalian NOP17 have been published to date. Its yeast homologue NOP17p is known to be located in nucleoli where it interacts with snoRNP protein NOP58p, exosome component Prp43p (homologous to mammalian OIP-2) and NOP53p. These proteins are involved in pre-rRNA processing [21].

GC BP is a C2H2-Zn-Finger transcription factor that suppresses interleukine-12 p35 gene in macrophages during phagocytosis of apoptotic cells [22]. GC BP stands for GC - binding protein because it binds a GC motif in the IL-12 p35 promoter. The GC BP gene is expressed in all tissues, therefore GC BP may have a broad physiological role not limited to regulation of interleukine-12.

Subsequent screenings using the hampin interactors as baits revealed several new interesting protein-protein interactions, for example, between MYST1 and NELF-C/TH1L. NELF-C/TH1L is a component of negative elongation factor (NELF) that is involved in promoter-proximal pausing by RNA polymerase II [23]. Also, NELF-C/TH1L negatively regulates protein kinase A-raf [24]. In this context, the interaction MYST1-NELF-C may link acetylation of H4 K16 to diverse processes including regulation of transcription elongation and A-raf signaling.

The identified binding of KIAA0103 to tumor suppressor RASSF1C [25] is especially interesting since it may reveal a liaison between H4 K16 acetylation and oncogenesis. Recently, RASSF1C was shown to localize to nuclei via anchoring to Daxx and to an unknown protein [26]. Hypothetically, RASSF1C translocation to nuclei may involve KIAA0103 and, indirectly, hampin A.

Our results together with published data allowed reconstruction of a mammalian nuclear interactome fragment centered on hampin (Fig. 3). Genes encoding homologs of KIAA0103, NOP17 and TTC4 exist in genomes from yeast to mammals, whereas true GC BP orthologs can be identified only in vertebrates. Therefore, many of these interactions may be well conserved. However, high-throughput screening of drosophila interactome yielded no high-confidence protein interactors for MSL1 [27]. Therefore, hampin appears to possess a functional profile more diverse than that of drosophila MSL1. This complexity may be even higher since hampin splice variants differ in domain composition.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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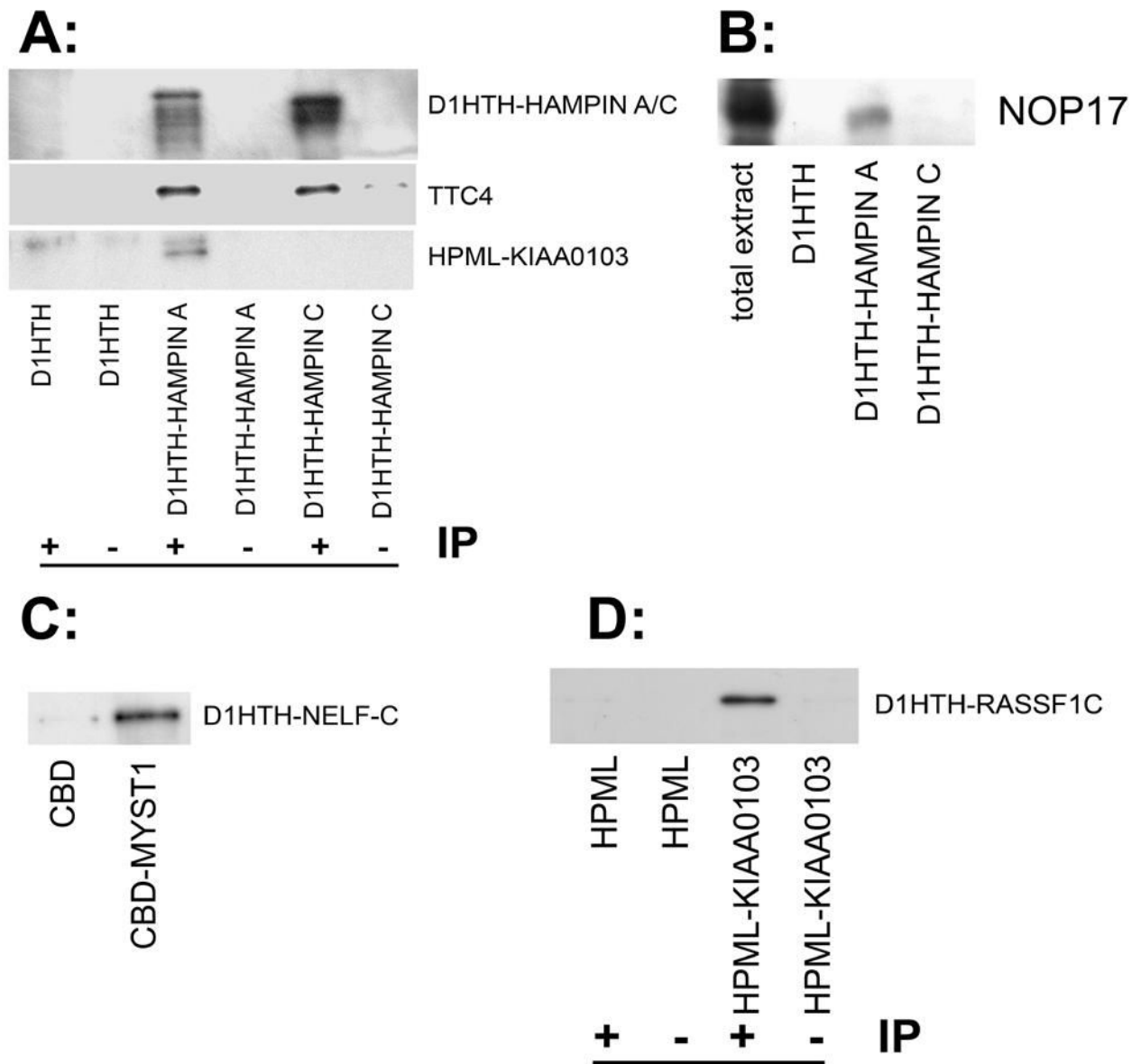
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## Abbreviations

