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TRAP150 在細胞核核醣核酸處理過程的功能探討

Characterization of the roles of TRAP150 in nuclear mRNA processing

李國銘

Kuo-Ming Lee

指導教授：譚婉玉 教授

Dr. Woan-Yuh Tarn

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國立臺灣大學博士學位論文
口試委員會審定書

論文中文題目:

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論文英文題目

Characterization of the roles of TRAP150 in nuclear mRNA processing

本論文係 李國銘 君（學號 D94448004）在國立臺灣大學分子醫學研究所完成之博士學位論文，於民國 99 年 06 月 03 日承下列考試委員審查通過及口試及格，特此證明

口試委員：

譚婉玉 （簽名）

（指導教授）

系主任、所長 （簽名）

中文摘要

TRAP150 起初被發現為轉錄活化複合體 TRAP150/Mediator 的一個次單位。此外，TRAP150 亦被發現為剪接體的其中一員，說明其可能在訊息核醣核酸剪接中扮演特定的角色。我們最近的報導指出，TRAP150 可與其他剪接分子共定位於細胞核內的核斑點中。TRAP150 不僅是活體內訊息核醣核酸剪接過程所必須，而其大量表現時，更可大幅活化此剪接過程。我們發現訊息核醣核酸剪接完成之後，TRAP150 仍與剪接產物結合，我們亦發現 TRAP150 可直接與數個 exon jounction complex 的組成蛋白及運送訊息核醣核酸出核的受器 TAP 進行結合。此觀察指出 TRAP150 可能具有調控訊息核醣核酸剪接後事件的功能。當 TRAP150 被束縛在先驅訊息核醣核酸上時，TRAP150 可促進剪接完成的訊息核醣核酸的降解。然而，與典型的無意義媒介訊息核醣核酸降解機制不同之處為，TRAP150 調控的訊息核醣核酸降解不依賴轉譯，且發生在細胞核中。儘管 TRAP150 可藉由不同的獨立區塊活化訊息核醣核酸的剪接以及訊息核醣核酸的降解，我們並不清楚此二功能確切的運作機制。最近，透過尋找 TRAP150 結合蛋白，我們發現數個腺苷酸化相關蛋白質與其結合。因此，TRAP150 可能會協調細胞核內核醣核酸處理過程的不同步驟，並且使異常加工的核醣核酸降解。

關鍵詞：先驅訊息核醣核酸剪接; 訊息核醣核酸降解; 剪接體; exon-junction complex; 訊息核醣核酸處理

Abstract

TRAP150 was initially identified as a subunit of the transcription coactivator TRAP/Mediator complex. In addition, TRAP150 has also been detected as a component of the spliceosome, suggesting its role in pre-mRNA splicing. We recently reported that TRAP150 colocalizes with splicing factors in nuclear speckles. TRAP150 is not only required for pre-mRNA splicing *in vivo*, but also substantially activates splicing when overexpressed. We found that TRAP150 remains bound to the spliced mRNA after splicing and accordingly interacts directly with several components of the exon junction complex and the mRNA export receptor TAP. This observation suggests that TRAP150 has a post-splicing function. When tethered to a precursor mRNA, TRAP150 could induce degradation of the spliced mRNA. However, in contrast to the canonical nonsense-mediated decay, TRAP150-mediated mRNA decay is translation independent and occurs in the nucleus. TRAP150 activates pre-mRNA splicing and mRNA degradation via distinct domains, but how it exactly acts in these two events is not yet clear. We recently screened for TRAP150-interacting proteins and found that TRAP150 also associated with polyadenylation factors. Therefore, TRAP150 may function in coordinating different steps of nuclear mRNA processing and perhaps target aberrantly processed mRNAs for degradation.

Keywords: pre-mRNA splicing; mRNA decay; spliceosome; exon-junction complex; nuclear mRNA processing

1. Introductions

Gene expression in eukaryotic cells is regulated by three different RNA polymerases, and each polymerase is dedicated to distinct sets of RNAs. RNA polymerase I (Pol I) is required for the expression of ribosomal genes except for that of the 5S RNA, which is synthesized by RNA polymerase III (Pol III) that is involved in the generation of some short transcripts like transfer RNAs (tRNAs). The spliceosomal small nuclear RNAs (snRNAs) and the bulk messenger RNAs (mRNAs) encoding for assorted proteins are transcribed by RNA polymerase II (Pol II) (1). Pre-messenger RNA (pre-mRNA) processing in eukaryotic cells is characterized by several distinct steps. After transcription initiation, 5' end capping, splicing and 3' end cleavage/polyadenylation help the maturation of mRNAs in the nucleus. Through specific interaction with nuclear pore complex (NPC) component, mRNA export receptor TAP could help the export of mature mRNAs to the cytoplasm where translation occurs to decode the message carried by individual mRNA (2). Different mRNA processing factors are involved in each step of mRNA metabolism and assembled dynamically into the messenger ribonucleoprotein particles (mRNPs). Although each RNA processing step could function independently *in vitro*, recent studies have provided the evidences that all pre-mRNA processing steps are intimately coordinated and coupled to transcription (2,3) as was revealed by extensive studies focused on the carboxyl terminal domain (CTD) of the largest Pol II subunit (Rab1).

1.1. Pol II CTD in transcription-coupled processes

The CTD of the largest Pol II subunit is a relatively unstructured domain located below the RNA exit channel and is composed of several heptapeptide ($Y_1S_2P_3T_4S_5P_6S_7$) repeats (26 repeats in *Saccharomyces cerevisiae* while 52 in

mammals). Residues within the heptapeptide are subject to several reversible post-translational modifications. For example, tyrosine residue at position 1 (Tyr1) and serine residues at position 2, 5 and 7 (Ser2, Ser5 and Ser7 respectively) within the hepatapeptide are subject to phosphorylation and glycosylation in a mutually exclusive manner (4). The phosphorylation status of Ser2 and Ser5 residues changes as transcription proceeds from initiation to termination. Upon transcription initiation, the general transcriptional factor TFIIH phosphorylates Ser5 residue through its CDK7 component. During the elongation stage, the elongation factor pTEFb phosphorylates Ser2 residue through its CDK9 component, and the previously phosphorylated Ser5 (Ser5P) is restored by several CTD phosphatases (5,6). Overall, the polarity of CTD phosphorylation, from Ser5P at transcription initiation to Ser2P during elongation and termination, is generated throughout the transcription.

The different phosphorylation status of CTD serves as a platform of recruiting various RNA processing factors to nascent transcripts. Pol II transcripts are characterized by unique m7GpppN cap structures at 5' end, which is the first step of pre-mRNA processing. Three enzymatic activities including RNA 5'-triphosphatase (RT), guanylyltransferase (GT) and methyltransferase (MT) mediate the formation of 5' cap. The RT cleaves the 5' triphosphate terminus of the pre-mRNAs to the diphosphate group, which is subsequently recognized and capped with GMP by GT, and the N7 position of guanine nucleotide could be methylated by MT (7). Once associated with the cap binding complex (CBC), nascent RNAs could avoid the access to nuclear 5'-to-3' exonucleases (8). The CBC also functions to recruit other RNA processing factors involved in the downstream processing events including splicing, 3' end formation and mRNA export, and thus serves as a check point to ensure that the transcripts could be further processed (9,10). In budding yeast, the capping enzyme is a heterodimer consisting of RT (Cet1) and GT (Ceg1) subunits,

however, the mammalian capping enzyme is a bifunctional protein with RT and GT activities (11,12). The GT domain of the mammalian capping enzyme specifically recognizes Ser5P CTD, which allosterically activates the activity of GT (12). Therefore, when transcription is initiated, subsequent recruitment of capping enzymes by Ser5P CTD could immediately modify the nascent transcripts.

Besides 5' end capping, 3' end processing of pre-mRNAs also depends on the presence of CTD. All mRNAs except for histone mRNAs contain the poly (A) tail, and the 3' end processing in mammalian cells is coordinated by several trans-acting factors and cis-acting elements. First of all, the AAUAAA cleavage and polyadenylation sequence (CPS) will be recognized by the cleavage/polyadenylation specificity factor (CPSF) while the downstream element (DSE) is bound to the cleavage stimulation factor (CstF). Later, cleavage that occurs predominately at the CA dinucleotide located between CPS and DSE is assisted by two essential factors, cleavage factor I (CFIm) and cleavage factor II (CF IIm) (13). Finally, the Poly (A) polymerase directs the addition of adenosine, and the poly (A) tails will be bound by poly (A) binding proteins (PABPs) to prevent RNAs from degradation by the 3'-to-5' exonuclease complex, exosome (8). In yeast, several 3' end processing factors including CstF50, Yhh1 and Rna15 have been described to interact with phosphorylated CTD. Moreover, the CPSF has been reported to function as a component of the active TFIID and remain with the elongating Pol II to the gene body (6,10,14,15). In contrast to the specific interaction between Ser5P CTD and capping enzyme, most of 3' end processing factors interact with phosphorylated CTD without any preference for distinct phosphoisoforms. However, Pcf11, the component of the yeast cleavage factor 1A (CF1A), preferentially interacts with Ser2P CTD through its CTD interacting domain (CID) (2,4,16). Furthermore, in cooperated with another CID containing factor Rtt103, Pcf11 functions in the transcription termination probably

through the specific recognition of Ser2P CTD, and subsequently recruits 5'-to-3' exonuclease Xrn2 to help the release of ongoing Pol II from the transcripts (17,18). Interestingly, recent studies in yeast identified another termination process triggered by another CID containing protein Nrd1. Nrd1 has been shown to form a complex with Nab3 and Sen1, and participate in the termination of both the cryptic unstable transcripts (CUTs) and stabilized small nucleolar RNAs (snoRNAs) (19). In contrast to the Ser2P CTD-dependent Pcf11-mediated cleavage, Nrd1 preferentially binds to the Ser5P CTD and dominates the cleavage of shorter transcripts (20,21).

In addition to the 5' end capping and 3' end processing, splicing also couples to the transcription, especially in the case of higher eukaryotic cells that usually contain numerous introns with huge sizes (> 10 kbs) in the genome. Introns are intervening sequence spanning the coding regions and are needed to be accurately excised from the pre-mRNAs by spliceosomes. The spliceosome is a large RNP complex composed of five snRNAs and nearly 150 protein factors. These proteins include the members of the serine-arginine rich (SR) protein family that could recognize splice site signals and mediate the interaction between snRNAs and pre-mRNAs to promote the spliceosome assembly (2,22). The truncation of the Pol II CTD inhibits not only the 3' end processing and termination but also splicing. The CTD is required for the recruitment of pre-mRNA splicing factors to active transcription sites *in vivo*, and overexpression of CTD-containing Pol II could result in the redistribution of splicing factors in the nucleus (3,23,24). Factors involved in the cross-talk between transcription and splicing include SR-like CTD associated factors (SCAFs), $p54^{nrb}$, PTB-associated splicing factor (PSF), CA150, UsnRNPs and SR proteins. Although $P54^{nrb}$ and PSF show interactions with CTD without any phosphoisoform preference, CA150 could recognize the phosphorylated CTD through the FF domain. Moreover, CA150 could mediate the splicing factors assembly at the 3' splice sites through the

WW domain (3,4,25). Besides CA150, SR proteins also function in the connection between transcription and splicing. By specific interaction with Pol II through SR proteins, U1 snRNP/SR proteins might involve in the early assembly of spliceosomes at the site where nascent pre-mRNAs are synthesized. The coupling between transcription and splicing prevents the formation of heterogeneous ribonucleoprotein particle (hnRNP) complex that might affect the stability or the downstream processing of the pre-mRNAs (26).

Therefore, through different phosphorylation status of CTD in the whole transcriptional process, different RNA processing factors are recruited cotranscriptionally, but temporally and spatially, to newly synthesized transcripts for efficient mRNA maturation. However, factors other than Pol II could also help the processing of nascent transcripts. The synthesis of pre-mRNAs is accompanied with the immediate loading of various RNA binding proteins, and the composition of binding proteins determines the fates of the specific transcripts (8). Examples of the cotranscriptional processes came from the studies on the transcription/export (TREX) complex and exon-junction complex (EJC).

1.2. Coupling TREX complex in mRNP synthesis

TREX is an evolutionary conserved complex composed of the multi-subunit THO complex (tetramer in yeast while pentamer in metazoans), the mRNA export protein UAP56 (Sub2 in yeast) and Aly/REF (Yra1 in yeast) (27). Aly/REF is a conserved mRNA export adaptor and enhances the interaction between mRNA and mRNA export receptor, TAP/P15 (Mex67/Mtr2 in yeast) (28-30). UAP56 belongs to the DEAD box family of ATP-dependent RNA helicase and mediates the assembly of spliceosome through its ATPase activity (31). It also involves in mRNA export through the direct interaction to Aly/REF. When overexpressed, excess UAP56

inhibits the recruitment of Aly/REF to the spliced mRNPs and can function as a dominant negative inhibitor of mRNA export. Consequently, loss function of Sub2 in yeasts results in the accumulation of poly (A) $^{+}$ RNAs in the nucleus, a similar phenotype revealed by Yra1 deficiency (32,33).

Although the function of THO complex in metazoan is less clear, extensive studies in yeast have been documented. Yeast THO complex is composed of four subunits including Tho2, Hpr1, Mft1 and Thp2. Transcription impairment, hyper-recombination and defective mRNA export could be observed with deficiency of either subunit, indicating the importance of the THO complex in transcription elongation and mRNA export (34,35). These phenotypes could be suppressed when the function of Sub2 or Yra1 was impaired and a stoichiometrical association between the THO complex and both Sub2 and Yra1 was identified. The THO/Sub2/Yra1 complex designated for TREX complex and its human counterpart with similar function has also been isolated (36,37). The THO complex mainly associates with DNA through the direct interaction with Pol II and the subsequently recruited Sub2 and Yra1 help the export of mRNAs (38). However, although Yra1 binds to the intronless RNAs as well as intron-containing ones in yeast, Aly/REF is specifically loaded to spliced RNAs but not unspliced ones in higher eukaryotic cells, indicating that the recruitment of the TREX complex depends on the occurrence of splicing (27,28). Furthermore, Cheng et al. demonstrated that the TREX complex recruitment in mammalian cells not only depended on the splicing but also on CBP80, a component of CBC. Therefore, the mammalian TREX complex is recruited to the 5' end of mRNAs (39). As the consequence, mRNAs are exported through the direction from 5' end to 3' terminus and the translational machinery could engage at the 5' end of the mRNA transcripts. The subsequently active translation could facilitate the mRNA export from the nucleus (40).

Similarly, another supramolecular complex, termed TREX2, also functions in the linkage between transcription and mRNA export. The TREX2 complex consists of the Sac3, Thp1 and SAGA histone acetylase complex. Sac3 is a NPC-associated protein while Thp1 is important for the transcription elongation and genome stability. Through the direct interaction with Sus1, a component of the SAGA complex, the actively transcribed genes are linked to the periphery of NPC and thus facilitate mRNA export, a process called NPC gene gating (41,42). Interestingly, Koptova et al. recently provided the evidence that the *Drosophila* Sus1, ENY2, could directly interact with the THO complex and mediate their recruitment onto newly synthesized RNAs. Therefore, the recruitment of the THO complex in higher eukaryotic cells also could occur cotranscriptionally (34,43).

In addition to the transcriptional regulation, splicing also results in the remodeling of mRNPs in higher eukaryotic cells, and the EJC serves as another example to illustrate the coordination of different RNA processing steps from nuclear to cytoplasmic events.

1.3. Versatile EJC in mRNP metabolism

EJC is a post-splicing complex deposited at position 20-24 nts upstream of the exon-exon junction (44). In contrast to other RNA binding proteins, the loading of EJC depends on the position rather than sequence. Therefore, all intron-containing transcripts would be decorated with EJC once splicing occurs. Four proteins including Y14, Mago, eIF4AIII and MLN51 form the heterotetrameric core of EJC which remains on the mRNA along with the export pathway to the cytoplasm until translation occurs. The EJC core component grips on the mRNA and serves as a dynamic interacting platform for various RNA processing factors (45).

A well known function of EJC is the regulation of nonsense-mediated decay

(NMD), which might be the most important function of EJC. NMD is a quality control system to detect the transcripts with the premature termination codon (PTC), which might be translated into C-terminal truncated proteins and become deleterious to the cells. The PTC is defined as the stop codon located at the position larger than 50-55 nts upstream of the last exon-exon junction, and consequently at least one EJC is positioned downstream of the stalled ribosome (46). When PTC is detected by the ribosome scanning during the pioneer round translation, the stalled ribosome will recruit the SURF complex consisting of SMG1, Upf1, eRF1 and eRF3. Upf1 belongs to the group 1 RNA helicase and could be phosphoryated by SMG1 on the C-terminal serine-glutamine (SQ) motif. Phosphorylation and dephosphorylation cycles of Upf1 contribute to the remodeling of mRNA surveillance complex and are required for NMD in mammals and *C. elegans* (47,48). The NMD factor Upf3b has been shown to be copurified with EJC, and the interaction between Upf3b and Upf1 through Upf2 activates SMG1 (45,49). The subsequent phosphorylation of Upf1 inhibits the additional rounds of translation through its direct interaction with translation initiation factor eIF3 and then triggers the decay of PTC-containing transcripts (50). So far, the downstream pathway to degrade PTC-containing mRNAs is not clear, but both 5'-to-3' and 3'-to-5' exonucleases are able to initiate the degradation (51).

Besides NMD, several splicing factors including SRm160m, RNPS1 and Pinin are also reported to interact with the core EJC component (46). Translation is enhanced when SRm160 and RNPS1 are tethered at 5' end of the intronless *Renilla* luciferase reporter, and translation activation might be correlated to the more efficient mRNA processes mediated by the deposited EJC (52,53). The importance of EJC in the translational control is further supported by the discovery that EJC might involve in the activation of mTOR signaling pathway which regulates protein expressions responding to different growth conditions (54). Via the EJC-bound SKAR (S6 Kinase

1 Aly/REF-like target), a substrate of the S6 kinase 1 (S6K1), EJC might involve in the translation initiation of mRNAs targeted by the mTOR signaling pathway through the recruitment and activation of S6K1(55). Therefore, the EJC that deposited after splicing could influence several cytoplasmic events for the regulation of gene expressions. A well known example is the expression of *oskar* mRNAs in *Drosophila* oocytes. *Drosophila* orthologs of Y14, Mago, MLN51 and eIF4AIII (Tsunagi, MagoNashi, Barentz and eIF4AIII respectively) are involved in subcellular localization and regulation of *oskar* mRNAs in the posterior pole of developing oocytes. The appropriate localization of *oskar* mRNAs depends on splicing, and the restricted translation of *oskar* mRNAs in the posterior pole is important for early pattern formation of the embryo (2,46).

In addition to the translation and splicing factors, several factors important for mRNA export are also identified as EJC interacting proteins. Although the contribution of EJC to the mRNA export remains less clear, EJC might be dispensable or insufficient for mRNA export in mammalian cells and *C. elegans* (56,57). Aly/REF, the subunit of TREX complex, has been regarded as a component of EJC outer shell and serves as the platform for subsequent recruitment of TAP/P15 (58). Therefore, a variety of RNA processing complexes not only involves in the efficient maturation of mRNPs but also in facilitating mRNA export through delivering mature mRNA to the mRNA export receptor TAP/P15, which plays an important role for the bulky mRNA export.

1.4. Unusual but critical TAP for the export of mRNAs

Eukaryotic cells are characterized by the compartmentalization of nucleus and cytoplasm. In contrast to the cotranscriptional translation in prokaryotic cells, gene expression in eukaryotes requires the dynamic shuttling of materials involved in gene

expressions through the NPC embedded in the nuclear envelope. The NPC is a macromolecular with a molecular weight of 125 MDa in metazoans (66MDa in yeast) and is composed of around 30 nuclear pore proteins (nucleoporins) classified into three groups. The first class includes Nups which are integral membrane proteins and function to anchor the NPC within the nuclear membrane. The second class includes FG nuclearporins whose domains contain distinctive sequence repeats with cores rich in phenylalanine and glycine (FG-repeats). The FG nuclearporins distribute in the active transport channel and function in the nucleocytoplasmic transport by direct interaction with soluble transport receptors. The third class includes nucleoporins without FG-repeats and is involved in the structural maintenance of the NPC.

The majority of nuclear transport pathways utilizes the members of β-karyopherins or importin-β family as conserved nuclear transport receptors, which could recognize either the nuclear localization signal (NLS) or the nuclear export signal (NES) of cargo proteins. β-karyopherins control the recognition and release of cargoes by the RanGTP-RanGDP gradient across the nuclear membrane (RanGTP in the nucleus and RanGDP in the cytoplasm). The Ran is a small GTPase whose nucleotide state is regulated by two regulators, nucleus RanGEF (Ran GDP exchange factor) and cytoplasmic RanGAP (Ran GTPase activator protein).

In contrast to the major protein transport pathway using β-karyopherins as carriers, the bulk mRNA export adopts the TAP (Mex67 in yeast) as their unique receptors (40,42). Without any domain structurally related to the β-karyopherins family, TAP mediates mRNA export through the direct interaction with the FG-repeats of FG nucleoporins. The N terminal region of TAP contains leucine-rich repeats (LRR domain) that could interact with Aly/REF. The middle domain of TAP is structurally related to NTF2 (NTF2-like domain) which is important for the heterodimer formation between TAP and P15. The C terminal of TAP possesses a short

ubiquitin-associated (UBA) domain. Whether the UBA domain of TAP in metazoan involves in the binding with ubiquitins is not clear. However, the UBA domain of yeast TAP, Mex67, could interact with ubiquitinated Hpr1, which is a component of THO complex. Ubiquitination of Hpr1 by E3 ligase Rsp5 depends on transcription, indicating that Mex67 recruitment by ubiquitinated Hpr1 might be cotranscriptionally. Besides the three major domains mentioned above, TAP also contains one mRNA-binding domain. However, due to the intrinsical lower RNA binding affinity of this mRNA-binding domain, additional adaptor protein such as Aly/REF is required to enhance the TAP-mRNA interaction (59,60). Although Yra1 is required for mRNA export in yeast, dysfunction of Aly/REF in metazoan has no obvious effect on the global mRNA export. Other than Aly/REF, several proteins including SR proteins, U2AF and FMRP have been reported to function as adaptors of mRNA export. Furthermore, the genome-wide screen for mRNA export in *Drosophila* also identified several factors involved in this process. However, whether they act as canonical adaptors of mRNA export or function as co-adaptors in processing specific transcripts remains to be characterized (60,61).

Another unaddressed issue is the remodeling of mRNPs during mRNA export. In contrast to the protein transport mediated by β-karyopherins family, TAP-mediated mRNP export does not depend on RanGTP-RanGDP gradients, and members of the DEAD-box protein family might play a more important role. For example, Dbp5, a RNA-dependent ATPase which involved in yeast gene expression pathway, could be recruited to the mRNAs during early transcription process. However, Dbp5 could only be activated at the cytoplasmic NPC face where two important factors including Gle1 and IP_6 are located. The restricted activation of Dpb5 subsequently removes a subset of proteins including Mex67 and poly (A) binding protein Nab2 from the mRNPs. The removal of Mex67 could prevent the re-entering of mRNAs into the nucleus and

confer the directionality of the export (40,62). In addition to the mRNP remodeling in the late stage of mRNA export, Sub2 could also regulate the earlier assembly of mRNPs as described above. Sub2 and Mex67 binds to the overlapping region of Yra1, and the interaction between Mex67 and Yra1 might displace Sub2 from the mRNAs. Furthermore, although the linkage between EJC and Aly/REF has been proposed, only the Upf3b and RNPS1 of the peripheral EJC proteins could be exported along with the mRNPs through the interaction with the EJC core (46). How the remodeling occurs remains to be elucidated. Furthermore, the dynamic interaction of different TAP-associated proteins might involve in the regulation of general and specific mRNA export.

Our laboratory previously set out to identify the TAP interacting proteins and thus identified some targets including several nucleoporins, RanGAP1 and RNA helicase RHA/DHX9 (Ref. 63, Appendix 1). Interestingly, one subunit of TRAP (Thyroid hormone receptor associated protein)/Mediator complex named TRAP150 was also detected (64). Little has been known about TRAP150 except that TRAP150 contained the RS domain and was identified as a component of splicing complex (9,22,65). Additionally, the identification of TRAP150 in several distinct mRNA processing complexes suggests that TRAP150 might involve in different mRNP maturation steps including transcription, splicing and mRNP export (66). Therefore, we decided to characterize the possible functions of TRAP150 in the pre-mRNA processing.

2. **Materials and methods**

2.1. Plasmids

The cDNA encoding full-length TRAP150 was PCR-amplified from a human fetal brain cDNA library (Clontech) using specific primers. The PCR product was inserted into a pcDNA3.1 (Invitrogen)-based vector in-frame with the sequence encoding the FLAG tag to generate pcDNA-TRAP150-FLAG. Analogously, expression vectors encoding each of the truncated TRAP150 proteins (Fig. 3B and 10) were generated. The cDNAs encoding full-length or truncated TRAP150 proteins were each cloned into pCEP4 (Invitrogen), yielding the expression vectors for hemagglutinin (HA)-tagged proteins. The DNA fragments containing the HA and full-length or truncated TRAP150 sequences were subcloned into pMCP (67) to generate the vectors for expressing the MS2 coat protein (MCP)-fusion proteins. To construct the siRNA-resistant TRAP150 expression vector containing HA-tag, we used site-directed mutagenesis strategy to change the siRNA targeting sequence. The siRNA-targeting sequence, 5'-ggtataagctccgagatga-3, was replaced by the following sequence, 5'-ggtataagcttagggacga-3'.

To raise an antiserum against TRAP150, the TRAP150ΔNC cDNA fragment was inserted in-frame into pGEX-5X (GE Healthcare) and pET29b (Novagen) to generate the bacterial expression vector for the glutathione S-transferase (GST) and 6X histidine (His)-fused TRAP150ΔNC protein, respectively.

The BCLAF1, MLN51 and CBP80 cDNAs were PCR-amplified and cloned into pcDNA3.1 and pCEP4 to generate the expression vectors of FLAG- and HA-tagged proteins, respectively. The cDNA fragment encoding the CPSF73 protein was PCR amplified and cloned into pcDNA3.1 vector to generate the FLAG-tagged CPSF73 expression vector.

The plasmids expressing FLAG-tagged Aly/REF, SRm160, Y14, TAP, Upf3B,

RNPS1, hUpf1, hUpf1-R844C and the reporter plasmids used for NMD assays (including βUAA-6bs, βUAC-6bs, βG, βwt and β39) were kind gifts from Jens Lykke-Anderson (University of Colorado, Boulder, CO; Ref. 68). The βΔ1 reporter was created by substitution of the *Hind* III/*BamH* I cDNA fragment corresponding to the spliced β-globin reporter mRNA. The βΔ1-5'm reporter was generated by using PCR based site-directed mutagenesis (Stratagene).

The pBS-PIP85a/4XMS2 for *in vitro* transcription contained four copies of MS2 sites, which were PCR amplified and inserted in the downstream region of the second exon of pBS-PIP85a (gift from Benjamin J. Blencowe, Toronto University).

2.2. Antibody preparation

The GST and 6X His-TRAP150ΔNC recombinant proteins were overexpressed in *Escherichia coli* and purified according to the manufacturer's instructions. 6X His-TRAP150ΔNC was used as antigen to immunize rabbits. Antibodies were affinity purified against recombinant GST-TRAP150ΔNC protein according to the method described previously (25). The specificity of purified anti-TRAP150 had been confirmed and shown in Supplemental Fig. 1.

2.3. Cell culture and transfection

HEK293, HeLa, Tet-Off HeLa and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin/glutamine (Invitrogen). Transfections were performed using Lipofectamine 2000 (Invitrogen) for 24 hrs. For immunoprecipitation experiments, HEK293 cells were transfected using calcium phosphate. At 16 hrs post-transfection, cells were placed in fresh medium and cultured for another 24 hrs before harvest.

2.4. Indirect immunofluorescence and heterokaryon assay

HeLa cells were fixed by 2% formaldehyde in PBS for 20 mins and subsequently permeabilized with 100% acetone at 4˚C for 3 mins. The primary antibodies we used included monoclonal anti-FLAG (M2; 1:300 dilution; Sigma) and anti-SC35 (4.6 μg/ml; Sigma), and polyclonal anti-HA (1:100 dilution; Covance Inc.) and affinity-purified polyclonal anti-TRAP150 (1:100 dilution). The secondary antibodies used were fluorescein-conjugated anti-rabbit IgG (12 μg/ml; Cappel Laboratories) for polyclonal primary antibodies, and rhodamine-conjugated anti-mouse IgG (7.5 μg/ml, Cappel Laboratories) for monoclonal primary antibodies. The specimens were observed using a Zeiss Axiovert 200M inverted research-grade fluorescence microscope coupled with an image analysis system.

The heterokaryon assay was performed as described (68) with a minor modification. Briefly, HeLa cells were cotransfected with the vectors encoding FLAG-TRAP150 and GFP-hnRNP A1 or GFP hnRNP C1. At 48 hrs post-transfection, HeLa cells were co-cultured with NIH3T3 cells and treated with cycloheximide (Sigma) at a concentration of 50 μg/ml for 3 hrs and 100 μg/ml for another 30 mins. To induce cell fusion, 50% polyethylene glycol 3350 (Sigma) was added to the co-culture for 2 mins. The cells were subsequently returned to fresh media containing 100 μg/ml cycloheximide for 3 or 16 hrs. Indirect immunofluorescence using anti-FLAG as the primary antibody was performed as described above. To distinguish HeLa from mouse NIH 3T3 cells, the cells were counterstained with Hoechst 33258 (5 μg/ml; Sigma).

2.5. In vivo splicing and RT-PCR

The splicing reporter pSV40-CAT(In) or pCEP4-E1A (25) was co-transfected with effector expression vectors (2 μg) into HeLa cells in 3.5-cm dishes at 80-90%

confluency. At 24 hrs post-transfection, RNA samples were harvested from cells using TRIzol reagent (Invitrogen) and treated with RQ-DNase1 (Promega). Subsequently, RNAs were converted to first-strand cDNAs using SuperScript III reverse transcriptase (Invitrogen) with specific 3' primer complementary to the CAT or E1A transcript. The primers for PCR amplification were listed below. Following, PCR and Sothern blotting were performed using specific primers as described (25).

To verify the result of microarray, oligo-dT (12-18 mers, Invitrogen) was used as the 3' primer for first-strand cDNA synthesis as described above. Specific primers for amplifying the mRNAs of each candidate gene were listed below:

CAT (f): 5'-ttttggaggcctaggctttt-3';

CAT (r): 5'-gtattcactccagagcgatg-3';

E1A (P2, f): 5'-gcaagcttgagtgccagcgagtag-3';

E1A (P1, r): 5'-ggtcttgcaggctccggttctggc-3';

BCLAF1 (f): 5'-tccaaacaactcaaatactacttttca-3';

BCLAF1 (r): 5'-gccgtcgacttccttttcttccttgcgtctg-3';

PSF (f): 5'-cttactgacgacaactcctc-3';

PSF (r): 5'-catgcgtcttaattcttcctgt-3';

Pinin (f): 5'-ccgctcgagatggaaaaggagtctgagg-3';

Pinin (r): 5'-cccaagcctttacttgctctctggatgtac-3';

hnRNPQ (f): 5'-tgggatcttcgtctaatg-3';

hnRNPQ (r): 5'-acctgggcagctgttttgtg-3';

GAPDH (f): 5'-cggagtcaacggatttggtcgtatg-3';

GAPDH (r): 5'-agccttctccatggtggtgaagac-3';

TAF5L (f): 5'-gcggctgtcacagactgctgaagag-3';

TAF5L (r): 5'-gctgctcaatgacatccttctggc-3';

TRAP150 (f): 5'-cagcacatacaatcagctcagtctcag-3';

TRAP150 (r): 5'-caaggcggagatcaacaggatcatc-3'.

2.6. In vitro splicing/tethering assay and immunoprecipitation of the spliceosome

Preparation of nuclear extracts was essentially according to Hirose et al. (69), except that calcium phosphate was used for cell transfection. In general, HEK293 cells grown in 15-cm dishes at 70-80% confluency were transfected with 50 μg of expression vector encoding either FLAG-tagged full-length or truncated TRAP150. For the *in vitro* tethering assay, the expression vector encoding MCP, MCP-TRA150 or MCP-TRAP150ΔN190 was transfected into HEK293 cells separately. At 16 hrs post-transfection, medium was refreshed and cells were incubated for another 24 hrs. For preparation of the TRAP150-depleted nuclear extract, HeLa cells grown in 15-cm dishes at around 60% confluency were transfected with 50 nM si-TRAP150-J5 using Lipofectamine 2000. At 48 hrs post-transfaction, cells were harvested for nuclear extract preparation as described (69).

In vitro splicing was performed essentially as described (70) using α- ^{32}P-labeled PIP85a, PIP85a/4XMS2 or AdML pre-mRNAs as substrates. For the immunoprecipitation of the spliceosomes, the splicing reaction was incubated at 30˚C for 90 mins, followed by subsequent incubation with anti-FLAG M2 agarose (Sigma) or anti-Sm (Y12, gift of Joan Steitz, Yale University) bound to protein A-Sepharose at 4˚C for 2 hrs (25). The beads were washed extensively with NET-2 buffer (50 mM Tris-HCl, pH 7.4, and 150 mM NaCl) containing 0.05% NP-40. Reactions were treated with 10 mg/ml proteinase K (Promega) prior to RNA recovery by ethanol precipitation. RNAs were analyzed by electrophoresis on 6% denaturing polyacrylamide gels.

To perform the *in vitro* splicing reaction without ATP (71), the nuclear extract was

incubated at room temperature for 20 mins to deplete ATP and subsequently used for the *in vitro* splicing reaction without extra addition of ATP and $MgCl_2$. The splicing reaction was performed at 30˚C for 1 hr. Immunoprecipitation and RNA analysis were performed as described above.

The *in vitro* tethering assay was performed as standard *in vitro* splicing assay except that the α- ^{32}P-labeled PIP85a/4XMS2 or PIP85a pre-mRNAs was incubated in the nuclear extract containing MCP-tagged proteins. At each time point from 0 to 120 mins, reaction was stopped by the Trizol reagent and the addition of α-^{32}P-labeled tRNAs.

2.7. Immunoprecipitation

For coimmunoprecipitation, HEK293 cells grown on a 10-cm dish were cotransfected with 14 μg of pCEP4-TRAP150-HA and 7 μg of vector encoding for a Flag-tagged EJC or NMD factor by calcium phosphate method. Cells were lysed in 1 ml of hypotonic lysis buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, and 1X protease inhibitor cocktail (Roche) on ice for 15 mins. Subsequently, additional NaCl was added to the lysate to a final concentration of 150 mM. The lysate was subjected to centrifugation at 13,400 X g at 4˚C for 20 mins. The supernatant was incubated with 30 μl anti-FLAG M2 agarose at 4˚C for 2 hrs. The beads were washed with NET-2 buffer as described above. Precipitated proteins were dissociated from the beads with sample buffer containing 50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2 % SDS, 20 % glycerol, and 0.2 mg/ml bromophenol blue.

To use mass spectrometry analysis identifying TRAP150-interacting proteins, HEK293 cells grown on a 15-cm dish at 60-70 % confluency were transfected with 20 μg of plasmids encoding Flag-tagged TRAP150 by calcium phosphate.

Immunoprecipitation using anti-FLAG was carried out as described above. Precipitated proteins were separated by 10 % SDS-PAGE, followed by SYPR Ruby staining (Bio-Rad) and detection with Typhoon 9410 (Amersham Biosciences). The following procedures including trypsinization and peptides recovery were essentially carried out as described (25).

To detect the interactions between TRAP150 and several endogenous proteins, 10 μg of expression plasmids encoding FLAG-TRAP150 was transfected into HEK293 cells at 60-70 % confluency by calcium phosphate. Proteins precipitated with M2 agarose were blotted with specific antibodies against Dcp1 (raised by our laberatory), hRrp6 (Sigma), CBP80 (Abcam), Xrn1, Xrn2 and CPSF73 (Bethyl).

To verify whether TRAP150 might be associated with the CBC or CPSF complex, 10 μg of the expression vectors encoding either FLAG-CBP80 or FLAG-CPSF73 was individually transfected into HEK293 cells at 60-70 % confluency by calcium phosphate. After immunoprecipitation by M2 agarose, the immunoprecipitates were subjected to immmunoblotting using the anti-TRAP150.

The RNA-immunoprecipitation was performed by transfection of expression vectors encoding FLAG-TRAP150 into HEK293 cells at 60-70% confluency by calcium phosphate. Immunoprecipitation by M2 agarose was performed as previous described. 10% of the immunoprecipitates was saved for immunoblotting with anti-FLAG while the remaining 90% was subjected to the proteinase K treatment. The following RNA recovery and RT-PCR analysis were performed as described above.

2.8. NMD assays and northern blotting

Tet-Off HeLa cells grown on a 3.5-cm dish were co-transfected with an MS2 site-containing NMD reporter (1 μg) and an expression vector encoding an MCP-effector (2.5 μg) as well as a control plasmid (βG; 0.5 μg). At 24 hrs

post-transfection, total RNA was isolated using TRIzol reagent and subjected to northern blotting analysis using a ^{32}P-labeled riboprobe complementary to exon 1 and exon 2 of β-globin. To determine thr mRNA half-life, 5 μg/ml actinomycin D (Sigma) was added to inhibit transcription at 10 hrs post-transfection. Cells were harvested at the indicated time points. For subcellular fractionation, cells were incubated with gentle lysis buffer (10 mM HEPES-KOH, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40) for 5 mins followed by centrifugation at 2,000 X g at 4˚C for 8 mins (68). The cytoplasmic and nuclear fraction was collected from the supernatant and the pellet, respectively. To examine the Upf1 dependency, pcDNA-hUpf1 or pcDNA-hUpf1-R844C (1 μg) was co-transfected with the vectors used in the tethering NMD assay. Cycloheximide treatment was peformed as described (68) at the concentration of 50 μg/ml for 2 hrs. For knockdown experiments, cells were transfected with 100 nM siRNA for the first 48 hrs followed by further transfection with the effector expression vector and the reporter as above. After incubation for another 24 hrs, total RNA was isolated for northern blotting. siRNAs si-TRAP150-J5 and -J8 were purchased from Dharmacon and si-Luciferase (si-Luc.), si-TRN, si-Xrn1, si-Xrn2, si-hRrp6 and si-CBP80 were from Invitrogen. Their sense-strand sequences were as follows:

si-TRAP150-J5: 5’-gguauaagcuccgagaugauu-3’;

si-TRAP150-J8: 5’-caaaugggagggccugguauu-3’;

si-Luc.: 5’-ggatttcgagtcgtcttaatgtata-3’;

si-TRN: 5’-acaggaaucaacuuaggaagaugcc-3’;

si-Xrn1: 5’-gggaucuggaaagaugcaauacuuu-3’;

si-Xrn2: 5’-gagaggagcauugaugacuggguuu-3’;

si-hRrp6: 5’-ucuucuauaggucuguauaacuggg-3’;

si-CBP80: 5’-aacagagggcaacagaauguccagg-3’

Monoclonal anti-TRAN1 was purchased from Sigma. Protein levels were quantified using ImageQuantTL software (GE Healthcare).

3. Results

3.1. Verification of TAP-TRAP150 interaction

3.1.1. TRAP150 is a TAP-interacting phosphorylated protein

TRAP150 has been identified as a putative TAP-associated protein by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis in our laboratory (Appendix 1; Ref. 63). To verify the interaction, FLAG-tagged TAP was overexpressed in HEK293 cells, followed by immunoprecipitation with M2 agarose beads, and consequently endogenous TRAP150 could be detected in the immunoprecipitates (Fig. 1A). The fact that RNase A treatment could not disrupt the interaction further demonstrated that it was RNA independent (Fig. 8).

TRAP150 contains an arginine/serine-rich sequence in the N-terminal region, and its C-terminal region has 48% overall identity with a cell death-promoting transcriptional repressor bcl2-associated factor (BCLAF1), also called bcl2-associated transcription factor (Btf; Ref. 64,72). In addition to the conserved region, BCLAF1 also contains an RS domain in the N-terminus, and a putative gene family consisting of BCLAF1 and TRAP150 has been proposed regarding the high similarity between these two proteins (64). Within the BCLAF1 homologous domain of TRAP150, an around 90-amino acids segment shares 30% similarity with MLN51, an EJC core component (58,73). Both TRAP150 and BCLAF1 are associated with the spliceosome, indicating their potential roles in pre-mRNA splicing (9,22).

TRAP150 was also predicted to be a phosphorylated protein by several groups using biochemical strategies to identify proteins with phosphorylated residues (74-78). To confirm the phosphorylation status of TRAP150 in our study, overexpressed FLAG-TRAP150 from HEK293 cells was immunocripitated and separated into two aliquots, mock treated and alkaline phosphatase (AP) treated. AP treatment resulted in

the increased mobility of the immunoprecipitated TRAP150, indicating that TRAP150 might be a phosphorylated protein (Fig. 1B). Comparing the relative position of input and immunoprecipitated TRAP150 with or without AP treatment further suggested that under steady state, TRAP150 might exist as a fully phosphorylated form in the cell.

3.1.2 TRAP150 is a nuclear-restricted protein with speckle localization

TRAP150 is characterized by the RS domain in the N-terminus, and its mouse homolog could be detected in the speckles, especially under the condition that transcription was inhibited (79). We performed the immunofluorescence staining to determine the subcellular localization of human TRAP150. Under steady state, endogenous TRAP150 predominately localized in the nucleoplasm with some punctuate stainings, and the dot-like structure of TRAP150 colocalized with the speckle marker, SC35 (Fig. 2A). Transiently expressed TRAP150 showed the similar subcellular localization pattern to that of the endogenous TRAP150, and higher expression levels resulted in more obvious speckle patterns. Furthermore, inhibition of transcription by 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) led to the rounded and enlarged speckles that are characteristic of speckle proteins (Ref. 80,81; Fig. 2B).

Many shuttling SR proteins including 9G8, SRp20 and ASF/SF2 function as mRNA export adaptors and could shuttle between the nucleus and cytoplasm according to the phosphorylation status of the RS domain (82,83). Given that TRAP150 was found to be a phosphorylated protein and capable of interacting with TAP, we were also curious about whether TRAP150 could shuttle in and out of the nucleus. To clarify this issue, we carried out the interspecies heterokaryon assay. For the purpose of avoiding cross-species signals of anti-TRAP150, FLAG-tagged

TRAP150 was co-expressed respectively with GFP-fused hnRNPA1 or hnRNPC1 in HeLa cells. The shuttling protein hnRNPA1 was a positive control while hnRNPC1 was expected to be nucleus-restricted. After transfection for 24 hrs, transfected HeLa cells were fused with NIH3T3 cells. 3 hrs post fusion, hnRNPA1 appeared in the mouse nuclei; however, no FLAG-TRAP150 signals could be observed in the mouse nuclei even after 16-hr incubation (Fig. 2C). Therefore, TRAP150 is probably a nuclear-restricted protein, albeit it could interact with the mRNA export receptor, TAP.

3.2. Characterization of TRAP150's function in splicing

3.2.1. TRAP150 activates splicing *in vivo*

Both TRAP150 and BCLAF1 were found to be in the spliceosome complex and reported to be mRNP-associated, which is independent of splicing (9). However, whether these two proteins participate in the regulation of pre-mRNA splicing is unknown. We first investigated whether TRAP150 and BCLAF1 could activate splicing. The reporter we used called CAT(In) encoding the chloramphenicol acetyl transferase (CAT) transcript inserted with the first intron of human β-globin (diagrammed in Fig. 3A). We also made several deletion mutants of TRAP150 to determine which region of TRAP150 might be important for the splicing regulation: ΔN190 represented that the N-terminal RS domain of TRAP150 was truncated; ΔC359 indicated that the conserved C-terminal fragment was removed; ΔNC only contained the central region of TRAP150 (Fig. 3B, upper panel). The reporter plasmid was respectively cotransfected with plasmids encoding different hemagglutinin (HA)-tagged effector proteins to HeLa cells, and the splicing pattern was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) using specific primers complementary to the SV40 promoter and the coding region of CAT transcripts.

Overexpressed TRAP150 obviously enhanced splicing in comparison with another splicing activator, CA150 (Fig. 3B, lane 2 and 3). In contrast to TRAP150, overexpressed BCLAF1 only resulted in about two folds activation similar to that by CA150 (Fig. 3B, lane 2 and 7). Given that BCLAF1 is a death-promoting transcription repressor and the higher expression level of BCLAF1 induces apoptosis, it is possible that the minor effect of BCLAF1 on splicing activation was related to its lower expression level (Ref. 72; Fig. 3B, lower panel). The ability of TRAP150 to enhance splicing activity depended on the N-terminal RS domain because overexpressed ΔN190 failed to activate splicing while ΔC359 acted as full-length TRAP150 (Fig. 3B, compare lane 3-5). Overall, our results demonstrated that overexpressed TRAP150 could activate splicing through its N-terminal RS domain.

3.2.2. TRAP150 is required for splicing *in vivo*

Since overexpressed TRAP150 could activate splicing *in vivo*, we also investigated whether TRAP150 might be required for splicing. SiRNA-mediated knockdown of TRAP150 proteins was achieved by si-TRAP150-J5 but not -J8 (Fig. 4A, lower panel). The CAT(In) reporter plasmid was cotransfected with individual siRNA group and splicing was analyzed by RT-PCR as previously described (25). To quantitate the splicing efficiency more precisely, southern blots of the RT-PCR products were performed. Splicing of reporter was inhibited when TRAP150 was down-regulated by si-TRAP150-J5, however si-TRN and the non-functional si-TRAP150-J8 had no obvious effect (Fig. 4A, compare lane 2-4). To rule out the possibility that splicing inhibition caused by si-TRAP150-J5 was non-specific by off-target effects, the plasmid encoding si-RNA resistant HA-TRAP150 was generated. HeLa cells were transfected with each siRNA group for two days, followed by further transfection of the reporter along with or without siRNA resistant effectors. Splicing defect was

restored in the presence of siRNA-resistant TRAP150 whose expression was confirmed by immunoblotting with anti-HA antibody (Fig. 4B, lane 3 and 4). These results indicated that TRAP150 was essential for splicing *in vivo*.

3.2.3. TRAP150 could not regulate alternative splicing

Previous results indicated that TRAP150 played an important role in constitutive splicing in an RS domain dependent manner. In addition to the constitutive splicing, SR proteins are also characterized as regulators of alternative splicing. Members of SR protein family could recognize critical cis-elements such as exonic splicing enhancer (ESE) or silencer (ESS) to mediate the exon inclusion or skipping, respectively. Besides the cis-element recognition, RS domain could also mediate spliceosome assembly through protein-protein interaction (84). Therefore, we used adenovirus E1A reporter to examine if TRAP150 could modulate alternative splicing. Three sorts of mRNAs could be generated from the E1A pre-mRNA using alternative 5' splice sites but the same splice acceptor (Fig. 5, upper panel). 13S and 12S mRNAs are synthesized throughout the adenovirus lytic cycle while the 9S mRNAs are characteristic of the late phase of adenovirus infection (85). SC35 and ASF/SF2 have been reported to enhance the proximal 5' splice site utilization while hnRNPA1 and RBM4 facilitate the utilization of the distal one (86,87).

Consistent with the previous report, we observed that overexpressed RBM4 modulated the production of 9S mRNA (Fig. 5, lane 5). However, neither overexpressed nor knockdown of TRAP150 affected the splicing pattern of E1A as compared to the group of vector alone (Fig. 5, compare lane 1, 2 and 6). Similar to the full-length TRAP150, overexpressed BCLAF1 or truncated TRAP150 failed to influence the splicing pattern of E1A (Fig. 5, compare lane 1 and 7-10). Furthermore, in contrast to the splicing of CAT(In) reporter, no obvious splicing defect on the

efficiently spliced E1A reporter could be observed when TRAP150 was down-regulated by siRNAs. These results suggested that the splicing activation mediated by TRAP150 might be gene-specific, especially for those characterized by poor splicing efficiency. Overall, our results indicated that TRAP150 might not involve in the modulation of alternative splicing.