**PBS**

phosphate buffered saline

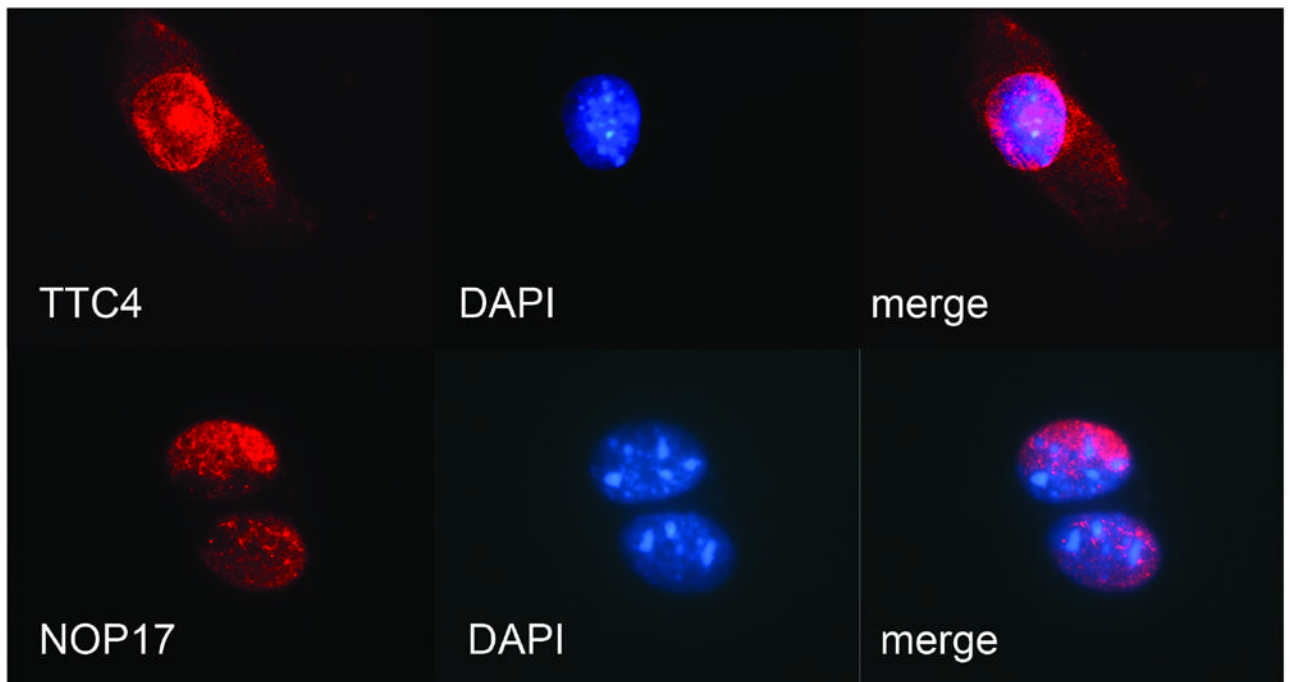




### Figure 1. Confirmation of observed interactions using *in vitro*-based methods

**A:** Results of co-immunoprecipitation (IP) of overexpressed D1HTH-hampin isoforms A and C His<sub>6</sub>-tagged proteins TTC4 and HPML-tagged KIAA0103 from *E. coli* extracts using monoclonal antibody to D1HTH (marked "+"). Co-precipitations with D1HTH polypeptide or with an unrelated antibody ("-") were used as controls. Western-blotting was done using rabbit anti-hampin, anti-TTC4 and anti-HPML antibodies (from top to bottom, respectively). Multiple bands of hampin isoforms A and C (upper panel) with lower molecular weights represent products of partial proteolytic degradation during bacterial expression. **B:** Pull-down of endogenous NOP17 protein from mouse myeloma nuclear extract on D1HTH-tagged hampin isoforms A and C, and D1HTH itself (negative control) immobilized on Ni-NTA-sepharose. Eluates from Ni-NTA agarose were stained with the help of mouse antibodies against NOP17. **C:** Pull-down of bacterially expressed D1HTH-tagged NELF-C on CBD-tagged MYST1 or CBD (negative control). Recombinant D1HTH-NELF-C was incubated together with CBD-MYST1 and then pulled down using calmodulin-sepharose. Beads were washed, and eluates

were probed with monoclonal antibody 2G10 specific to D1HTH. **D:** Co-immunoprecipitation of D1HTH-tagged RASSF1C with HPML-tagged KIAA0103 from bacterial extracts. Proteins HPML or HPML-tagged KIAA0103 were precipitated with monoclonal anti-HPML antibody 2D8 from bacterial extract containing D1HTH-RASSF1C. Eluates from protein-G-sepharose were analyzed using rabbit antibodies against D1HTH.