3.3. In vitro analysis of the properties of TRAP150 in splicing regulation

3.3.1. TRAP150 is not required for *in vitro* splicing

Previous results of *in vivo* splicing demonstrated the importance of TRAP150 in splicing under physiological conditions. We further used the cell free system to dissect the mechanism of TRAP150-mediated splicing activation. TRAP150 depleted nuclear extract was prepared for *in vitro* splicing to examine whether TRAP150 might involve in the spliceosome assembly. Immunoblotting results showed that the protein level of TRAP150 was reduced to less than 5% of the control nuclear extract (Fig. 6A). Two substrates derived from adenovirus including adenovirus major late (AdML) and PIP85a were used for *in vitro* splicing assays. Kinetic analysis of splicing at different time points indicated that both substrates showed the similar splicing efficiency no matter which nuclear extract was used (Fig. 6B). So far, we could not rule out the possibility that the remaining TRAP150 was sufficient to support the splicing, or gene-specific splicing regulation of TRAP150 might exist. Our current results indicated that TRAP150 was dispensable for *in vitro* spliceosome assembly, although it was critical for *in vivo* splicing (Fig. 4). TRAP150 has been reported to function as a transcription factor, and the coupling of transcription and splicing *in vivo* could greatly enhance the processing and stability of pre-mRNAs (26,88). Therefore, TRAP150 might play a role in the event of coupling transcription and splicing at the earlier stage of spliceosome assembly.

3.3.2. TRAP150 joins the spliceosome at the earlier stage and associates with spliced RNA

Spliceosome is an energy-dependent stepwise assembly by U1, U2, U4/U6 and U5 snRNPs. However, the assembly begins with the ATP-independent base pairing between U1 snRNA to the 5' splice site (5' SS) and the recognition of the branch point sequence (BPS) by the splicing factor 1 (SF1, also called branch point binding protein, BBP). Accompanied with the binding of U2AF65 and U2AF35 to the polypyrimidine tract downstream of BPS and AG dinucleotides at 3' splice site (3'SS), respectively, these interactions result in the formation of the E complex. Subsequent to the E complex formation is a series of ATP-dependent remodeling. Base pairing between U2 snRNA with the BPS leads to the formation of the A complex, followed by the B complex formation that involves the loading of U4/U6.U5 tri-snRNP. The B complex remains inactive until the release of the U1 and U4 snRNPs, and then the formed activated spliceosome (the B* complex) could initiate the first transesterification that generates the C complex. After the second catalytic step, the spliceosome including the U2, U5 and U6 snRNPs dissociates and the mRNA is released in the form of mRNP (22).

It has been reported that when ATP-depleted nuclear extract was used for *in vitro* splicing without addition of extra ATP and magnesium chloride, the spliceosome assembly could be blocked at the stage that only the E complex formed (71). We followed the above condition to allow the E complex formation only, and determine if TRAP150 could join the spliceosome assembly at the earlier stage. We prepared the TRAP150 overexpressing nuclear extract from HEK293 cells transfected with the expression vector encoding FLAG-TRAP150. The expression of TRAP150 was confirmed by immunoblotting using anti-FLAG (Fig. 7A). Splicing products with or

without ATP were immunoprecipitated by M2 beads. At the same time, we also verified whether TRAP150 might associate with intronless RNAs (ΔiPIP85a). TRAP150 could associate with the pre-mRNAs without ATP addition, indicating that TRAP150 could join spliceosomes prior to the formation of the A complex (Fig. 7B, compare lane 3 and 4). Consistent with previous reports, our result also showed that TRAP150 could bind to the intronless RNAs, which further confirmed that the RNA binding of TRAP150 did not depend on splicing and could occur without the assembly of spliceosome (Fig. 7B, compare lane 11 and 12; ref. 9).

Interestingly, under normal conditions that ATP was supplemented, although all sorts of spliced products could be immunoprecipitated by FLAG-TRAP150, it seems that TRAP150 preferentially associated with pre-mRNAs as well as fully spliced ones (Fig. 7B, compare lane 7 and 8). To further confirm that the so-called fully spliced products were not contaminated with other splicing intermediates, we compared the amounts of RNAs immunoprecipitated by anti-FLAG M2 beads with those by snRNP-recognizing Y12 antibody. We also prepared the nuclear extract expressing nonfunctional TRAP150 fragment, ΔNC, to rule out the possibility that interaction between TRAP150 and RNAs might be nonspecific. In contrast to the TRAP150, ΔNC did not pull down significant levels of either spliced product (Fig. 7C, compare lane 2, 5 and 8). Furthermore, the predicted signal of fully spliced products immunoprecipitated by FLAG-TRAP150 was absent in Y12 immunoprecipitates (Fig. 7C, compare lane 5 and 6). These results demonstrated that TRAP150 could bind to the mature spliced mRNAs, suggesting that TRAP150 might associate with the post-splicing complex, EJC.

3.3.3. TRAP150 associates with components of EJC

In order to demonstrate that TRAP150 could associate with EJC, HA-tagged

TRAP150 was co-transfected with individual EJC component with FLAG-tag. After immunoprecipitation by M2 beads, immunoblotting using anti-HA was performed to verify whether TRAP150 could interact with each EJC component (Fig. 8). At the same time, we determined the interaction between TRAP150 and another transcription elongation factor, CA150ΔN. We also compared the conditions with or without RNaseA treatment to determine whether the interaction might be mediated by RNAs. Factors involving in mRNA export such as FLAG- tagged TAP and Aly/REF efficiently precipitated TRAP150. Splicing factors including SRm160 and RNPS1 also showed good interaction with TRAP150. A weak interaction could be observed between TRAP150 and the EJC core component Y14, yet another core factor MLN51 efficiently immunoprecipitated TRAP150. We failed to detect any signal of TRAP150 from eIF4AIII immunoprecipitates, but the reciprocal interaction using FLAG-TRAP150 to immunoprecipitate HA-eIF4AIII was observed by our colleagues (Appendix 2). Furthermore, the interaction between TRAP150 and NMD factors including Upf3b and RNPS1 indicated that TRAP150 might play a role in the regulation of nonsense mediated decay (NMD).

3.4. Investigation of RNA decay mediated by TRAP150

3.4.1. TRAP150 induces mRNA decay in tethering NMD assay

To investigate whether TRAP150 might participate in NMD pathway, we adopted the strategy called tethering NMD assay based on the coat protein derived from the bacterialphage MS2 (68). The minimum binding site recognized by the MS2 coat protein (MCP) is a particular secondary structure with a stem-loop formed by twenty one nucleotides (89). Because the onset of NMD requires the interaction of SURF recruited by the stalled ribosome and the downstream EJC, the insertion of multiple copies of MS2-binding sites into the downstream of the stop codon of the wide type

transcripts will introduce the NMD pathway in the presence of any NMD or EJC factor fused with MCP (90). The tethering reporter we used was the β-globin reporter inserted with 6X MS2 binding sites in the 3'UTR (βUAA-6bs), and another β-globin/GAPDH chimeric transcript (βG) was co-expressed to serve as the transfection and loading control (diagrammed in Fig. 9A, upper panel). Northern blotting was carried out to evaluate the effect of the tethered proteins on the reporters. Consistent with the previous results, tethered-Upf3b resulted in the decay of βUAA-6bs (Fig. 9A, lane 2); we found that both tethered TRAP150 and BCLAF1 also caused the degradation of the reporter, although a less extent was achieved by BCLAF1 and probably due to its lower expression level (Fig. 9A, lane 3 and 4).

To rule out the possibility that earlier RNA processing steps such as transcription might be affected by tethered TRAP150, we determined the half life of the reporter mRNAs. Actinomycin D was used to shut down the transcription, and both the βUAA-6bs and βG showed similar degradation rates in the presence of MCP only. However, the level of βUAA-6bs decreased to less than 50% within 1 hr in comparison with the βG control transcripts in TRAP150 tethering group (Fig. 9B).

To specifically determine the possible effect of the tethered TRAP150 on the pre-mRNAs, other reporters either lacking the intron 1 of β-globin (βΔ1) or the intron 2 5' splice site mutant without intron 1 (βΔ1-5'm) were generated (Fig. 9C, upper panel). Consistent with the previous report, the 5' splice site mutation greatly inhibited the splicing and only a few spliced products could be observed (Ref. 91; Fig. 9C, rectangle). However, tethered TRAP150 only specifically decreased the level of spliced products of both βΔ1 and βΔ1-5'm, and no coincident effect on pre-mRNAs could be observed (Fig. 9C). Overall, these results suggested that tethering of TRAP150 might specifically result in the degradation of spliced mRNAs but not pre-mRNAs.

3.4.2. C-terminal conserved region of TRAP150 is required for mRNA degradation

We next investigated which region of TRAP150 might involve in the mRNA decay. We compared the effects of different deletion clones as illustrated in Figure 3B on the degradation of the βUAA-6bs reporter. In contrast to the results of domain mapping on splicing regulation, deletion of N-terminal RS domain of TRAP150 had no obvious effect on its degradation ability. However, the C-terminal conserved domain truncation of TRAP150 dramatically blocked its capacity for mRNA decay (Fig. 10A). To further examine the importance of the TRAP150 C-terminal conserved domain for mRNA degradation, we constructed additional deletion clones spanning the amino acids from the 359 to 625 on the C-terminal region of TRAP150 (Fig. 10B, upper panel). The smallest fragment, ΔN596 containing the C terminal 359 amino acids compensatory to the ΔC359, also could induce the degradation of β6UAA-6bs, suggesting that the conserved C-terminal region was critical to elicit the mRNA degradation (Fig. 10B).

3.4.3 TRAP150-mediated degradation occurs in a translation-independent manner

Active translation is required to initiate nonsense-mediated decay, and any EJC that failed to be removed by pioneer round scanning could also trigger NMD. We tried to determine whether TRAP150-mediated degradation depended on translation. First of all, we tested another reporter on which the wide type stop codon UAA was mutated to UAC (βUAC-6bs). This reporter contains no stop codon upstream of the MS2 binding sites; as a result, the scanning ribosome will theoretically remove all tethered proteins and prevent the NMD degradation pathway. However, in contrast to the

canonical NMD factor Upf3b that failed to degrade the βUAC-6bs, the MCP-TRAP150 group could still efficiently degrade this ribosome read-through reporter, and this activity was inhibited when the C-terminal conserved region was deleted (Fig. 11A).

Next we determine if the translation inhibitor cycloheximide (CHX) that could block the elongation of ribosomes might influence the ability of TRAP150 to degrade mRNAs. As reported before, CHX treatment inhibited Upf3b-mediated degradation of βUAA-6bs; however, no obvious effect on TRAP150-mediated decay could be observed, indicating that this effect might occur prior to the translation (Fig. 11B, upper panel). Finally, we examined whether TRAP150-mediated decay depends on Upf1, the key factor for NMD. So far, many alternative NMD pathways including Upf2-dependent, RNPS1-dependent and EJC independent pathways have been proposed, however all of these pathways depend on Upf1 (92-94). Upf1R844C contains the amino acid substitution of arginine to cytosine at position 844 within the helicase domain, and could function as the dominant negative effector of NMD (68). Consistently, in the presence of Upf1R844C, Upf3b-mediated decay was inhibited. However, TRAP150 functioned well even in the presence of Upf1 dominant negative mutant, indicating that TRAP150-mediated decay might differ from the canonical NMD (Fig. 11B).

3.4.4. TRAP150 is not required for NMD and induces mRNA degradation in the nucleus

Previous results indicated that tethered TRAP150 might degrade mRNA through non-NMD pathway. To further examine whether TRAP150 involved in NMD, we characterized the effect of down-regulated TRAP150 on the non-tethering reporter containing authentic premature termination codon (PTC). β39 represents the β globin

transcript containing one premature stop codon in the penultimate exon. Under normal conditions, β39 transcripts were efficiently cleared out by NMD, and the expression of Upf1R844C inhibited β39 degradation (Fig. 12A, compare lane 2 and 6). However, knockdown of TRAP150 had no obvious effect on the stability of β39, indicating that TRAP150 might be not necessary for NMD or might not involve in the NMD (Fig. 12A, compare lane 2 and 10). Considering our findings that TRAP150-mediated decay was translation- and Upf1-independent, the later possibility was preferred.

Given that TRAP150 was a nuclear-restricted protein and might trigger the mRNA decay prior to the initiation of translation, it raised the possibility that TRAP150 might work within the nucleus. To verify the hypothesis, the tethering NMD assay was performed with RNAs prepared either from the nuclear or cytoplasmic fraction. To ensure the fractionation step, we performed immunobloting using antibodies against tubulin and laminA/C as the marker for cytoplasmic and nuclear fraction, respectively. The shuttling protein Upf3b could be detected in both cytoplasmic and nuclear compartment; however, the distribution of MCP-TRAP150 could only be detected in the nucleus as revealed by anti-HA immunoblotting, consistent with the result of heterokaryon analysis (Fig. 12B and 2C). Results of northern blotting indicated that the NMD factor Upf3b enhanced the degradation of βUAA-6bs but not βUAC-6bs in the cytoplasm, yet a minor effect could also be observed in the nucleus (Fig. 12C, lane 2 and 5). However, TRAP150 only significantly degraded both βUAA-6bs and βUAC-6bs in the nucleus but no further degradation could be observed in the cytoplasm (Fig. 12C, lane 3 and 6). Taken together, these results suggested that TRAP150-mediated decay might be distinct form NMD and only occur in the nucleus.

3.4.5. Neither 5’-to-3’ nor 3’-to-5’ exonucleolytic attack involves in

TRAP150-mediated decay

Several RNA destabilization factors function through the recruitment of nucleases for the subsequent RNA degradation. For example, tethered KSRP could result in the degradation of βUAA-6bs by bringing the 3'-to-5' exonuclease and exosome complexes. Either *in vitro* immunodepletion or *in vivo* siRNA-mediated down-regulation of exosome components could inhibit the degradation (51). Since TRAP150 might represent another nuclear mRNA decay system, we next characterized whether TRAP150-mediated mRNA degradation might recruit any nuclease.

In yeast, mRNA turnover predominantly occurs in the cytoplasm and is initiated by Ccr4p/Pop2p complex-mediated deadenylation. Subsequent to the deadenylation is the decapping reaction carried out by Dcp1p/Dcp2p complex, followed by 5'-to-3' exonucleolytic degradation executed by Xrn1p. Exosome also involves in the general mRNA decay but plays a minor role. However, in higher eukaryotic cells the exosome-mediated degradation seems to be the major pathway (95). Although whether Xrn2 involves in the mRNA decay in higher eukaryotic cells is not clear, the yeast Xrn2, Rat1, was reported to mediate the mRNA degradation in the nucleus in addition to its important role in transcription termination (96). Therefore, we tested whether TRAP150 could mediate mRNA decay through interactions with these general mRNA turnover factors. To determine whether TRAP150 could associate with the respective exonuclease, FLAG-TRAP150 was overexpressed in HEK293 cells, and the M2-immunoprecipitates were subjected to immunobloting using specific antibodies for Xrn1, Xrn2, decapping factor Dcp1, and exosome component hRrp6. Interestingly, Xrn2 and hRrp6 that both predominantly locate in the nucleus showed better interaction with TRAP150 than that of Xrn1 (Fig. 13A). Next, we investigated if these factors were required for TRAP150-mediated mRNA degradation.

As demonstrated by immunoblotting, each siRNA successfully decreased the expression of its target gene (Fig. 13B). We then combined the siRNA transfection with tethering NMD assay to examine the nuclease dependence. To achieve higher knockdown efficiency, siRNAs targeting Xrn1, Xrn2 and hRrp6 were transfected individually or combinationarily into Tet-Off HeLa cells. Transfected cells were incubated for 48 hrs, followed by another transfection with reporter and effector plasmids. Consistent with the previous report that NMD required both 5'-to-3' and 3'-to-5' exonucleolytic activities, knockdown of all three nucleases inhibited Upf3b-mediated degradation, although si-hRrp6 exhibited a less activity (Ref. 51; Fig. 13C, upper panel). In contrast to Upf3b-meiated degradation, no significant effect could be found on TRAP150-mediated decay when these nucleases were down-regulated (Fig. 13C, lower panel). We could not rule out the possibility that the remaining proteins were sufficient to assist TRAP150-mediated mRNA degradation, but our current results indicated that the decay might though a novel pathway that needs to be further identified.

3.4.6. The mRNA decay regulated by TRAP150 might be distinct from DRN

Several defects of mRNA processing could turn on the nuclear mRNA surveillance system. These defects could occur during capping, splicing, polyadenylation and mRNA export (96). In yeast, decay of RNA in the nucleus (DRN) is a Cbc1p-dependent degradation system. It has been proposed that all RNAs are subjected to the control of DRN, and the extent is determined by the level of nuclear retention. The retained RNAs are degraded predominantly by Rrp6p, and to some extent by Rat1p (95). Although whether a similar quality control exists in higher eukaryotic cells remains unclear, we tested the dependence of TRAP150-mediated decay on CBC80 and hRrp6, the corresponding proteins of yeast Cbc1p and Rrp6p,

respectively. Since the interaction between TRAP150 and hRrp6 had been demonstrated as shown in Fig. 13A, we only verified the interaction between TRAP150 and CBP80, and an RNA-independent interaction between CBP80 and FLAG-TRAP150 could be observed by immunoprecipitation (Fig. 14A). When we tested the reciprocal interaction, however, endogenous TRAP150 failed to be immunoprecipitated by FLAG-CBP80. This suggested that TRAP150 might interact with CBP80 transiently and might be an unstable component in the cap binding complex (Fig. 14B).

We then performed siRNA transfection with the tethering NMD assay as mentioned above. The knockdown efficiency was shown in Fig. 14C. Down-regulation of hRrp6 and CBP80 slightly affected Upf3b-mediated degradation (Fig. 14D, upper panel, compare lane 2, 4-6). The CBP80 dependence of Upf3b-mediated decay was not unexpected because CBP80 could interact with Upf1 and participate in the NMD during pioneer round translation (97). However, no significant change could be observed for TRAP150-mediated decay even when both hRrp6 and CBP80 were co-repressed (Fig. 14D, lower panel). Therefore, TRAP150-mediated degradation was not due to the nuclear retention of reporters trapped by tethered TRAP150.

3.4.7. TRAP150-mediated degradation is not coupled to splicing

Our current results have shown that TRAP150 is an RS domain-containing splicing activator that also induces mRNA degradation through the C-terminal conserved domain. However, what remained to be elucidated was whether these two activities functioned cooperatively or independently. Another splicing factor RNPS1 has also been reported to degrade mRNA through NMD pathway when tethered (98). However, RNPS1-mediated splicing and mRNA degradation take place in different compartments of the cell while the activities mediated by TRAP150 might limit in the

nucleus. Although our previous results demonstrated that only the amount of spliced RNAs but not the precursor ones was decreased by TRAP150, we could not conclude that the degradation was coupled to splicing. In order to answer this question, the *in vitro* system that could dissect each step of mRNA metabolism was adopted. Four copies of MS2 binding sites were inserted downstream of the second exon of PIP85a (PIP85a-4XMS2). This substrate was incubated with the standard splicing reaction using nuclear extracts overexpressing MCP, MCP-TRAP150 and MCP-ΔN190 respectively. In the presence of MCP-TRAP150, but not MCP or MCP-ΔN190, the splicing of PIP85a-4XMS2 was enhanced as revealed by the accumulation of spliced products when incubation time was prolonged (Fig. 15B, lane 6-10). However, the accumulated spliced products indicated that no degradation occurs after splicing as compared to the loading control, tRNAs. The difference in splicing efficiency was not due to the different batches of nuclear extracts we used because similar splicing efficiency among three nuclear extracts could be observed using PIP85a as the substrate (Fig. 15C). Therefore, although we could observe the splicing activation caused by tethered TRAP150, the mRNA degradation mediated by TRAP150 could not be reconstituted under this *in vitro* condition. Therefore, additional factors either absent from the nuclear extract or loaded cotranscriptionally might be critical for TRAP150-mediated mRNA degradation. However, our current results suggested that splicing might not be sufficient to trigger TRAP150-mediated mRNA degradation, or TRAP150 might promote mRNA decay independent of the splicing.

3.5. Identification of TRAP150 associated protein

3.5.1. TRAP150 interacts with several groups of mRNA processing factors

So far, our current results suggested that TRAP150 might mediate the mRNA degradation through a novel pathway remained to be revealed. In order to find out

how TRAP150 could regulate the stability of mRNAs, we searched for the TRAP150 interacting proteins. We overexpressed FLAG-tagged TRAP150 and ΔN190 in HEK293 cells, and the immunoprecipitates using M2 beads were separated by 10% SDS-PAGE and visualized by SYPRO Ruby staining. The reason we chose ΔN190 was that it also enhanced the mRNA degradation. We found that the staining pattern of TRAP150 interacting proteins was similar to that of ΔN190 (Fig. 16A). Furthermore, some interacting proteins co-migrated with the Flag-TRAP150 could be clarified when ΔN190 was used as the bait. The interested bands were in-gel digested and analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS). The identified peptides of TRAP150 immunoprecipitated proteins were summarized in Table 1.

Accordingly, several TRAP150 interacting proteins including SRm160, Aly, eIF4AIII and Y14 were detected (Fig. 8 and 16A). Interestingly, factors belonging to distinct mRNA processing complexes were also identified. In addition to several EJC components that had been confirmed before, we also detected Acinus and pinin, another two nuclear restricted proteins that form the sub-complex with RNPS1 (99). As our expectation, lots of RNA binding proteins including members of the heterogeneous nuclear ribonucleoprotein (hnRNP) and SR protein family were also detected. However, because the immunoprecipitated products were not treated with RNase, we could not rule out that some interactions might be bridged by RNAs. Besides the UAP56 and Aly, another two components of THO complex, hTho2 and fSAP35 (hTho6), were identified. Given that TRAP150 is a TAP-interacting protein, it might cooperate with TREX in the related mRNP processing steps. However, the hypothesis remains to be validated.

Consistent with the function of TRAP150 in splicing activation, several components of snRNPs were identified. Interestingly, many of these factors belong to

the U2 snRNPs (100). Furthermore, we also got the peptides of CPSF73 and CPSF160, two important components of pre-mRNA 3' end processing. It has been reported that the subunits of CPSF could directly interact with the components of U2 snRNPs, and depletion of CPSF affected the splicing efficiency in the coupled 3' end cleavage and splicing assay, and vice versa (101). CPSF73 is the endonuclease responsible for not only the 3' end cleavage of poly $(A)^+$ RNAs but also the 3' end processing of non-polyadenylated histone pre-mRNAs. Besides, CPSF73 could also function as the 5'-to-3' exonuclease and help the release of U7 snRNPs, which play an important role in the maturation of histone mRNAs. Moreover, U2 snRNPs were reported to bind to the histone pre-mRNAs and facilitate the 3' end formation (102-104). Overall, these two mRNA processing complexes might cooperate with each other and mediate the maturation of distinct classes of mRNAs. Therefore, we were interested in that TRAP150 might regulate mRNA stability through the interaction with the U2 snRNPs as well as the CPSF complex.

3.5.2. TRAP150 associates with CPSF73

The immunoprecipitation experiments were performed to verify the interaction between TRAP150 and CPSF73. As shown in Fig. 16B, FLAG-TRAP150 could immunoprecipitate a significant level of endogenous CPSF73 (Fig. 16B). However, we failed to detect endogenous TRAP150 co-purified with FLAG-CPSF73 (Fig. 16C). These suggested that although TRAP150 could interact with CPSF73, the interaction might be transient and dynamic, and TRAP150 might not be the bona fide subunit of the CPSF complex. However, whether TRAP150-mediated degradation depends on the CPSF73 remains to be elucidate.

3.6. Global analysis of gene-expression profiles under knockdown of TRAP150

3.6.1. TRAP150 affects the mRNA level of several mRNA processing factors

Although we have used the tethering system to demonstrate the function of TRAP150 in mRNA degradation, however, an un-answered question is whether TRAP150 really participates in mRNA stability regulation under physiological conditions. To address this question, our colleagues initiated a microarray analysis to find out whether gene expression profiles could be affected under different expression levels of TRAP150. Theoretically, any gene whose stability is subject to the regulation of TRAP150 might be up-regulated when TRAP150 is down-regulated. Among the 23,279 analyzed genes, only 209 genes showed increased signals in the si-TRAP150-J5 treated group (Appendix 3A). Interestingly, when we surveyed the functions of these up-regulated genes, nearly 30% are involved in the gene expression, *i.e.* transcription, mRNA processing and translation (Appendix 4, table).

TRAP150 has been reported to form another complex called SNARP (SNIP1/SkIP-associated RNA processing) that could regulate the expression level of cyclin D1. The SNARP is composed of SNIP1 (Smad nuclear interacting protein 1), TRAP150, BCLAF1, Pinin and SkIP (Ski-interacting protein; Ref. 105,106). Interestingly, genes encoding BCLAF1 and pinin were also increased under lower expression level of TRAP150, indicating that TRAP150 might affect the amount of the SNARP complex trough regulating the level of its transcripts. Therefore, these two genes were selected for validation. In addition, we selected PSF which is the marker of paraspeckle and modulates both transcription and splicing, and hnRNPQ which plays an important role in splicing regulation of SMN transcripts (107,108). Usp9X gene was also chosen because it could interact with DDX3, another TAP-interacting protein (63). To more precisely determine the difference in the expression levels of the above interested genes, the quantitative real-time RT-PCR was carried out to compare the mRNA levels between mock and si-TRAP150-J5 treated cells. Among the five

selected genes, however, only the transcriptional levels of BCLAF1 and PSF increased when TRAP150 was down-regulated (Appendix 3B). In brief, our preliminary result indicated that TRAP150 might not be a general regulator for gene expression and only affect a subset of genes, especially those involving in the gene expression.

3.6.2. TRAP150 associates with the mRNPs of each candidate gene

Since our results have shown that TRAP150 could promote mRNA degradation through the tethering assay and specifically regulate the expression level of the candidate genes by microarray analysis, we wondered if TRAP150 might be detected in the mRNP of each candidate gene. In order to test this possibility, the RNP immunoprecipitation (RNP IP) was performed to determine whether the candidate mRNAs could be detected within TRAP150-associated mRNPs. FLAG-TRAP150 was overexpressed in the HEK293 cells and immuoprecipitated by M2 agarose beads. The precipitated proteins were confirmed by immunoblotting with anti-FLAG and around 10% FLAG-TRAP150 was immunoprecipitated (Fig. 17B). The mRNAs were extracted from the FLAG-TRAP150 immunoprecipitates and analyzed by RT-PCR with specific primers for each candidate gene. In addition to the selected candidate genes including BCLAF1, PSF, Pinin and hnRNPQ, we also picked out the GAPDH and TAF5L that showed no obvious expression difference upon si-TRAP150-J5 treatment for the control of nonspecific association. All candidate transcripts except for hnRNPQ could be immunoprecipitated by TRAP150 to a significant extent (Fig. 17A). TRAP150 could also immunoprecipitate hnRNPQ transcripts (Fig. 17A, hnRNPQ), yet in comparison with those of GAPDH and TAF5L, the interaction between TRAP150 and hnRNPQ mRNAs might be much less or just result form a background signal.

3.6.3. An inverse relationship stand for the expression of TRAP150 and BCLAF1

As mentioned before, TRAP150, BCLAF1 and Pinin are the components of the SNARP complex that regulates the expression of cyclin D. Interestingly, except for TRAP150, the protein levels of all SNARP components varied during the cell cycle. Furthermore, their highest expressions occurred in the G_1-phase, indicating that the coordination between the mRNA transcription and processing could guarantee the appropriate expression of cyclin D during the cell cycle (105). Our previous results showed that the mRNA expression of BCLAF1 could be affected by different expression levels of TRAP150, so we next simply examined whether the protein level of BCLAF1 also correlated to the TRAP150 expression level.

HeLa cells were either transfected with si-TRAP150-J5 or control siRNA. 48 hrs post transfection, cells were harvested and subjected to the immunoblotting with antibodies against BCLAF1, hnRNPQ, PSF and TRAP150. RNA samples were also collected at the same time and the mRNA level of candidate genes was evaluated by RT-PCR. The si-TRAP150-J5 siRNA successfully down regulated TRAP150 to a pretty low level. Interestingly, a significant increase of BCLAF1 proteins was observed. On the contrary, the protein expression level of other candidates showed no obvious difference (Fig. 18A). The result of RT-PCR was similar to that of immunoblotting, and only BCLAF1 showed the inverse relationship to the expression level of TRAP150 (Fig. 18B). The result of RT-PCR in PSF was not consistent with that of real-time RT-PCR, only by which the different expression of PSF could be detected. Considering the sensitivity of the two methods, the differential expression of PSF was probably minor and could only be revealed by the strategy with higher sensitivity. Overall, our results suggested that an inverse relationship existed for the TRAP150 and BCLAF1, both containing a similar domain structure and delivering similar molecular functions.

4. Discussion

In this study, we characterized the function of the RS-domain containing TRAP150 in nuclear mRNA processing. We showed that the overexpressed TRAP150 could activate the pre-mRNA splicing, and such an activity depended on the N-terminal RS domain (Fig. 3). Furthermore, siRNA-mediated knockdown of TRAP150 greatly inhibited the splicing of reporter pre-mRNAs, indicating the critical role of TRAP150 in splicing regulation *in vivo* (Fig. 4). Also, we provided the evidences that TRAP150 might serve as a novel regulator for nuclear mRNA surveillance. When tethered to the 3'-UTR of an mRNA, TRAP150 could trigger mRNA degradation through the C-terminal domain (Fig. 10). However, the degradation was not affected by the translation impairment or the presence of the Upf1 dominant negative mutant (Fig. 11). Moreover, knockdown of TRAP150 had no influence on the degradation of the transcripts containing authentic PTC. We also found that TRAP150-mediated mRNA degradation occurred in the nucleus (Fig. 12). All results we have provided supported that TRAP150 might involve in the nuclear mRNA degradation irrelative to the classical NMD pathway. Overall, our results suggested that TRAP150 is a modular protein and might participate in different steps of nuclear mRNA metabolism.

4.1. Role of TRAP150 in pre-mRNA splicing

Several hints provided by other laboratories indicated that TRAP150 might involve in the splicing regulation. First of all, the mouse TRAP150, the RS domain-containing protein that showed 91% identity to the human homologue, was demonstrated to locate in the interchromatin granules (79). Later, the identification of human TRAP150 associated with some pre-mRNA splicing factors led to the hypothesis that TRAP150 might function as the linkage between the synthesis and maturation of mRNAs (66,109). Subsequent biochemical analysis of mRNPs assembled *in vitro*

indentified TRAP150 as a novel component of the mRNPs, and its RNA association was splicing- and CBC-independent (9). However, whether TRAP150 really involves in the regulation of pre-mRNA splicing remains unknown until we began our investigation.

Extensive characterizations of the composition of different sub-complexes assembled during spliceosome formation were performed by the Lűhrmann's group. Accordingly, TRAP150 was regarded as the complex B proteins as it was only detected in the complex B but absent in the complex A. In addition, TRAP150 might be released from the spliceosome during the transition from complex B to complex C (22,65,110). However, our results indicated that TRAP150 could associate with pre-mRNAs prior to the formation of complex A. Moreover, TRAP150 could bind to the fully spliced RNAs in addition to the pre-mRNAs (Fig. 7). Given that the interaction between TRAP150 and mRNAs was sensitive to the heparin treatment (9), the binding might be weak and transient. Therefore, we speculated that in the previous compositional analyses, TRAP150 might be washed out during the purification of the sub-spliceosome complex and thus failed to be detected.

In addition to the RNA binding properties of TRAP150, we also provided evidences that TRAP150 was required for the splicing regulation at the cellular level. However, no obvious splicing defect could be observed when the TRAP150-depleted extract was used in the cell-free system (Fig. 6). But the ability of TRAP150 to join the spliceosome at the earlier stage suggested its role in coupling transcription and splicing (Fig. 7). Smith et al. showed that both BCLAF1 and TRAP150 might interact with the FF domain of CA150 as detected by the LC/MS/MS analysis (109). The interaction between TRAP150 and CA150 verified in this study (Fig. 8) suggested that TRAP150 might participate in the connection between transcription and splicing. An important function of the splicing factors recruited by elongating Pol II is to help

the spliceosome assembly (26). Indeed, our results of *in vitro* tethering assay showed that tethered-TRAP150 enhanced the splicing of MS2 site-containing pre-mRNAs (Fig. 15). Whether the enhanced splicing is due to the efficient spliceosome assembly remains to be elucidated. To further study whether TRAP150 plays any role in the coordination of transcription and splicing, the *in vitro* coupling assay of transcription and splicing might be introduced (111). It has been reported that both the splicing efficiency and stability of mRNAs were greatly improved in the coupling system because of the appropriate formation of the mRNPs, in contrast to the hnRNPs assembled on the naked transcripts synthesized by viral polymerases. Therefore, such a strategy might be suitable to answer whether TRAP150 plays an important role in bridging the transcription and splicing.

Besides the constitutive splicing, we also investigated if TRAP150 could modulate alternative splicing. However, no obvious effect on the splicing pattern of the E1A reporter could be observed (Fig. 5). Furthermore, TRAP150 also failed to activate the inclusion of exon 5 of the CD44 variant as demonstrated by our colleagues. The exon 5 inclusion had been shown to be regulated by SRrp86, which could interact with TRAP150 even under high salt wash condition (112). In addition to these *in vivo* assays, the elevated concentrations of SR proteins could affect the splicing of AdML substrates *in vitro* by enhancing the utilization of the proximal 5' splice sites (86). However, in our *in vitro* results, similar to the *in vivo* ones, no obvious effect on the splicing of AdML pre-mRNAs was seen when the overexpressed TRAP150-containing nuclear extract was used (Supplemental Fig. 2). Taken together, our results, from both *in vivo* and *in vitro* analyses, indicated that TRAP150 might not involve in the regulation of alternative splicing, although we could not rule out the possibility that specific genes might be targeted to the regulation by TRAP150.

4.2. Role of TRAP150 in mRNA decay

Another important function of TRAP150 characterized in this study is to promote mRNA decay. Although TRAP150 could preferentially associate with the spliced mRNAs and several components of EJC factors (Fig. 8), TRAP150 apparently did not involve in the regulation of NMD. Indeed, TRAP150 promoted mRNA degradation when tethered to the 3' UTR of the transcripts. However, the upstream stop codon and the presence of Upf 1 were not required for the TRAP150-mediated degradation (Fig. 11). These features reminded us of another NMD factor, SMG7. SMG7 is a 14-3-3 like adaptor that forms a complex with SMG5 and protein phosphatase 2A. This complex has been proposed to target to the phosphorylated Upf1 and mediate its translocation to the cytoplasmic mRNA decay bodies for mRNA degradation. Tethering of SMG7 induced mRNA degradation irrespective of the presence of the stop codon. Therefore, SMG7 might act at the downstream stage of PTC definition in the NMD pathway (113,114). However, in contrast to the SMG7 that could be detected in the cytoplasmic foci, TRAP150 predominantly localized to the nucleus. Although TRAP150 interacted with Upf3b, no interaction between TRAP150 and Upf1 could be identified (Supplemental Fig. 3). Therefore, we conclude that TRAP150-mediated mRNA decay is dissimilar to the NMD pathway.

Since TRAP150 might represent a novel nuclear surveillance pathway, we were curious about how it could regulate mRNA stability. One possibility is TRAP150 could recruit the nucleases. However, in spite of the ability of TRAP150 to interact with nucleases Xrn2 and hRrp6, knockdown of these and other nucleases involved in the general mRNA decay could not alter TRAP150-medidated mRNA degradation (Fig. 13). We could not rule out the possibility that the remaining nucleases caused by incomplete knockdown were sufficient to promote mRNA degradation. Another explanation is that additional nucleases remained to be tested could cooperate with

TRAP150 to degrade mRNAs. For example, although Rrp6 was regarded as the catalytic subunit of the nuclear exosome complex in yeast, another catalytic subunit, Dis3 (also known as ribosomal RNA-processing protein 44, Rrp44), also involved in the processing or degradation of different classes of nuclear RNAs such as rRNAs and snoRNAs, etc (115,116). Therefore, it might be worth testing if knockdown of both catalytic subunits of the exosome complex might affect TRAP150-mediated mRNA degradation.

In addition to the exonucleases, several endonucleases were recently identified to cleave specific targets (117). Interestingly, among the peptides identified through TRAP150-interacting proteins, RasGTPase activating protein SH3 domain-binding proteins (G3BPs) were also included. Studies from the G3BPs deficient mice indicated that its endonucleolytic activity may play an important role in the post-transcriptional regulation of targeted mRNAs in response to the alteration of growth conditions and extracellular stimuli. Although G3BPs could be detected in the stress granules, phosphorylation could mediate the translocation of G3BPs from cytoplasm to nucleus and the subsequent integration to mRNPs. G3BPs contain two RNA-binding motifs, RNA recognition motif and C-terminal arginine-glycine (RGG) rich box. However, no endonuclease domain has been characterized within G3BPs. It has been shown that site-specific cleavage of RNAs by G3BPs is dependent on their phosphorylation and interaction with the substrate RNAs through the recognition of the specific consensus sequence: ACCCA(U/C)(A/C)(C/G)G(C/A)AG (118-120). Therefore, it will be intriguing to determine whether TRAP150 and G3BPs might involve in the degradation of some specific mRNAs under stress conditions.

Considering that TRAP150 was a nuclear-restricted protein and the tethering NMD assay was adopted for the analysis of mRNA stability, TRAP150 might delay the export of the mRNAs and turn on the nuclear mRNA surveillance systems as the case

of DRN (95). However, no obvious effect on TRAP150-mediated mRNA decay was observed upon the downregulation of CBP80 and hRrp6, two critical components for the yeast DRN (Fig. 14). A critical unresolved question is whether the same surveillance system operates in higher eukaryotic cells. Furthermore, considering the importance of CBC in mRNA maturation, the knockdown efficiency of CBP80 might not be sufficient to inhibit its function and thus a significant level of mature mRNAs was still generated. Therefore, other more direct methods, for example, expressing a chimeric TRAP150 with efficient NES-signal peptides, could be used to characterize the possible effects on the trapped mRNAs by TRAP150 in the nucleus. Taken together, although in several directions we have tried to discover how TRAP150 could trigger mRNA decay, the exact mechanism remains further testified.

4.3. Roles of TRAP150 in other mRNA processing steps

Interestingly, when we searched for the associated proteins of TRAP150, we found several components of the 17S U2 snRNPs which are critical for the branch point selection and the catalysis steps (100). Moreover, we also identified some subunits of the CPSF complex. Kybyrz et al. identified a supracomplex with U2 and CPSF complexes and proposed the mechanism to couple splicing and 3' end processing by the recruitment of the supracomplex to the RNAs through elongating Pol II and the mutual stabilization of the splicing and 3' end processing machineries (101). If TRAP150 really interacts with both complexes, it might also involve in the 3' end processing of mRNAs. In fact, when we reexamined the results of the tethering assays with MCP-TRAP150, we found that although TRAP150 could result in the efficient degradation of reporter transcripts, some smearing signals could still be observed (Fig. 14D for example). Such signals could result either from the degradation of βG transcripts or from the aberrant 3' end processing that led to the heterogenous lengths

of poly (A) tails, which could be sensed and degraded through nuclear surveillance systems (96). Our laboratory has started to test the latter possibility and the preliminary results indicated that the smearing signals were as a result of the irregular poly (A) tails. Whether this feature is correlated to the mRNA degradation remains further investigation.

In this study, TRAP150 was initially identified as the TAP-interacting protein. Because of the nucleus-restricted property of TRAP150, however, we did not pursue the possible role of TRAP150 in mRNA export. Interestingly, LC/MS/MS analysis of TRAP150-associated proteins showed that four of the seven subunits of the mammalian TREX complex might be TRAP150-associated. In addition to the CBC and splicing, recent study suggested the matozoan TREX could be recruited cotranscriptionally through Sus1 (43). Since TRAP150 is a putative transcriptional factor and modulates splicing, it is reasonable to persume that TRAP150 might involve in the loading of the TREX complex during the mRNA maturation process and the subsequent export efficiency of the mature mRNAs. Moreover, large numbers of ribosomal proteins as well as some translation factors have also been identified. So far, it is hard to explain how a nuclear-restricted TRAP150 could involve in the translational regulation. However, if TRAP150 could facilitate the efficient export of the mRNAs, this could result in the onset of the pioneer round translation from the cytoplasm to the nuclear-associated, a similar mechanism that was adopted by ASF/SF2 to regulate NMD (121).

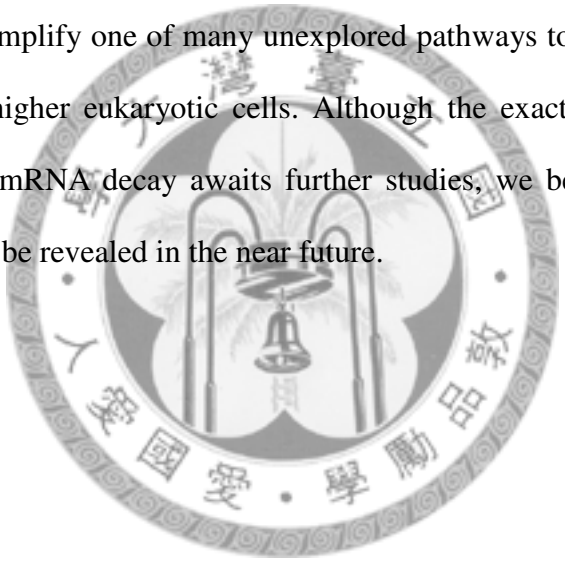
In addition to the importance of the TREX complex in coordinating transcription and mRNA export, other roles of TREX, especially those involved in the nuclear RNA surveillance, have been revealed using yeast THO/Sub2 mutants. A phenotype called differential chromatin fractionation (DCF), which represented a transcription-dependent complex at the 3’ end of THO/Sub2 target genes, was

observed by Rougemaille et al. from strains deficient in THO or Sub2. DCF formation is due to the defective 3' end processing and results in the loss of 3' end fragments from chromatin preparation (122). The inefficient 3' end polyadenylation could trigger the nuclear surveillance pathway mediated by a non-canonical poly (A) polymerase-containing TRAMP complex and the Rrp6-containing nuclear exosome complex (123). The interrelationship between the 3' end processing and mRNA export was further sustained by recent studies from two groups. Johnson et al. provided the evidence that the cleavage/polyadenylation factor CF1A, but not the previously thought Sub2, was required for the recruitment of Yra1. Moreover, Sub2 and Pcf11, the Pol II CTD-binding subunit of CF1A, have been found to bind to the Yra1 in a mutually exclusive manner (124). Moreover, Qu et al. found that the mutations of factors required for mRNA export including THO/Sub2, Yra1p, Sac3p and the Mex67p/Mtr2 heterodimer caused not only the nuclear retention of mRNAs, but also the failure in the release of 3' end processing factors from the polyadenylated transcripts (125). Taken together, these findings suggested that the correct 3' end processing might assist the remodeling and formation of the export-competent mRNPs, which in turn led to the release of 3' end processing factors. It might be interesting to examine whether TRAP150 is involved in such kind of remodeling, of which aberrations could trigger the mRNA decay.

In addition to the exonuclease activity employed by the THO/Sub2 complex, a recent report also indicated that the NPC-associated-endonuclease Swt1 involved in the perinuclear mRNP surveillance. By specific interactions between TREX and TREX2 complexes, PIN (PilT N terminus) domain-containing Swt1 might recognize and degrade any defective pre-mRNAs or mRNAs trapped at the nuclear periphery to avoid the subsequent cytoplasmic translation (126). Whether such kind of regulations also functions in higher eukaryotic cells remains to be demonstrated in the future.

Also, the connection of TREX complex to several different kinds of mRNA surveillance pathways highlights future works for us to study how TRAP150 might help the turnover of mRNAs.

As we know, the majority of findings about the nuclear mRNA surveillance was based on the studies summary in yeast (127). Interestingly, various aberrant transcripts have recently been identified in human transcriptome by high-throughput analyses (128-131), indicating the existence of the nuclear surveillance system in mammalian cells to clear out these transcripts. Here we provide the evidences that TRAP150 might exemplify one of many unexplored pathways to regulate the nuclear mRNA stability in higher eukaryotic cells. Although the exact mechanism of how TRAP150 mediates mRNA decay awaits further studies, we believe this and other pending puzzles will be revealed in the near future.



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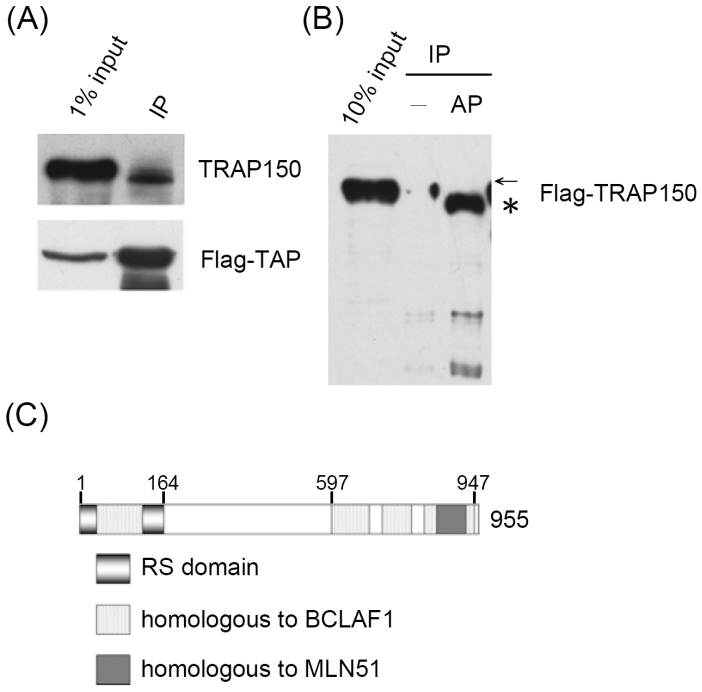


Figure 1. TRAP150 is a TAP-interacting phosphorylated protein. (A) FLAG-tagged TAP was transiently overexpressed in HEK293 cells. The cell lysates were subjected to the immunoprecipitation by agarose beads conjugated with anti-FLAG. The precipitated proteins were immunoblotted with anti-TRAP150 (upper) or anti-FLAG (lower). (B) Overexpressed FLAG-TRAP150 from HEK293 cells was immunoprecipitated by anti-FLAG. The immunoprecipitated FLAG-TRAP150 was treated with alkaline phosphatase (AP) or not (-) and detected by immunoblotting with anti-FLAG antibody. The arrow and asterisk indicated the immunoprecipitated TRAP150 with or without the AP treatment, respectively. (C) Schematic representation of the TRAP150 domain structure. The numbers represent the relative positions of amino acid residues corresponding to each indicated domain.