**Figure 2. Subcellular localization of endogenous proteins TTC4 and NOP17 in 3T3 fibroblasts**  
Mouse 3T3 fibroblasts were labeled with polyclonal antibodies against TTC4 (top) or NOP17 (bottom) followed by secondary antibodies conjugated with Alexa Fluor (red fluorescence). Nuclei counterstained with DAPI (blue fluorescence). From left to right: antibody labeling, DAPI and merged images.



**TABLE 1**

Identification of protein-protein interactions from two-hybrid library screenings

Bait	Interactor	GenBank number	Amino acid coordinates	Length; Domains	Known or hypothetical functions of the interactors
Hampin	KIAA0103/ TTC35	NP_080012	Full length	297; TPR2	None
	TTC4	NP_082485	Full length	386; TPR	Putative co-chaperone and tumor suppressor
	GC BP NOP17	NP_666371 NP_083682	P83-C terminus Full length	619; C2H2 Zn finger 290; NOP17	Transcription factor, regulates IL12 p35 gene in macrophages Putatively involved in pre-rRNA processing
	MYST 1	NP_080646	G39-C terminus	458; MYST; C2HC Zn Finger; chromodomain;	Histone acetyl transferase
KIAA0103	RASSF1C	NP_062687	Full length	270; RA	Tumor suppressor
	PRP3	NP_081817	E291-C terminus	683; PWI	Splicing factor
MYST1	NELF-C/ THIL	NP_065605	Full length	592	Negative regulator of RNAPII transcription elongation and A-raf protein kinase
	Hampin A	AK_014463	Y437-K616	616	Component of histone acetyl transferase complex
RASSF1C	RNF10	NP_057907	G372-C terminus	805	None