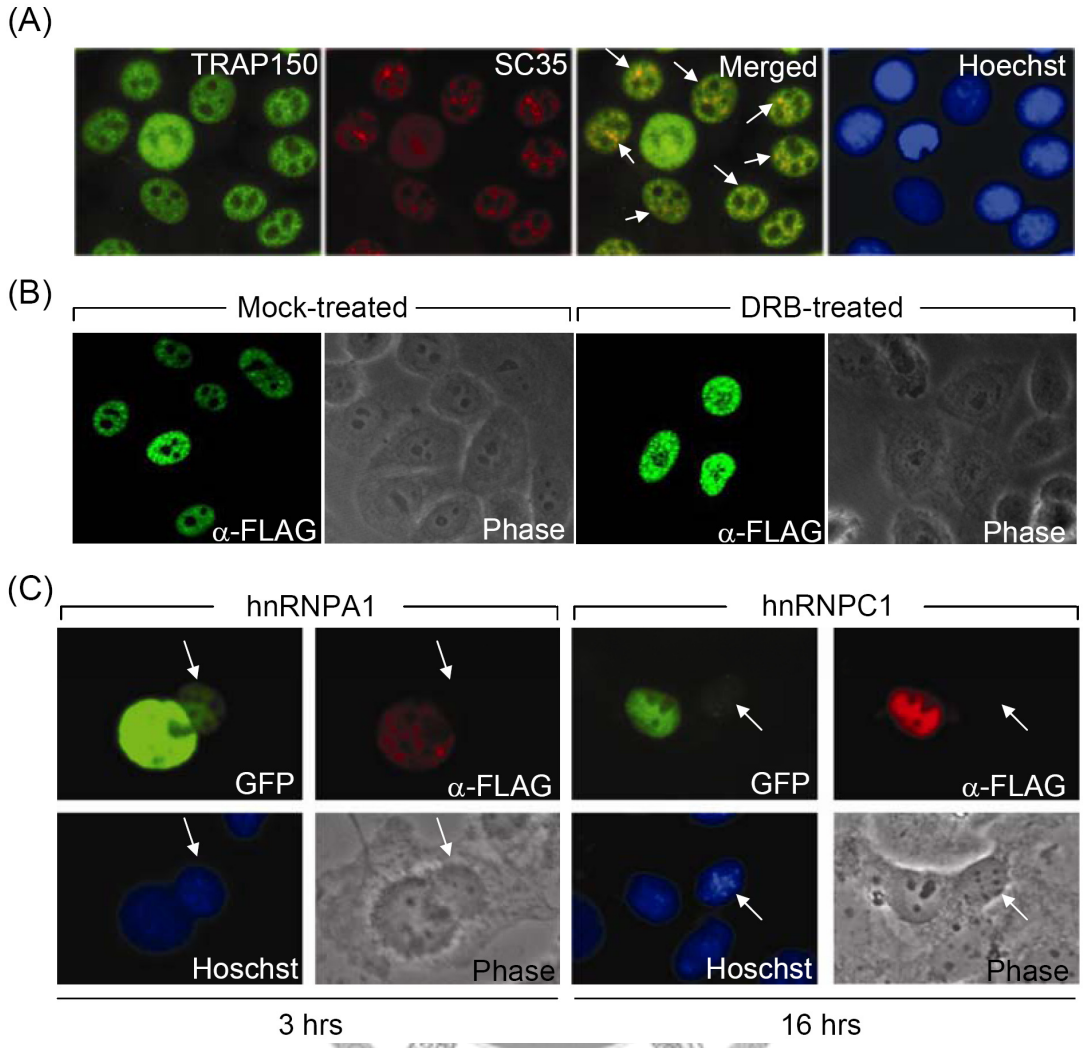


Figure 2. Cellular localization of human TRAP150. (A) The double immunofluorescence staining of HeLa cells were performed with anti-TRAP150 and anti-SC35. Co-localized signals were indicated by arrows. Nuclei were stained with Hoechst dye 33258. (B) HeLa cells were transfected with expression vectors encoding FLAG-TRAP150. The transfected cells were either mock- or DRB-treated. Immunofluorescence staining with anti-FLAG was performed to detect the overexpressed TRAP150. (C) HeLa cells were co-transfected with the expression vectors encoding FLAG-TRAP150 and GFP-hnRNP A1 or GFP-hnRNP C1. Transfected HeLa cells were fused with mouse NIH3T3 cells for 3 or 16 hrs in the presence of cycloheximide. Immunofluorescence was performed with anti-FLAG; GFP fusion proteins were directly visualized by fluorescence microscopy. Arrows indicated mouse cell nuclei.

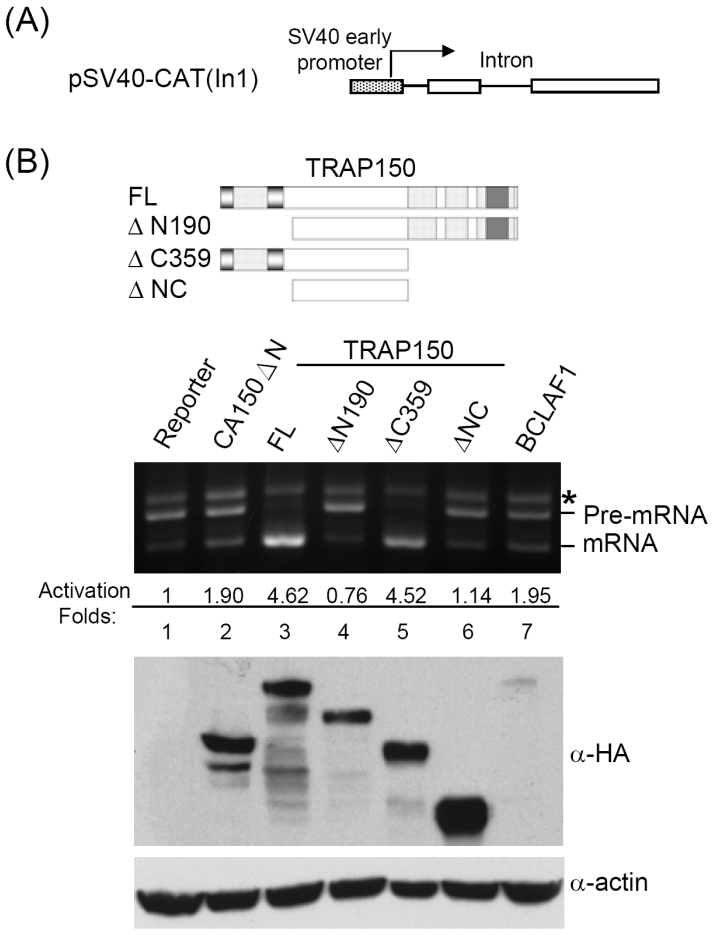


Figure 3. TRAP150 activates pre-mRNA splicing. (A) The splicing reporter pSV40-CAT(In1) encoded the chloramphenicol acetyl transferase, in which human β-globin intron 1 was inserted. (B) Schematic representation of full-length and truncated TRAP150 proteins. Domains were depicted as in Figure 1. (C) The splicing reporter was cotransfected with an expression vector encoding the indicated effector into HeLa cells. RT-PCR was performed with primers specific for the reporter exons (upper). Band identity was indicated; the asterisk denoted the precursor/spliced RNA hybrid. Splicing efficiency in individual transfectants was calculated as the intensity of the spliced RNAs over the sum of the spliced RNAs and the precursor. Splicing activation folds were obtained from three independent experiments. The immunoblotting with anti-HA was performed to confirm the expression level of each effector. The actin served as the loading control (lower).

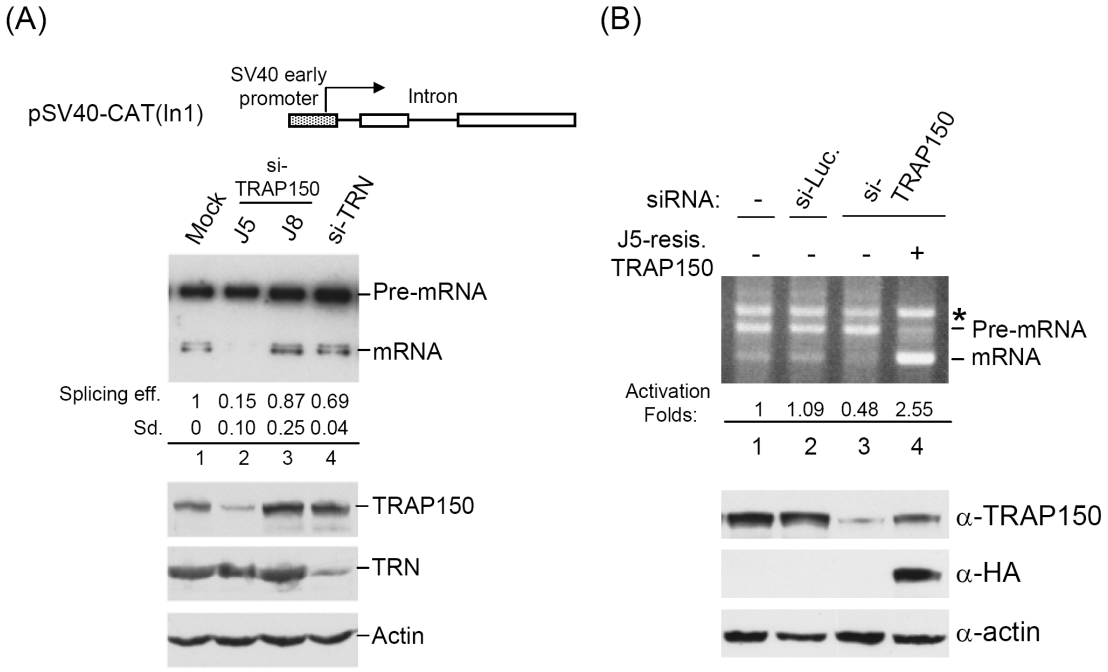


Figure 4. TRAP150 is essential for *in vivo* splicing. (A) The pSV40-CAT(In1) reporter was co-transfected with indicated siRNAs into HeLa cells. RT-PCR was performed with primers specific for the reporter exons; the amplified products were analyzed by Southern blotting with the primer specific for the exon (upper). Splicing efficiency in individual transfectants was calculated as the intensity of the spliced RNAs over the sum of the spliced RNAs and the precursor and the ratios were obtained from three independent experiments. Immunoblotting with antibodies specific to indicated proteins was performed to reveal the knockdown efficiencies (lower). (B) HeLa cells were transfected with siRNAs (si-Luc. or si-TRAP150). 48 hrs post-transfection, the pSV40-CAT(In1) reporter was co-transfected with empty vector (-) or the vector encoding HA-tagged J5-resistant TRAP150 (+) into siRNA-transfected HeLa cells. The results of RT-PCR and calculated folds of splicing activation were described as Figure 3C. The elevated expression level of TRAP150 was confirmed by immunoblotting with anti-TRAP150, and anti-HA was used to detect the expression of J5-resistant TRAP150. The actin represented the loading control (lower).

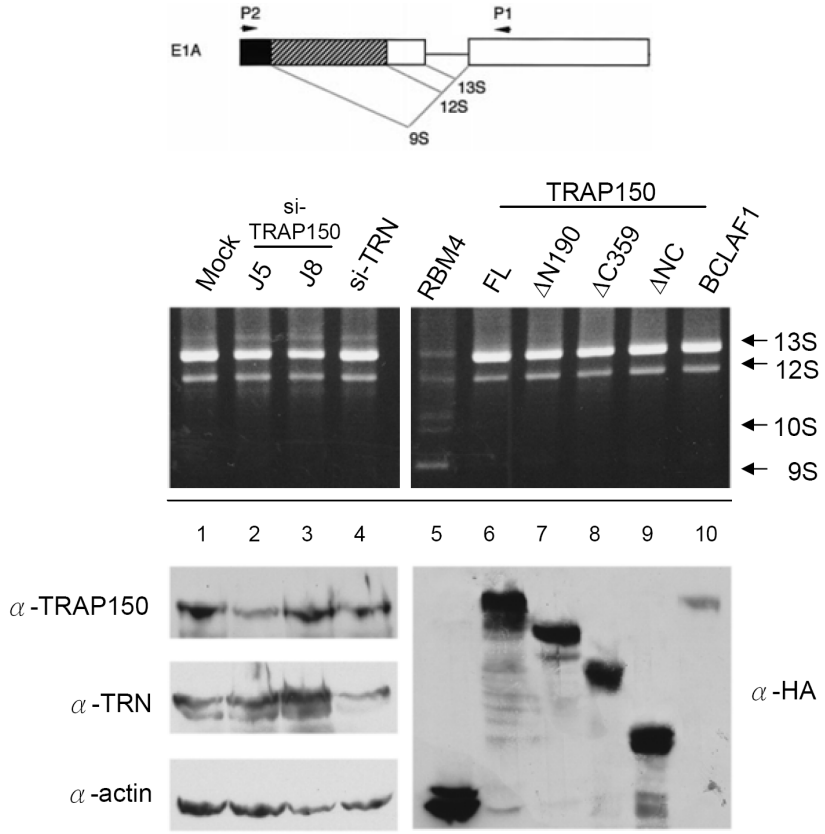


Figure 5. TRAP150 could not modulate the alternative splicing. The diagram showed the major splicing products of adenovirus E1A pre-mRNA encoded by pCEP4-E1A plasmid (upper). The reporter plasmid was co-transfected with either indicated siRNAs (middle, lane1-4) or expression vectors encoding HA-tagged effectors as indicated (middle, lane 5-10). Total RNA was isolated and subjected to the RT-PCR analysis with specific primers as indicated (P1 and P2). The Immunoblotting with indicated antibodies was carried out to verify the knockdown efficiency of each siRNA (lower, lane 1-4), or the expression level of each effector (lower, lane 5-10).

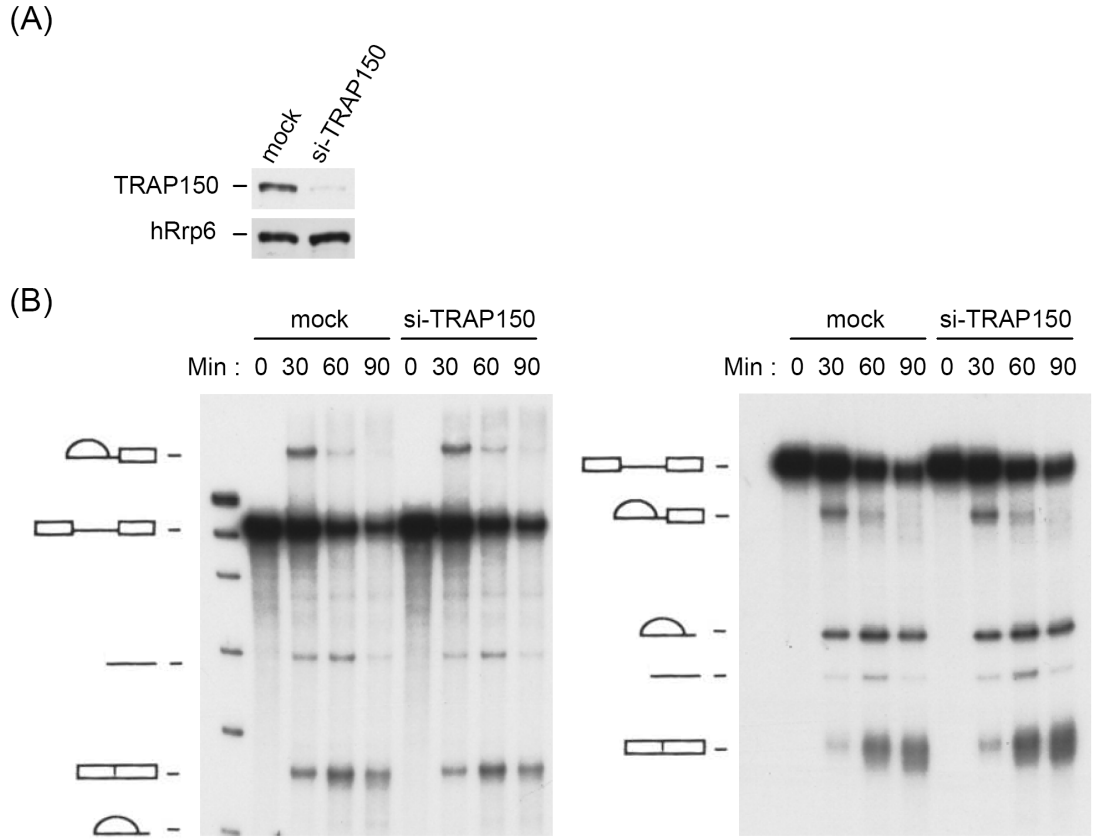


Figure 6. TRAP150 is not required for pre-mRNA splicing in a cell-free system. (A) HeLa cells were mock or si-TRAP150 (J5) transfected. The nuclear extract was prepared from the transfected cells and analyzed by immunoblotting with anti-TRAP150 (upper). The hRrp6 was used as the loading control (lower). (B) *In vitro* splicing using α-^{32}P-labeled AdML (left) or PIP85a (right) pre-mRNAs as the substrate was performed in the mock- or TRAP150-depleted nuclear extract.

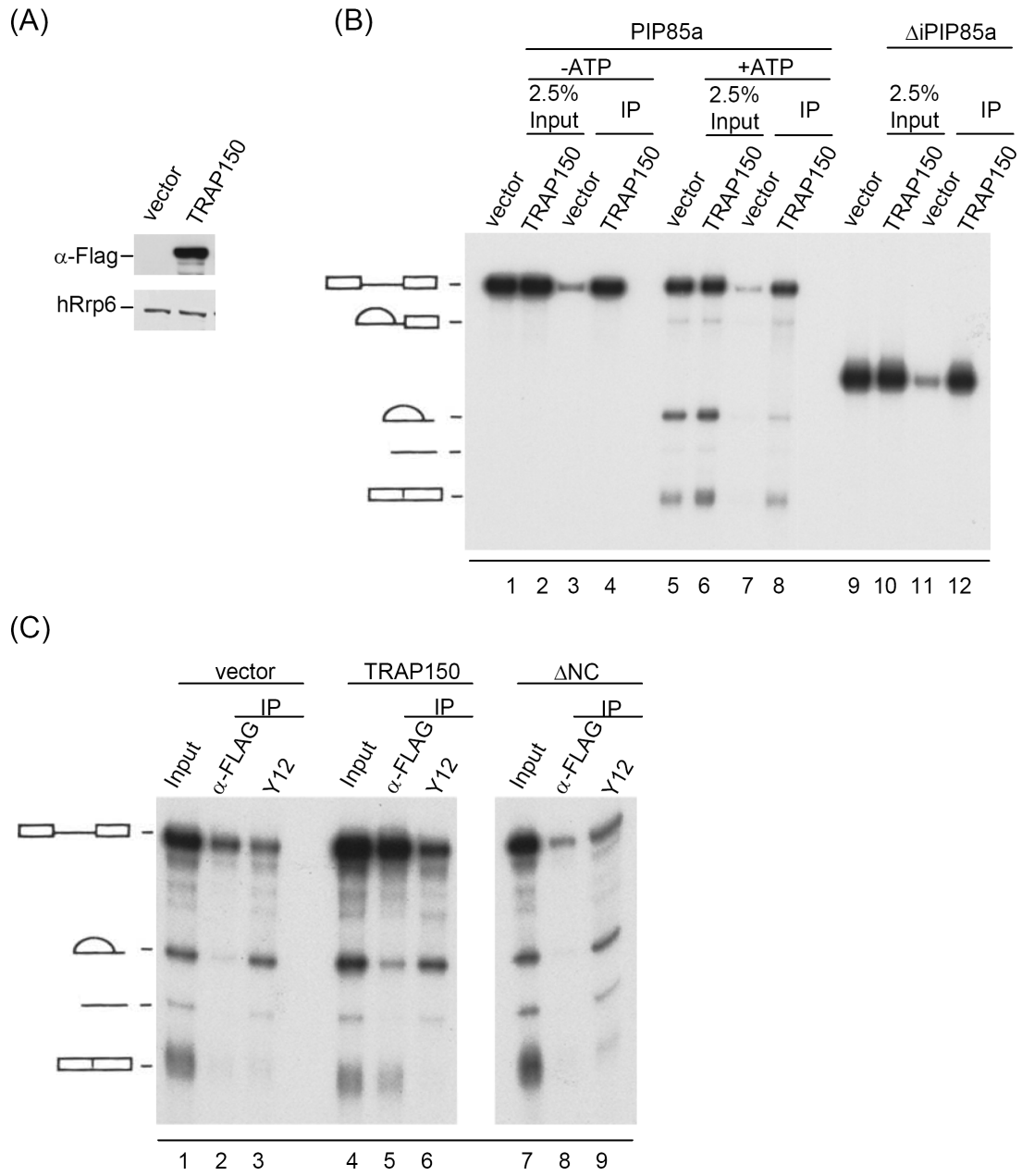


Figure 7. The RNA binding properties of TRAP150. α-^{32}P-labeled PIP85a pre-mRNAs or intronless PIP85a (ΔiPIP85a) mRNAs were subjected to *in vitro* splicing reaction using HEK293 cell nuclear extracts with or w/o FLAG-TRAP150. (A) Immunoblotting using anti-FLAG was performed to detect the expression of FALG-TRAP150. The hRrp6 was served as the loading control. (B) The reaction was performed in the absence (-) or presence (+) of ATP, followed by immunoprecipitation using anti-FLAG. The autoradiogram showed the splicing reaction and precipitated RNAs. (C) Standard *in vitro* splicing using α-^{32}P-labeled PIP85a was performed. The subsequent immunoprecipitation was performed using anti-FLAG or anti-Sm protein (antibody Y12).

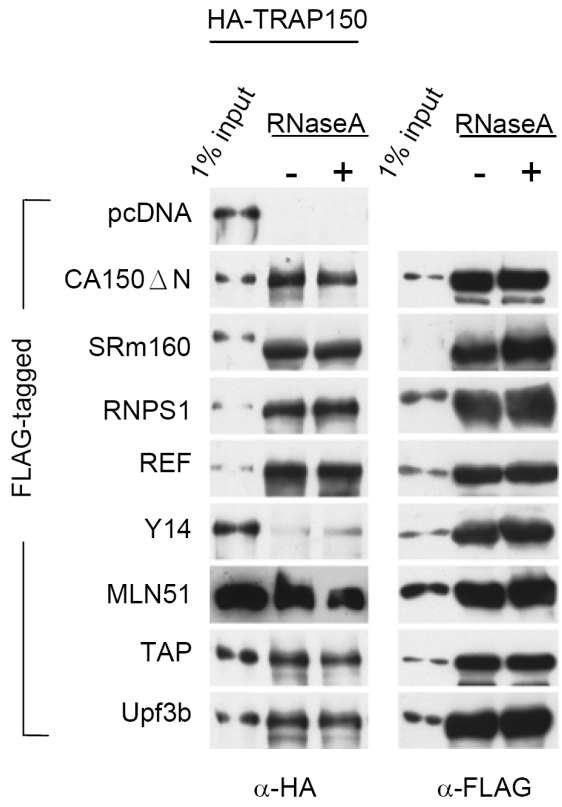


Figure 8. TRAP150 is associated with the EJC. HEK293 cells were transfected with the expression vectors encoding HA-TRAP150 and indicated FLAG-tagged proteins. Immunoprecipitation was performed using anti-FLAG antibody in the presence of RNaseA (+) or not (-). The immunoblotting using anti-HA was performed to detect the HA-TRAP150 co-purified with FLAG-tagged proteins (left). The immunoprecipitated FLAG-tagged EJC component was confirmed by immunoblotting with anti-FLAG (right).

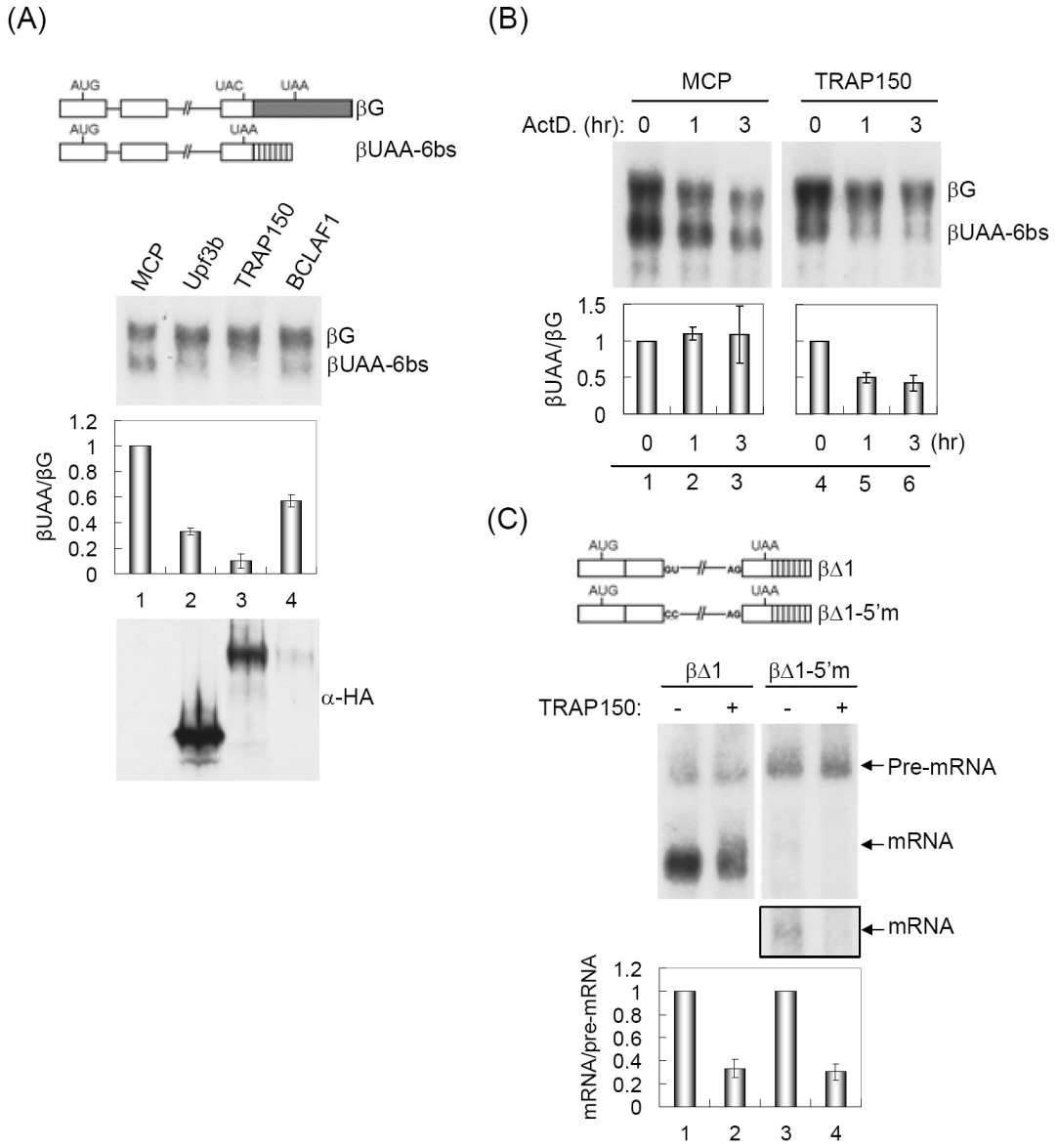


Figure 9. TRAP150 promotes mRNA degradation in the tethering NMD assay. (A) The schematic representation of the β-globin reporter βUAA-6bs that contains the UAA stop codon upstream of the six copies of the MS2 coat protein (MCP)-binding site; βG without MCP-binding sites was used as a co-transfection reference. The βUAA-6bs and βG vectors were cotransfected with expression vectors encoding the indicated effectors. Northern blotting was performed using α-^{32}P-labeled β-globin probe. In individual transfectants, the intensity of steady state βUAA-6bs mRNA was normalized to that of βG. Bars showed the relative level of the βUAA-6bs mRNAs from MCP-effector transfectants to that from the MCP only group and the ratios were obtained from three independent experiments. (B) The βUAA-6bs and βG vectors were cotransfected with the MCP or MCP-TRAP150 expression vector for 10 hrs.

Cells were collected after additions of actinomycin D for 0, 1 or 3 hrs. Northern blotting was performed as in panel A. Bars showed the relative mRNA level of βUAA-6bs to βG from each transfectant normalized to that of time zero. (C) Schematic representation of the βΔ1 and the 5' splice site mutant βΔ1-5'm reporters. These two reporters were individually cotransfected with the MCP or MCP-TRAP150 expression vector. Northern blotting was performed as in panel A; a longer exposure of the spliced products of βΔ1-5'm was shown in the outlined rectangle. The steady state level of the spliced mRNAs was normalized to that of the precursor mRNAs in individual transfectants; relative levels of the above ratios from MCP-TRAP150 transfectant to the corresponding mock were indicated.

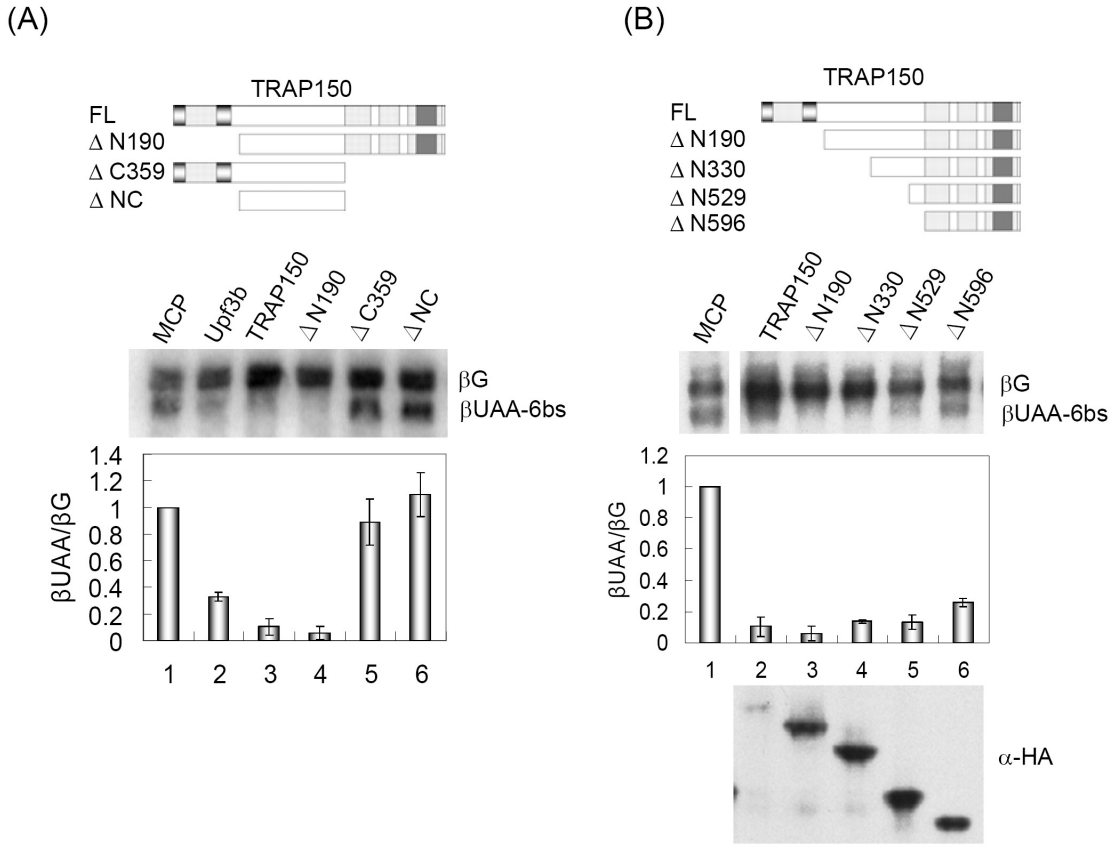


Figure 10. The C-terminal conserved region is critical for mRNA degradation. (A) The tethering NMD assay was performed by co-transfection of the reporter as in panel A and the vector encoding MCP or MCP-fused proteins as indicated. Domains were depicted as Figure 1. The relative mRNA level of βUAA-6bs to βG and its bar representation from the transfectants were described as Figure 9A. (B) Additional deletion constructs encompassing different lengths of C-terminal TRAP150 were generated (upper) and used for the tethering NMD assay (middle). The expression of each effector was verified by immunoblotting with anti-HA.

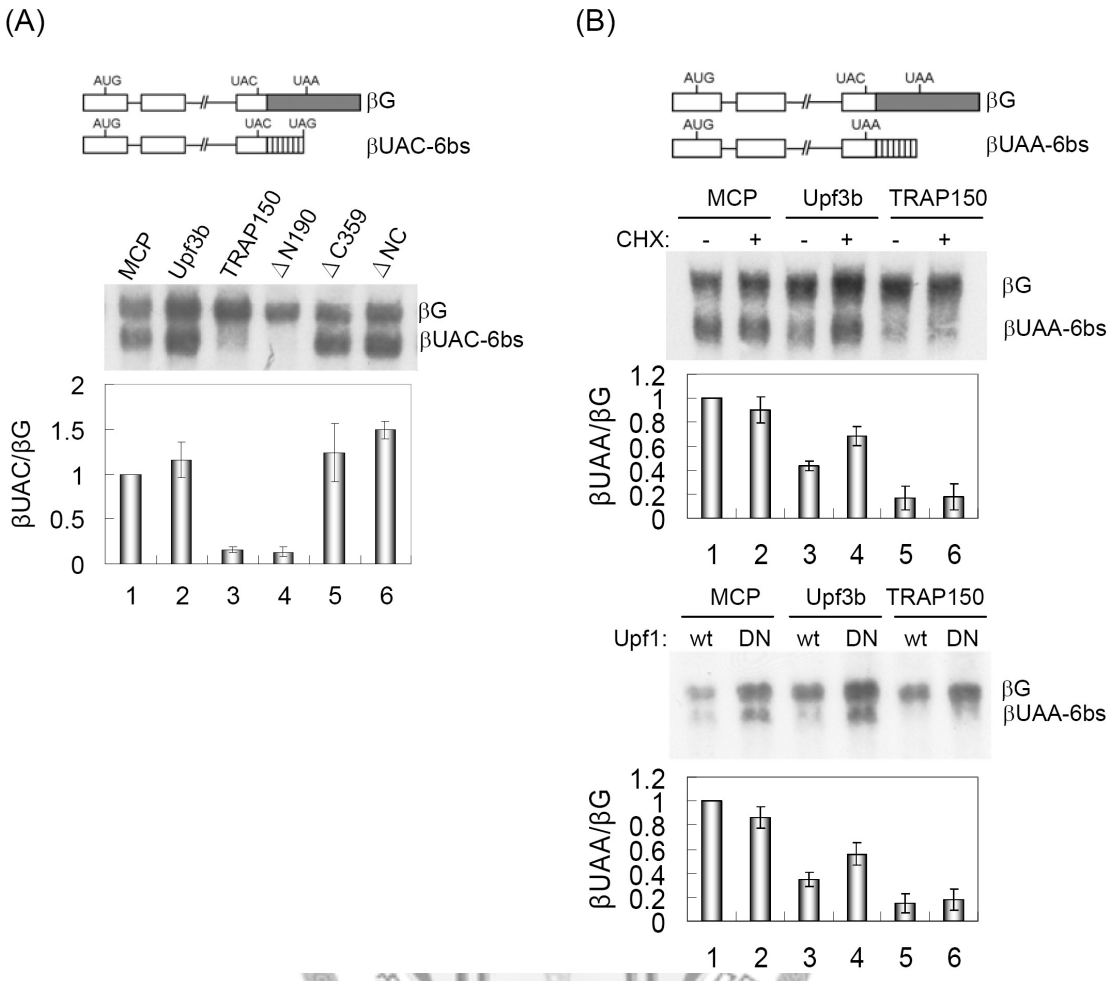


Figure 11. TRAP150-mediated mRNA decay is Upf1- and translation-independent. (A) The scheme showed the βUAC-6bs reporter that is similar to βUAA-6bs except that its UAA codon was mutated to UAC. The TRAP150-tethering assay and the relative mRNA level of βUAC-6bs to βG from the transfectants were shown as Figure 9A. (B) Tet-Off HeLa cells were transfected with the βUAA-6bs and βG reporters and the MCP or MCP-effector expression vector. 24 hrs post-transfection, cells were mock (-) or cycloheximide treated (+) and then incubated for another 2 hrs (upper). To determine the Upf1 dependence (lower), the βUAA-6bs and βG plasmids were cotransfected with the expression vector encoding MCP, MCP-Upf3b or MCP-TRAP150 as well as the wild-type or dominant-negative (DN) mutant Upf1. Northern blotting and calculated relative levels of steady state βUAA-6bs mRNAs were shown in Figure 9A.

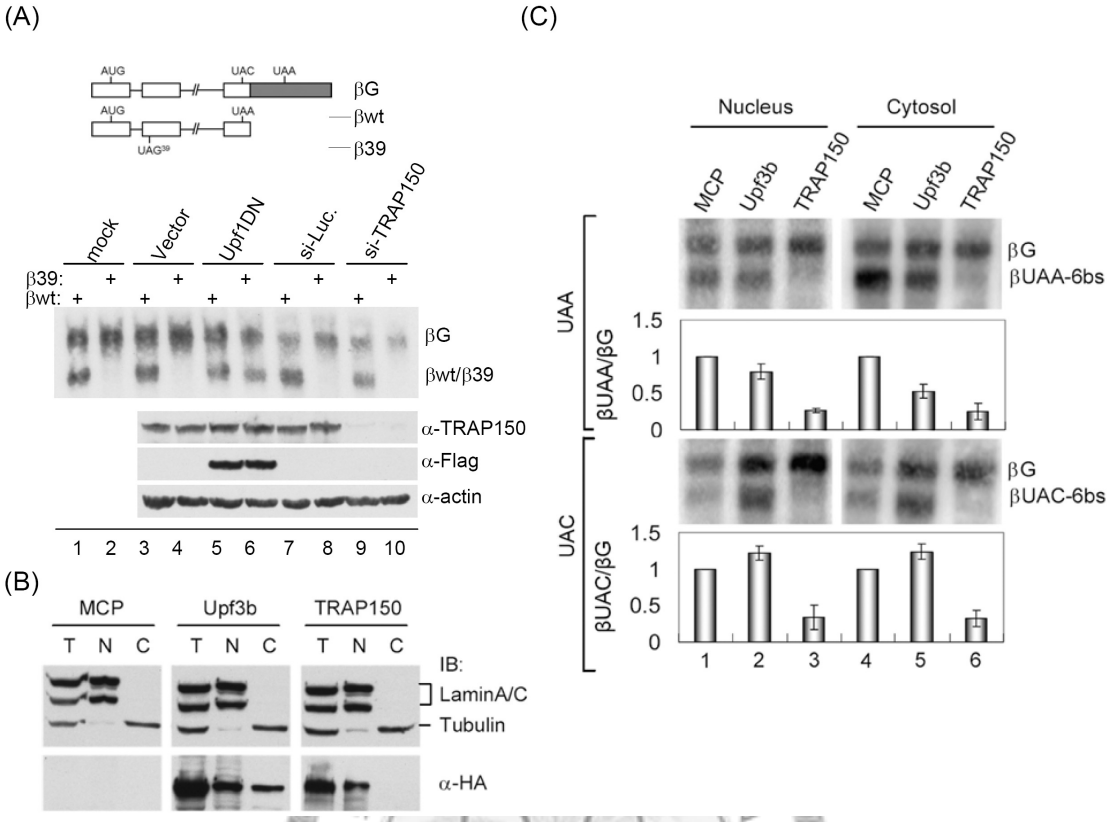


Figure 12. TRAP150 is not essential for NMD and promotes mRNA decay in the nucleus. (A) HeLa cells were transfected with the indicated siRNAs for 48 hrs. Subsequently, the β-globin reporter containing a translation termination codon UAG in exon 2 (β39) or not (βwt) was co-transfected with the reference βG. For lanes 5 and 6, the expression vector of the dominant-negative Upf1 was co-transfected with the reporters. Northern blotting was performed as Figure 9A. Immunoblotting of cell lysates was performed using anti-TRAP150, anti-FLAG and anti-actin (bottom). (B) Tet-Off HeLa cells were transiently transfected with an expression vector encoding MCP, MCP-Upf3b or MCP-TRAP150. Immunoblotting of total (T), nuclear (N) or cytoplasmic (C) extract of transfected HeLa cells was performed using respective antibodies against lamin A/C, tubulin and HA epitope for MCP-fusions. (C) The βUAA/C-6bs reporter assay was performed essentially as Figure 9A except that the RNAs for northern blotting were prepared from subcellular fractions. Bars showed the relative mRNA level of βUAA-6bs or βUAC-6bs to βG.

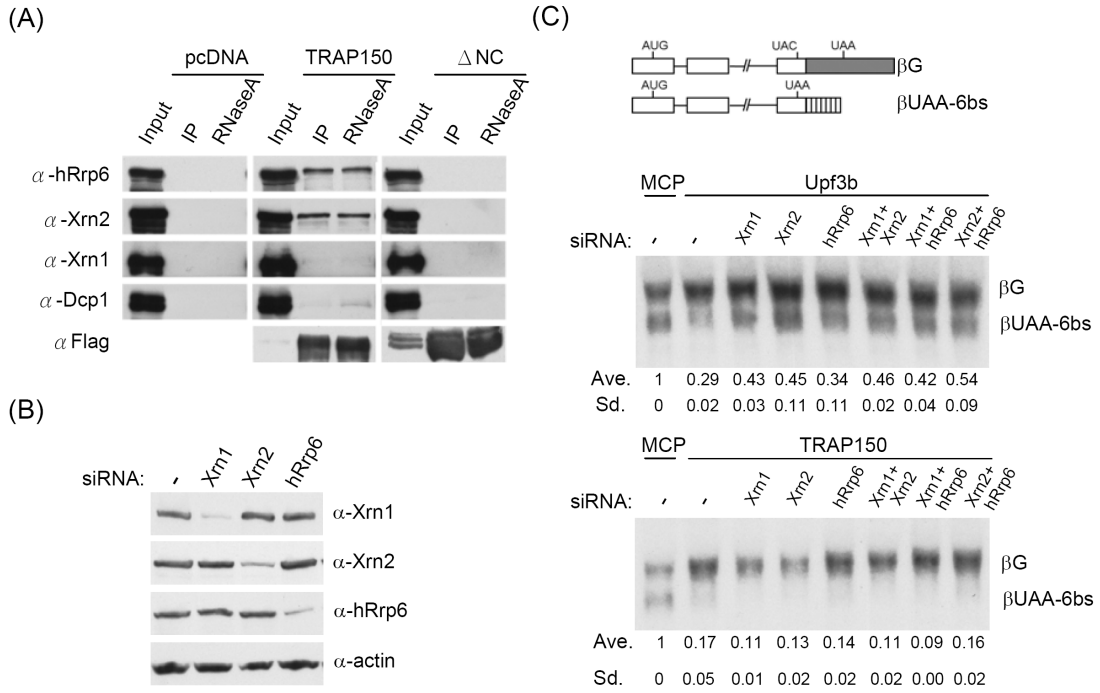


Figure 13. Knockdown of the exonuclease Xrn1, Xrn2 and Rrp6 does not interfere with TRAP150-mediated mRNA degradation. (A) The expression vector encoding FLAG-TRAP150 or FLAG-ΔNC was transfected to HEK293 cells. Cell lysates were subjected to the immunoprecipitation with anti-FLAG and subsequent immunoblotting with indicated antibodies. (B) Knockdown of indicated exonucleases was achieved by specific siRNAs. Immunoblotting with indicated antibodies showed the protein depletion efficiency. (C) At 48 hrs post-transfection of siRNAs, the βUAA-6bs reporter and the MCP, MCP-Upf3b or MCP-TRAP150 expression vector were transfected. Northern blotting was performed as Figure 9A; values below the autoradiograms indicated the relative level of steady state βUAA-6bs mRNAs.

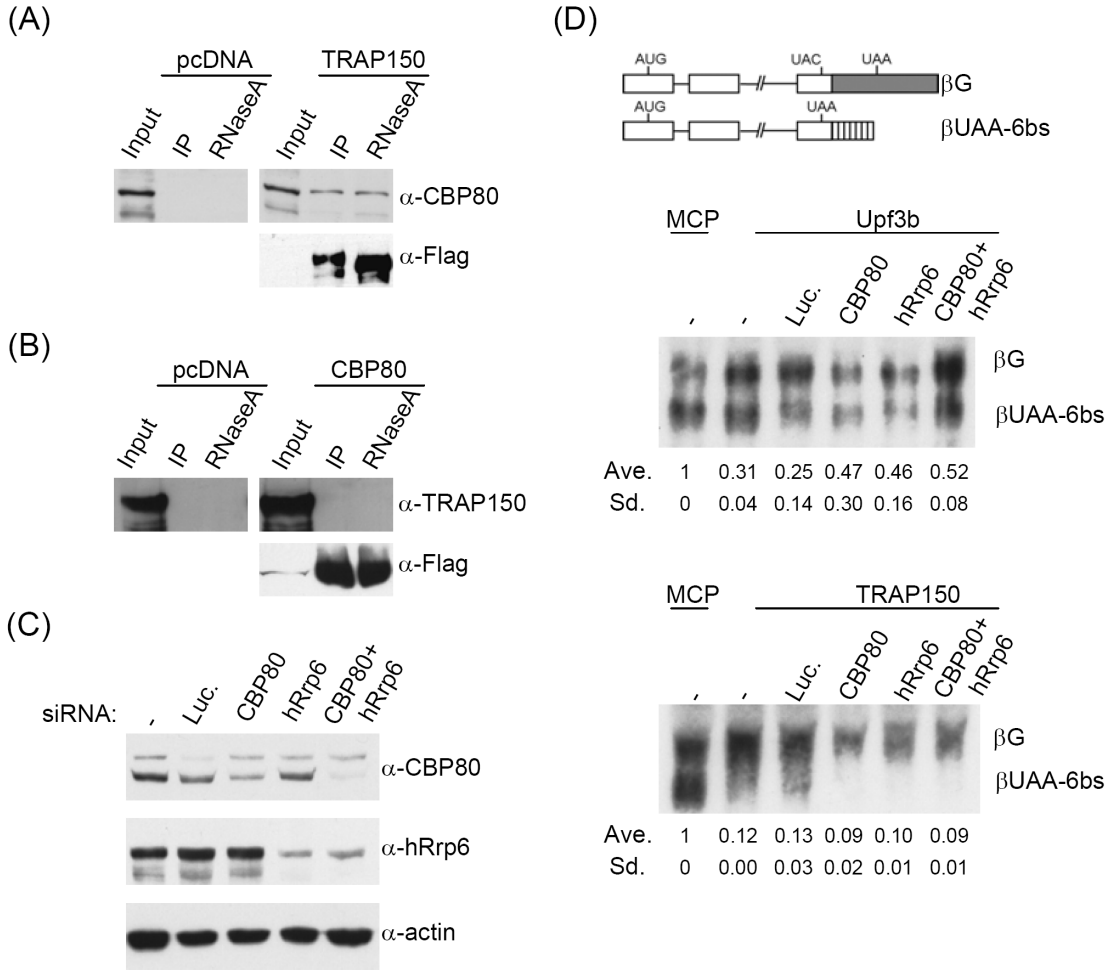


Figure 14. TRAP150-meidated mRNA decay is distinct from DRN. HEK293 cells were transfected with expression vectors encoding FLAG-TRAP150 (A) or FLAG-CBP80 (B). The anti-FLAG immunoprecipitated proteins were subjected to the immunblotting using indicated antibodies. (C) Knockdown of indicated exonucleases was achieved by specific siRNAs. Immunoblotting with indicated antibodies showed the protein depletion efficiency. (D) At 48 hrs post-transfection of siRNAs, the βUAA-6bs reporter and the MCP, MCP-Upf3b or MCP-TRAP150 expression vector were transfected. Northern blotting was performed as Figure 9A; values below the autoradiograms indicated the relative level of steady state βUAA-6bs mRNAs.

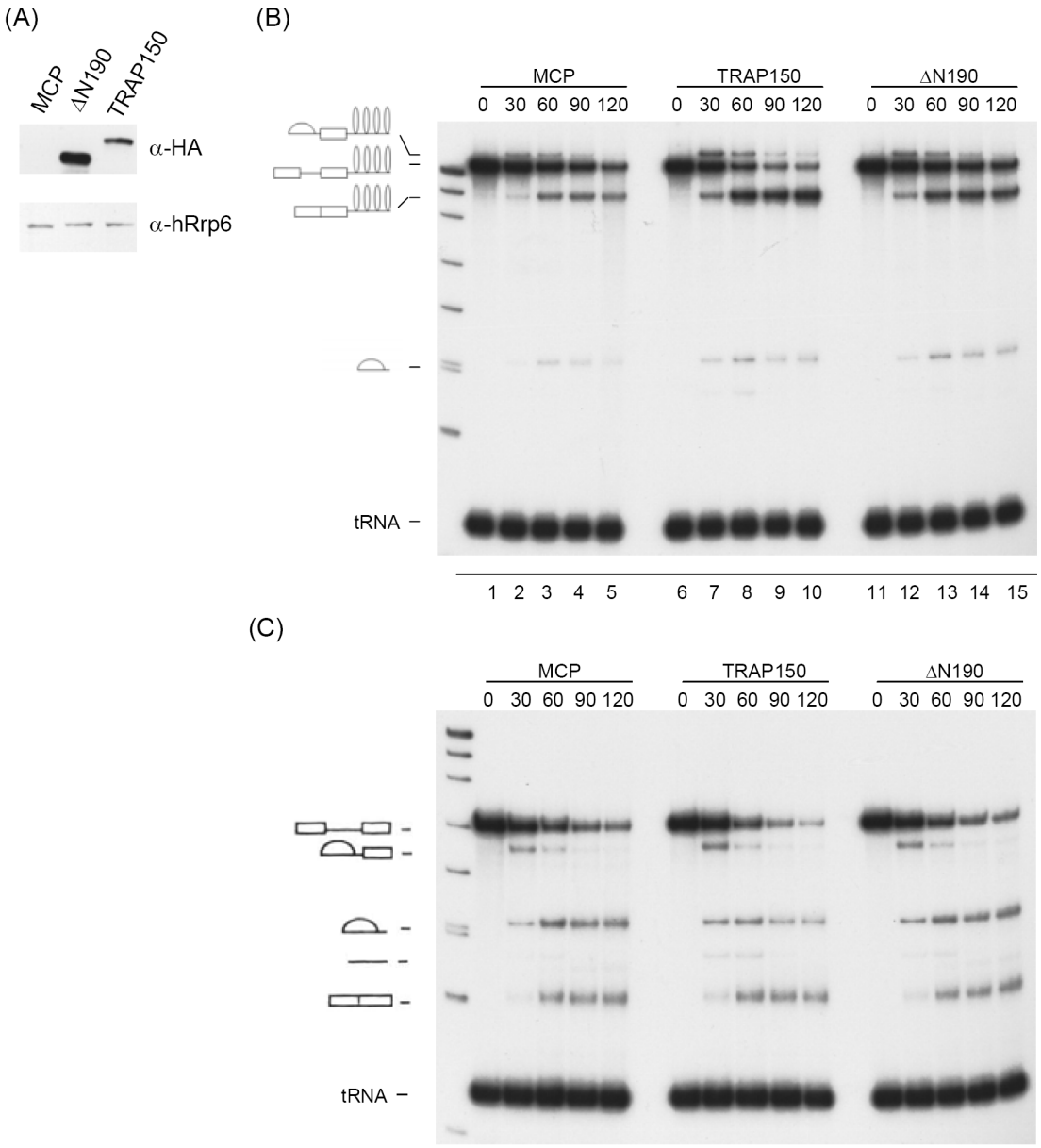


Figure 15. Tethered TRAP150 enhances pre-mRNA splicing *in vitro*. (A) MCP, MCP-TRAP150 or MCP-ΔN190 was overexpressed respectively in HEK293 cells for nuclear extract preparation. The expression was verified by immunoblotting with anti-HA, and the hRrp6 was used for the loading control. (B) *In vitro* splicing was performed essentially as Figure 6 except that the substrate was changed to a modified PIP85a which was inserted with four copies of MCP-binding sites (PIP85a-4XMS2) into the downstream of its second exon. When splicing reaction was stopped, tRNA was added to serve as mRNA recovery and loading control. (C) Similar to (B), except that α-^{32}P-labeled PIP85a pre-mRNA was used as the substrate.

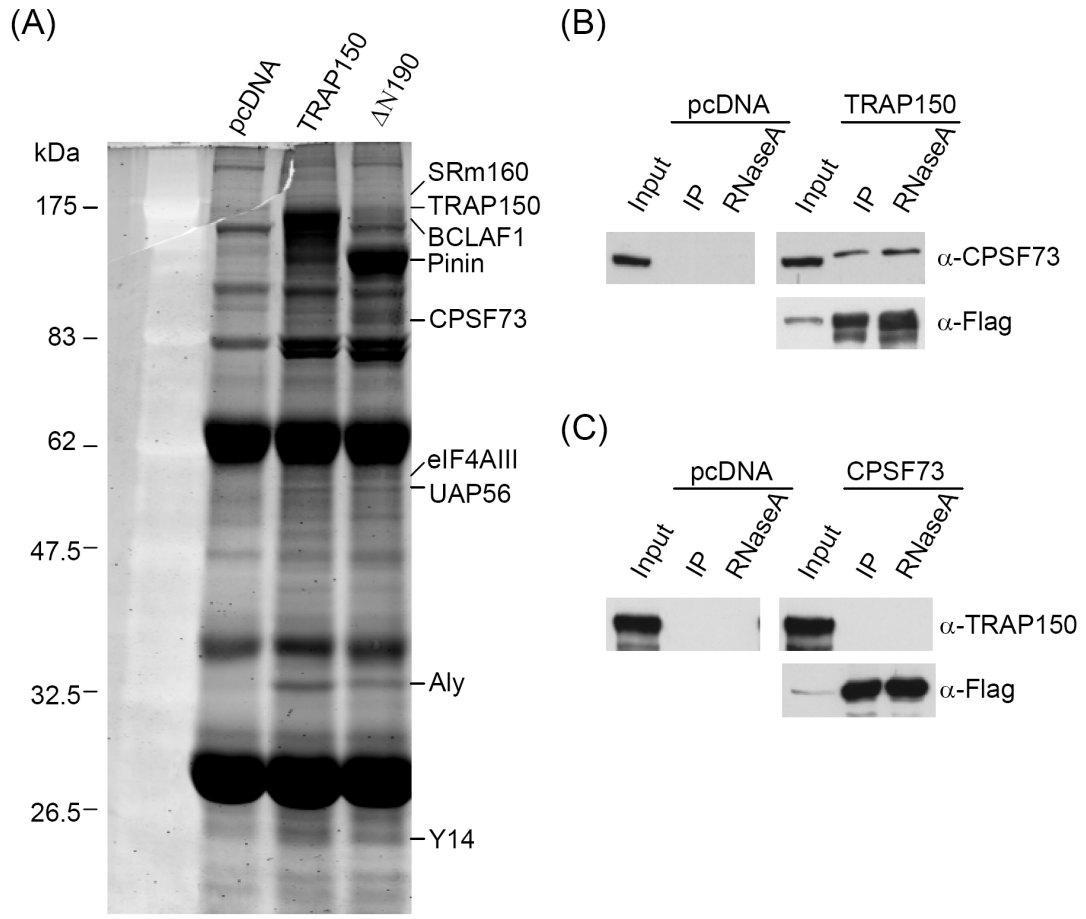


Figure 16. TRAP150 associates with CPSF73. HEK 293 cells were transiently transfected with an expression vector encoding FLAG-tagged TRAP150, TRAP150ΔN190 or mock. Cell lysates were subjected to immunoprecipitation with resins conjugated with anti-FLAG. (A) Co-precipitated proteins were detected by SDS-PAGE stained with SYPRO Ruby. FLAG-TRAP150-associated proteins were identified by mass spectrometry. (B) The immunoprecipitates of FLAG-TRAP150 was subjected to the immunoblotting with anti-CPSF73. (C) Anti-TRAP150 was used to perform the immunoblotting of FLAG-CPSF73 immunoprecipitates.

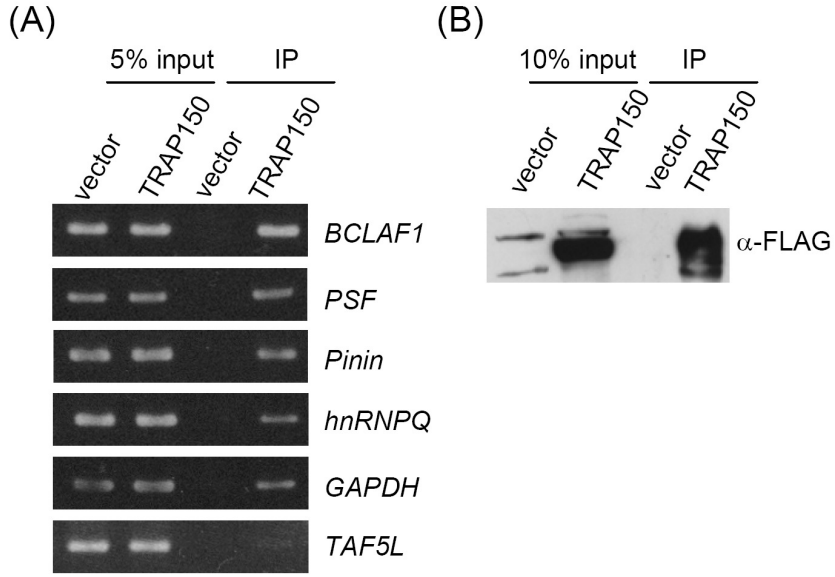


Figure 17. TRAP150 binds to the mRNAs that are subject to the expression regulation of TRAP150. HEK293 cells were transfected with vector alone or the vector encoding FLAG-TRAP150. (A) RNAs were extracted from the anti-FLAG immunoprecipitates and RT-PCR was performed to amplify the indicated mRNAs with specific primers. (B) The immunoblotting using anti-FLAG was performed to confirm the expression and immunoprecipitation efficiency.

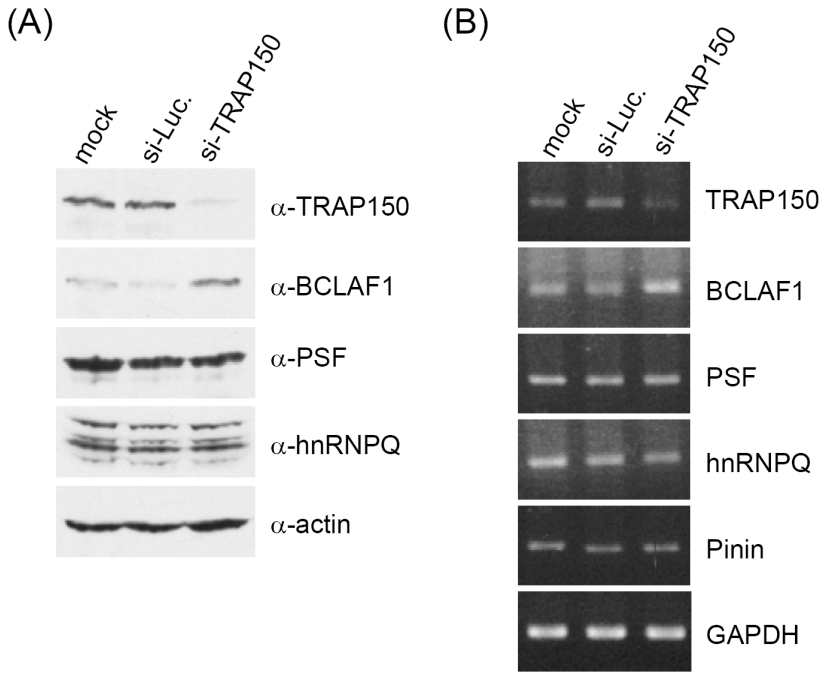


Figure 18. TRAP150 regulates both the mRNA and protein level of BCLAF1. HeLa cells were transfected with the indicated siRNAs for 48 hrs. (A) Cell lysates were subjected to immunoblotting using indicated antibodies. (B) RT-PCR analysis of RNA samples from siRNA-transfected cells.

Table 1. Protein composition of the TRAP150-associated proteins

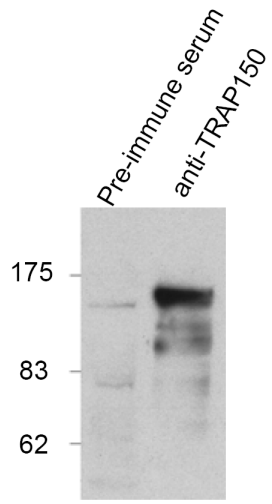
Protein Name	Protein accession#	Cal. Mass	# of peptides	Score
Transcription factors				
Interleukin enhancer-binding factor 2 (ILF2)	Q12905	43035	7	237
Transcriptional activator protein Pur-alpha (PURA)	Q00577	34889	3	110
Zinc finger protein 326	Q5BKZ1	65613	1	61
Components of snRNPs				
U4/U6 small nuclear ribonucleoprotein Prp31 (hPrp31)	Q8WWY3	55421	9	164
Splicing factor 3B subunit 1 (SF3b155, SAP155)	O75533	145723	7	119
Splicing factor, arginine/serine-rich 1 (ASF-1)	Q07955	27728	7	82
Component of gems 4 (Gemin-4)	P57678	119913	2	72
Splicing factor 3B subunit 4 (SF3b50, SAP49	Q15427	44386	1	67
Splicing factor 3A subunit 1 (SF3a120, SAP114)	Q15459	88831	1	54
Splicing factor 3B subunit 2 (SF3b145, SAP145)	Q13435	97596	1	49
U5 small nuclear ribonucleoprotein 200 kDa helicase (U5-200KD)	O75643	244353	1	47
116 kDa U5 small nuclear ribonucleoprotein component (U5-116 kDa)	Q15029	109366	1	46
Pre-mRNA 3' end processing factors				
Polyadenylate-binding protein 4 (PABP4, iPABP)	Q13310	70738	9	79
Polyadenylate-binding protein 2 (PABP-2)	Q86U42	32729	5	84
Cleavage and polyadenylation specificity factor subunit 1 (CPSF160)	Q10570	160782	4	82
Cleavage and polyadenylation specificity factor subunit 3 (CPSF73)	Q9UKF6	77436	2	72
Polyadenylate-binding protein 1 (PABP-1)	P11940	70626	1	68
WD repeat-containing protein 33 (WDR33)	Q9C0J8	145799	1	52
Reported components of EJC				
Eukaryotic initiation factor 4A-III (eIF4-AIII)	P38919	46841	13	249
Apoptotic chromatin condensation inducer in the nucleus (Acinus)	Q9UKV3	151797	7	185
Serine/arginine repetitive matrix protein 1 (SRm160)	Q8IYB3	102274	4	89
Polymerase delta-interacting protein 3 (S6K1 Aly/REF-like target, SKAR)	Q9BY77	46089	3	92
Pinin	Q9H307	81565	1	78
Partner of Y14 and mago (PYM)	Q9BRP8	22642	1	57
RNA-binding protein 8A (Y14)	Q9Y5S9	19877	1	51
Subunits of TREX				
Spliceosome RNA helicase BAT1 (UAP56)	Q13838	48960	3	78

THO complex subunit 2 (Tho 2)	Q8NI27	169475	3	56
THO complex subunit 4 (Aly)	Q86V81	26872	2	124
THO complex subunit 6 homolog (fSAP35)	Q86W42	37511	2	59
Members of SR protein family				
Bcl-2-associated transcription factor 1 (BCLAF1)	Q9NYF8	106059	14	121
Serine/arginine repetitive matrix protein 2 (SRm300)	Q9UQ35	299438	7	77
RNA-binding protein 39 (RBM39)	Q14498	59343	6	179
Putative RNA-binding protein Luc7-like 2 (LUC7L2)	Q9Y383	46486	4	130
Splicing factor, arginine/serine-rich 7 (9G8)	Q16629	27350	3	87
Splicing factor, arginine/serine-rich 6 (SRp55)	Q13247	39563	3	70
Luc7-like protein 3 (CREAP-1)	O95232	51435	2	74
Splicing factor, arginine/serine-rich 3 (SRp20)	P84103	19318	1	65
Transformer-2 protein homolog beta (hTRA2-beta)	P62995	33646	1	64
Splicing factor, arginine/serine-rich 4 (SRp75)	Q08170	56645	1	61
Splicing factor, arginine/serine-rich 13A (SRp38)	O75494	31282	1	55
Splicing factor, arginine/serine-rich 5 (SRp40)	Q13243	31245	1	52
Members of hnRNP family				
Heterogeneous nuclear ribonucleoprotein M (hnRNP M)	P52272	77464	21	401
Heterogeneous nuclear ribonucleoproteins C1/C2 (hnRNP C1/C2)	P07910	33650	16	239
Heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2/B1)	P22626	37407	6	132
Heterogeneous nuclear ribonucleoprotein G (RBMX)	P38159	42306	5	100
Putative heterogeneous nuclear ribonucleoprotein A1-like 3 (hnRNP A1-like 3)	P0C7M2	34202	5	89
Heterogeneous nuclear ribonucleoprotein F (hnRNP F)	P52597	45643	4	106
Heterogeneous nuclear ribonucleoprotein Q (hnRNP Q)	O60506	69560	4	87
Heterogeneous nuclear ribonucleoprotein U (hnRNP U)	Q00839	90457	3	135
Heterogeneous nuclear ribonucleoprotein D0 (hnRNP D0, AUF1)	Q14103	38410	2	72
Heterogeneous nuclear ribonucleoprotein R (hnRNP R)	O43390	70899	2	51
Heterogeneous nuclear ribonucleoprotein A0 (hnRNP A0)	Q13151	30822	1	71
Other RNA binding proteins				
Insulin-like growth factor 2 mRNA-binding protein 1 (IMP-1)	Q9NZI8	63417	11	218
Nuclease-sensitive element-binding protein 1 (YB-1, EFI-A)	P67809	35903	10	198
Fragile X mental retardation 1 protein (FMRP)	Q06787	71131	8	83
Insulin-like growth factor 2 mRNA-binding protein 3 (IMP-3)	O00425	63681	7	123
Nucleophosmin (B23, NO38)	P06748	32555	6	65
Ras GTPase-activating protein-binding protein 1 (G3BP-1)	Q13283	52132	5	189
ELAV-like protein 1 (HuR)	Q15717	36069	5	134

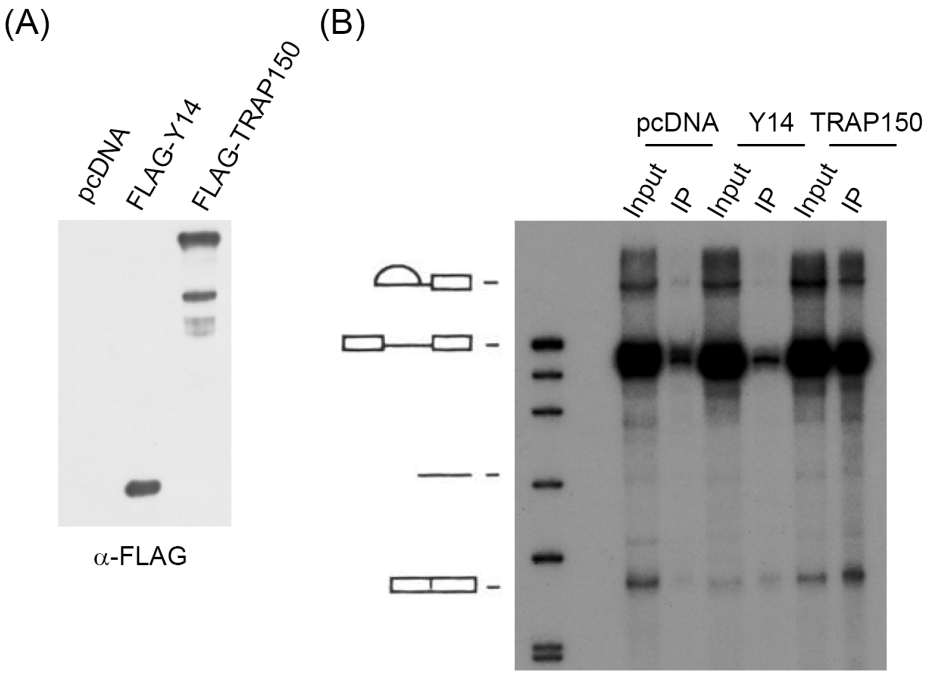
RNA-binding protein 10 (RBM10)	P98175	103396	5	88
52 kDa Ro protein (Ro, SS-A)	P19474	54135	5	45
La-related protein 1 (LARP1)	Q6PKG0	123434	4	64
Ras GTPase-activating protein-binding protein 2 (G3BP-2)	Q9UN86	54088	3	100
Lupus La protein (La, SS-B)	P05455	46808	3	64
Fragile X mental retardation syndrome-related protein 1 (hFXR1p)	P51114	69678	3	58
Guanine nucleotide-binding protein-like 3 (Nucleostemin)	Q9BVP2	61958	2	49
RNA-binding protein 14 (RBM14)	Q96PK6	69449	1	57
Serine/threonine-protein phosphatase 1 regulatory subunit 10 (PPP1R10)	Q96QC0	98996	1	46
Members of DEAD box helicase family				
ATP-dependent RNA helicase A	Q08211	140869	15	228
Probable ATP-dependent RNA helicase DDX5 (p68)	P17844	69105	6	127
Nucleolar RNA helicase 2 (DDX21, Gu)	Q9NR30	87344	6	63
Probable ATP-dependent RNA helicase DDX17	Q92841	80222	5	104
Factors with other enzymatic activities				
Protein phosphatase 1B (PP2C-beta)	O75688	52609	17	238
Protein arginine N-methyltransferase 1 (PRMT1)	Q99873	41489	15	160
Protein arginine N-methyltransferase 5 (PRMT5)	O14744	72638	14	204
Casein kinase II subunit alpha' (CK II alpha')	P19784	41187	8	192
Peroxiredoxin-1 (PRDX1)	Q06830	22096	6	88
Poly [ADP-ribose] polymerase 1 (PARP-1)	P09874	113012	3	68
Serine/threonine-protein phosphatase PGAM5, mitochondrial	Q96HS1	31985	3	58
Ribose-phosphate pyrophosphokinase 1 (PRS-I)	P60891	34812	2	61
Serine/threonine-protein kinase 38-like (STK38L)	Q9Y2H1	53968	1	63
Protein phosphatase 1A (PP2C-alpha)	P35813	42421	1	51
Glycerophosphodiester phosphodiesterase domain-containing protein 3 (GDPD3)	Q7L5L3	29605	1	46
Translation factors				
Elongation factor Tu, mitochondrial (EF-Tu)	P49411	49510	2	95
Eukaryotic translation initiation factor 4B (eIF4B)	P23588	69183	1	69
Eukaryotic translation initiation factor 4 gamma 1 (eIF4G1)	Q04637	175426	1	52
Ribosomal proteins				
40S ribosomal protein S9 (RPS9)	P46781	22578	13	97
40S ribosomal protein S3 (RPS3)	P23396	26671	11	150
60S ribosomal protein L6 (RPL6)	Q02878	32708	11	130
40S ribosomal protein S4, X isoform (RPS4X)	P62701	29579	10	131

60S ribosomal protein L3 (RPL3)	P39023	46080	9	72
28S ribosomal protein S27, mitochondrial (MRP-S27)	MRPS27	47581	8	192
60S ribosomal protein L4 (RPL4)	P36578	47667	8	151
60S ribosomal protein L7a (RPL7a)	P62424	29977	8	147
60S ribosomal protein L7 (RPL7)	P18124	29207	8	97
60S ribosomal protein L18 (RPL18)	Q07020	21621	6	142
40S ribosomal protein S3a (RPS3A)	P61247	29926	6	121
60S ribosomal protein L17 (RPL17)	P18621	21383	6	82
60S acidic ribosomal protein P0 (RPLP0)	P05388	34252	5	145
28S ribosomal protein S22, mitochondrial (MRP-S22)	P82650	41254	5	129
40S ribosomal protein S6 (RPS6)	P62753	28663	5	70
60S ribosomal protein L11 (RPL11)	P62913	20240	5	69
40S ribosomal protein S7 (RPS7)	P62081	22113	5	58
40S ribosomal protein S2 (RPS2)	P15880	31305	4	103
60S ribosomal protein L24 (RPL24)	P83731	17768	3	85
60S ribosomal protein L8 (RPL8)	P62917	28007	3	66
60S ribosomal protein L10 (RPL10)	P27635	24588	3	62
28S ribosomal protein S2, mitochondrial (MRP-S2)	Q9Y399	33228	2	61
60S ribosomal protein L5 (RPL5)	P46777	34341	2	60
28S ribosomal protein S31, mitochondrial (MRP-S31)	Q92665	45290	2	66
60S ribosomal protein L15 (RPL15)	P61313	24131	2	46
Ribosomal L1 domain-containing protein 1 (RSL1D1, PBK1)	O76021	54939	1	70
60S ribosomal protein L29 (RPL29)	P47914	17741	1	61
28S ribosomal protein S9, mitochondrial (MRP-S9)	P82933	45794	1	53
40S ribosomal protein S5 (RPS5)	P46782	22862	1	51
Stress responsive proteins				
Heat shock 70 kDa protein 1A/1B (HSPA1A)	P08107	70009	48	619
Heat shock 70 kDa protein 9 (HSPA9)	P38646	73635	8	219
Heat shock 70 kDa protein 4 (HSPA4)	P34932	94271	5	133
Heat shock cognate 71 kDa protein (HSPA8)	P11142	70854	4	74
Heat shock protein 105 kDa (HSPH1)	Q92598	96804	1	72
Heat shock 70 kDa protein 1-like (HSP70-Hom)	P34931	70331	3	66
Heat shock 70 kDa protein 5 (HSPA5, BiP)	P11021	72288	2	50
STIP1 homology and U box-containing protein 1 (CHIP)	Q9UNE7	34834	3	46
Miscellaneous				
Complement component 1 Q subcomponent-binding protein, mitochondrial (SF2p32)	Q07021	31343	6	104

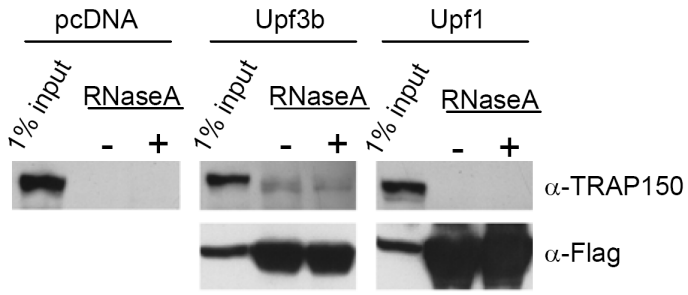
Calmodulin-regulated spectrin-associated protein 3 (CAMSAP3)	Q9P1Y5	134666	4	90
Pentatricopeptide repeat-containing protein 3 (TRG-15)	Q96EY7	78500	3	123
ADP/ATP translocase 2 (SLC25A5)	P05141	32874	3	108
Phosphoribosyl pyrophosphate synthase-associated protein 2 (PRPSAP2)	O60256	40899	3	55
Methylosome subunit pICln (pICln)	P54105	26199	2	86
Methylosome protein 50 (WDR77)	Q9BQA1	36701	1	83
Zinc finger CCHC domain-containing protein 3	Q9NUD5	43591	1	69
Probable rRNA-processing protein EBP2 (EBNA1BP2)	Q99848	34798	1	68
Serrate RNA effector molecule homolog (ARS2)	Q9BXP5	100604	1	66
Cell growth-regulating nucleolar protein (LYAR)	Q9NX58	43588	1	56
T-complex protein 1 subunit epsilon (CCT-epsilon)	P48643	59633	1	52
T-complex protein 1 subunit alpha (CCT-alpha)	P17987	60306	1	49
Ribosome-binding protein 1 (RRp)	Q9P2E9	152381	1	47



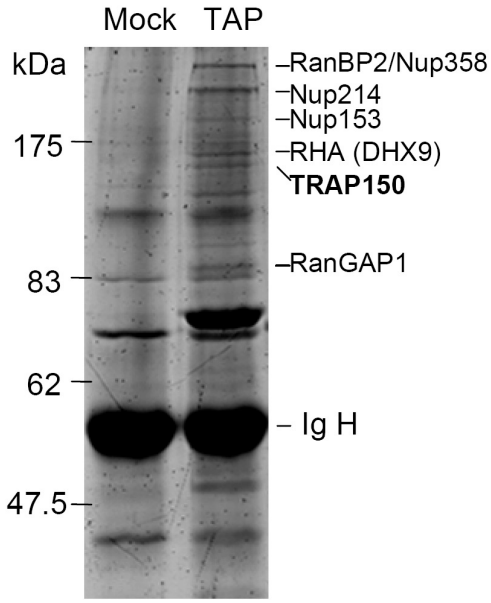
Supplemental figure 1. The specificity of anti-TRAP150 antibody. Total cell lysates prepeared from HeLa cells were subjected to the immunobolttting either with the pre-immune serum from the immunized rabbit or with purified anti-TRAP150 antibody.



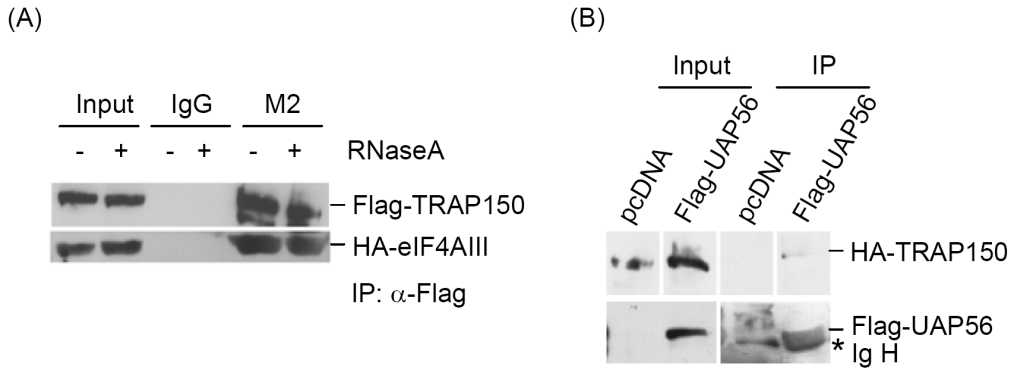
Supplemental figure 2. Overexpressed TRAP150 has no effect on the splicing of AdML pre-mRNA . α-^{32}P-labeled AdML pre-mRNAs were subjected to *in vitro* splicing reaction using mock nuclear extracts prepared form HEK293 cells or extracts containing FLAG-Y14 or TRAP150. (A) Immunoblotting using anti-FLAG was performed to detect the expression of FALG-Y14 and TRAP150. (B) Standard *in vitro* splicing using α-^{32}P-labeled AdML pre-mRNAs was performed. The subsequent immunoprecipitation was performed using anti-FLAG.



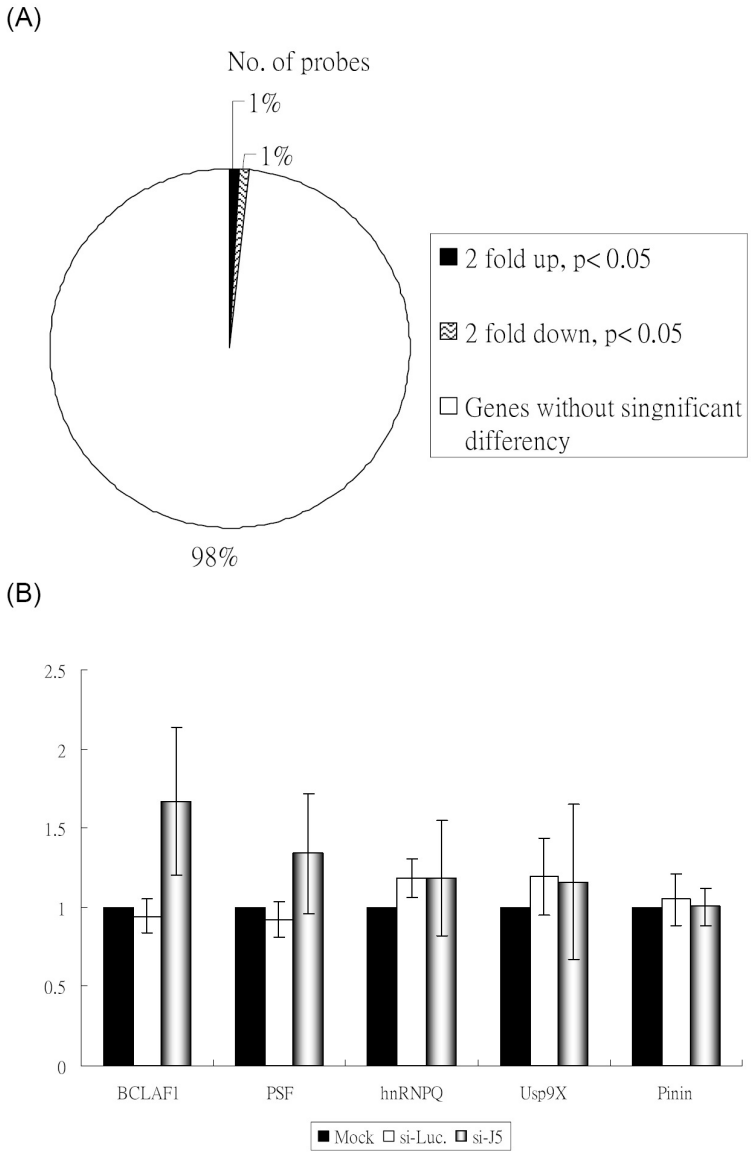
Supplemental figure 3. Upf3b but not Upf1 interacts with TRAP150. HEK293 cells were transfected separately with the vector alone or expressing vectors encoding FLAG-Upf3b or FLAG-Upf1. Total cell lysates were subjected to the immunoprecipitation with anti-FLAG-conjugated resins and the subsequent immunoblotting with anti-TRAP150 and anti-FLAG, respectively.



Appendix 1: TRAP150 associates with TAP. Co-precipitated proteins were detected by SDS-PAGE using SYPRO Ruby. FLAG-TAP-associated proteins were identified by mass spectrometry. This work was done by Dr. Ming-Chih Lai in our lab.



Appendix 2: TRAP150 interacts with eIF4AIII and UAP56. (A) HEK293 cells were co-transfected with expression vectors encoding FLAG-TRAP150 and HA-eIF4AIII. Total cell lysates were subjected to immunoprecipitation by anti-FLAG beads or protein A-Sepharose conjugated with the control IgG antibody. To detect the HA-eIIF4AIII in the immunoprecipitates, the immunoblotting with anti-HA was performed. (B) The expression vector encoding HA-TRAP150 was co-transfected with either pcDNA vector alone or the expression vector encoding FLAG-UAP56 into HEK293 cells. The co-precipitated HA-TRAP150 by anti-FLAG-conjugated beads was verified by immunoblotting with anti-HA. (A) and (B) were performed by Dr. Kang-Mai Wu and Ia-Wen Hsu in our lab, respectively.



Appendix 3: Microarray analysis of genes responding to the expression of TRAP150. (A) The diagram showed the ratio of genes either up- or down-regulated by knockdown of TRAP150. (B) HEK293 cells were transfected with indicated siRNAs, and total RNA was subjected to the real-time RT-PCR analysis using specific primers recognizing the mRNA of each candidate gene. Bars showed the mRNA level of each candidate gene under differential expressions of TRAP150 by siRNA-mediated knockdown. These works were performed by Dr. Kang-Mai Wu in our lab.

Appendix Table. Genes up-regulated in TRAP150-knockdown cells

		log2 (Ratio)	P-value			Annotation
	ID	T/C	T/C	Gene symbol	Accession	Function
	PH_hs_0010283	1.841894628	1.75398E-11	BHLHB2	NM_003670.2	Transcription
Transcription	PH_hs_0032031	1.461041475	0.000246117	GABPB2	NM_005254.5	Transcription
	PH_hs_0028423	1.419571372	0.000276214	HMGB2	NM_002129.3	Transcription
	PH_hs_0033036	1.406132555	5.59307E-05	ZNF45	NM_003425	Transcription, zinc ion binding
	PH_hs_0028851	1.403327755	0.000181995	ZEB2	NM_014795	Transcription, zinc ion binding
	PH_hs_0025208	1.308470078	0.001363363	CHD1L	NM_004284.3	Chromatin remodeling, DNA repair, helicase
	PH_hs_0022608	1.307924581	0.01712925	KLF3	NM_016531.5	Transcription, zinc ion binding
	PH_hs_0032324	1.260437184	2.9219E-08	POLR3C	NM_006468.6	Transcription, Innate immunity
	PH_hs_0014800	1.211722965	0.044003658	MED21	NM_004264.3	Transcription (mediator complex)
	PH_hs_0030215	1.182961838	1.49605E-05	ZNF254	NM_203282.2	Transcription, zinc ion binding
	PH_hs_0025396	1.175223211	0.011446822	ACTL6A	NM_178042.2	Transcription, neurogenesis, chromatin remodeling
	PH_hs_0033388	1.149693383	0.000187778	ASCC2	NM_032204.3	Transcription
	PH_hs_0026937	1.134484231	0.008294386	ZNF569	NM_152484.2	Transcription, zinc ion binding
	PH_hs_0035417	1.124649453	0.004277576	HOXA5	NM_019102.2	Transcription
	PH_hs_0035603	1.112869006	0.043862063	ZNF226	NM_001032372.1	Transcription, zinc ion binding
	PH_hs_0025115	1.105567375	0.009857676	ATXN3, SCA3	NM_001127697	Transcription, hydrolase, thiol protease
	PH_hs_0025404	1.105108008	0.033046186	CCNC	NM_001013399.1	Transcription
	PH_hs_0030249	1.096494608	1.21456E-05	SMARCE1	NM_003079.4	Neurogenesis, transcrpition (chromatin remodeling)
	PH_hs_0035848	1.073580194	0.000166935	ZNF561	NM_152289.2	Transcription, zinc ion binding

	PH_hs_0035640	1.068056226	0.002854495	PWP1	NM_007062.1	Transcription
	PH_hs_0032384	1.034445525	0.025551867	XRN2	NM_012255.3	Transcription, mRNA processing
	PH_hs_0022880	1.03307713	0.014687463	BCOR	NM_001123385.1	Transcription
	PH_hs_0023538	1.028711379	0.00013121	ASF1A	NM_014034.2	Transcription, chromatin remodeling
	PH_hs_0032212	1.028244984	1.97047E-05	CSDA	NM_003651	Transcription
	PH_hs_0024053	1.025456913	0.001981101	ZNF586	NM_017652.2	Transcrpition, metal ion binding
	PH_hs_0008731	1.022930304	0.001295172	ZFP64	NM_199426.1	Transcription, metal ion binding
	PH_hs_0006563	1.022721016	5.19151E-05	FUBP1	NM_003902.3	Transcription, RNA binding
	PH_hs_0008788	1.022439652	0.010416944	PPRC1	NM_015062.3	Transcription, RNA binding
	PH_hs_0004386	1.01932478	0.020721776	PNRC1	NM_006813.2	Transcription
	PH_hs_0024669	1.01201691	0.009450059	EYA3	NM_001990.2	Transcription, metal ion binding , DNA repair
	PH_hs_0005744	1.010622873	0.012303807	L3MBTL3	NM_001007102.1	Transcription
mRNA	PH_hs_0027942	1.627775292	5.94351E-05	SFPQ	NM_005066.2	Transcription, mRNA processing, mRNA splicing
processing	PH_hs_0024815	1.580176716	0.011753556	PNN	NM_002687.3	Transcription, mRNA processing, mRNA splicing
	PH_hs_0039330	1.557325957	0.00140946	SYNCRIP	NM_006372.4	mRNA processing, mRNA splicing
	PH_hs_0004625	1.484918182	0.00207321	BCLAF1	NM_001077441.1	Transcription, mRNA splicing, cell death
	PH_hs_0000418	1.361445703	0.0279702	FUSIP1	NM_054016.1	mRNA processing, mRNA splicing
	PH_hs_0016616	1.318631557	0.00838808	PRPF38B	NM_018061.2	mRNA processing, mRNA splicing
	PH_hs_0016478	1.307713709	0.000884373	FIP1L1	NM_001134938.1	mRNA processing
	PH_hs_0031073	1.302932743	0.01291979	BCAS2	NM_005872.2	mRNA processing, mRNA splicing
	PH_hs_0029403	1.300882529	0.000168487	TARDBP	NM_007375.3	Transcription, mRNA processing, mRNA splicing
	PH_hs_0022174	1.210348235	0.00013308	SFRS1	NM_001078166.1	mRNA processing, mRNA splicing
	PH_hs_0030938	1.206149099	0.000524474	SFRS6	NM_006275.5	mRNA processing, mRNA splicing

	PH_hs_0023651	1.198950922	0.024285106	PRPF18	NM_003675.3	mRNA processing, mRNA splicing
	PH_hs_0029849	1.150597957	3.67559E-05	RNPS1	NM_006711.2	mRNA processing, mRNA splicing, NMD
	PH_hs_0041191	1.149598442	0.000526546	HNRNPR	NM_001102399.1	mRNA processing, mRNA splicing
	PH_hs_0037359	1.017033201	0.001446205	HNRPA3	NM_194247.2	mRNA processing, mRNA splicing
Metabolism	PH_hs_0033324	1.463100008	8.00384E-06	PDK1	NM_002610.3	Carbohydrate metabolism, signal transduction (small GTPase mediated signal transduction)
	PH_hs_0026700	1.455400785	0.004889565	GBE1	NM_000158.3	Glycogen biosynthesis
	PH_hs_0040251	1.387406089	0.015398955	CEL	NM_001807.3	Lipid degradation, lipid catabolic process
	PH_hs_0028376	1.251023757	2.50888E-05	INSIG1	NM_198337	Metabolic process (cholesterol, lipid, steroid)
	PH_hs_0018454	1.248955225	0.017775776	ACOX1	NM_007292.4	Fatty acid, lipid metabolism, oxidation reduction
	PH_hs_0010761	1.221397061	0.00979848	SUCLA2	NM_003850.2	Metabolic process, tricarboxylic acid cycle
	PH_hs_0023444	1.184190045	0.003458727	RPE	NM_199229.1	Carbohydrate metabolism, isomerase activity
	PH_hs_0024663	1.175451211	0.005309748	MDH2	NM_005918.2	Tricarboxylic acid cycle, carbohydrate metabolism
	PH_hs_0013723	1.160882002	0.013371692	ACSL3	NM_203372.1	Metabolic process (fatty acid, lipid), ATP binding
	PH_hs_0001366	1.113310354	0.00973367	LYCAT	NM_182551.3	Phospholipid biosynthesis, acyltransferase activity
	PH_hs_0029286	1.056198956	0.001187629	MOCS2	NM_176806.2	Molybdenum cofactor biosynthesis, Transferase activity, nucleotide binding
	PH_hs_0023784	1.052645406	0.022875106	ACO1	NM_002197.2	Tricarboxylic acid cycle, RNA binding, metal ion binding
	PH_hs_0022201	1.032477482	0.021169387	TYMS	NM_001071.2	Nucleotide biosynthesis, DNA repair
	PH_hs_0025237	1.002604373	0.010965591	EHHADH	NM_001966	Metabolic process (fatty acid, lipid), oxidation reduction
Transoport	PH_hs_0031188	1.737227026	3.42704E-08	KIAA1012	NM_014939.3	Transport (ER-Golgi transport)
(protein)	PH_hs_0035446	1.196182454	0.010482423	SCYL2	NM_017988.4	Transport activity
	PH_hs_0033467	1.195192244	0.006063382	IPO11	NM_001134779.1	Transport, protein transport

	PH_hs_0017180	1.166293901	0.010158982	RAB39	NM_017516.1	Transport, protein transport, signal transduction (small GTPase mediated signal transduction)
	PH_hs_0025110	1.152682327	0.001958144	ATG16L1	NM_030803.6	Autophagy, protein transport, transport
	PH_hs_0001635	1.103223522	0.000237658	GOLPH3	NM_022130.3	Trans-Golgi network, signal transduction (mTOR signaling)
	PH_hs_0003684	1.442127799	0.037744019	SLC11A2	NM_000617.2	Transporter, Iron transport
Transport (ion)	PH_hs_0014719	1.270439274	0.000775921	SLC39A8	NM_022154.5	Transporter, Iron transport (zinc ion)
	PH_hs_0005938	1.187126519	4.04341E-06	ARMC1	NM_018120.3	Transport, metal ion binding
	PH_hs_0001375	1.152201083	0.007778574	SCO1	NM_004589.2	Cellular copper ion homeostasis , copper ion transport
	PH_hs_0024991	1.089385632	0.017087126	SLC44A1	NM_080546.3	Transport, choline transport
	PH_hs_0033622	1.083076882	0.001053318	TIMM8A	NM_004085	Transport, Transmembrane transport, metal ion binding
Transport (others)	PH_hs_0032536	1.193123812	3.77907E-07	SLC35B4	NM_032826.4	Transport, sugar transport
	PH_hs_0025694	1.069806171	0.002922092	ABCF2	NM_007189.1	Transport, ATP binding, ATPase activity
Signal transduction	PH_hs_0014696	2.099984942	3.23486E-06	OR51B4	NM_033179.2	Signal transduction (G-protein coupled receptor)
	PH_hs_0025281	1.575353379	0.000187477	KIFAP3	NM_014970.2	Signal transduction
	PH_hs_0022601	1.196525593	6.61356E-05	MKLN1	NM_013255	Signal transduction
	PH_hs_0027331	1.195713584	4.96531E-06	PLCG1	NM_002660.2	Signal transduction (phosphoinositide phospholipase C , receptor signaling protein activity)
	PH_hs_0026463	1.189259005	4.31346E-05	RSU1	NM_012425.3	Signal transduction
	PH_hs_0022676	1.104648147	0.037178271	DPYSL3	NM_001387.2	Dihydropyrimidinase activity, signal transduction, nervous system development
	PH_hs_0001219	1.09945217	0.00015115	MARK1	NM_018650.3	Signal transduction
	PH_hs_0004462	1.081006965	0.004250628	EGFR	NM_005228.3	Signal transduction
	PH_hs_0033345	1.058564687	6.13393E-05	PRKD3	NM_005813.3	Signal transduction (G-protein coupled receptor)

	PH_hs_0004215	1.026339111	0.001928454	GNAI3	NM_006496.2	Signal transduction (G-protein coupled receptor), GTP binding
	PH_hs_0001785	1.015346714	0.003698353	BCKDK	NM_001122957.1	Signal transduction, kinase activity
	PH_hs_0009752	1.001764646	0.008973693	LGR4	NM_018490.2	Signal transduction (G-protein coupled receptor)
Cell cycle	PH_hs_0004570	1.495762078	5.53873E-05	CCNG1	NM_199246.1	Cell cycle
	PH_hs_0031197	1.421041834	0.003605463	SGOL2	NM_152524	Cell cycle, cell division
	PH_hs_0028219	1.261219196	2.33631E-06	septin-10	NM_144710.2	
	PH_hs_0030635	1.240847724	0.000233756	SASS6	NM_194292.1	Cell cycle, centriole replication
	PH_hs_0029480	1.235746688	7.17571E-08	CEP110	NM_007018.4	Cell cycle
	PH_hs_0023490	1.184571199	0.001003913	BUB1	NM_004336.3	Apoptosis, cell cycle, mitotic cell cycle spindle assembly checkpoint
	PH_hs_0033108	1.147079553	1.1782E-05	SEPT7 (CDC10)	NM_001788.4	Cell cycle, mitosis, GTP binding
	PH_hs_0018127	1.145753632	5.40604E-08	CD2AP	NM_012120.2	Cell cycle, mitosis
	PH_hs_0040791	1.023076133	0.008762505	CEP76	NM_024899.2	Regulation of centriole replication
Ribosome biogenesis	PH_hs_0005095	1.276514379	0.000383546	EXOSC9	NM_001034194.1	Exonuclease, rRNA processing
	PH_hs_0032189	1.27118693	2.41417E-05	UTP6	NM_018428.2	rRNA processing
	PH_hs_0010481	1.241292138	0.000114074	WDR36	NM_139281.2	Sensory transduction, rRNA processing
	PH_hs_0023336	1.192335565	0.000467438	SDAD1	NM_018115.2	Ribosome biogenesis,transport, protein transport
	PH_hs_0035418	1.074679503	0.000637869	BXDC2	NM_018321.3	Ribosome biogenesis
	PH_hs_0034552	1.032955558	0.005075261	BMS1	NM_014753	Ribosome biogenesis, nucleotide binding
	PH_hs_0011834	1.012836934	7.65955E-05	MPHOSPH10	NM_005791.1	Ribosome biogenesis, rRNA processing
	PH_hs_0022478	1.00444114	3.08005E-05	PES1	NM_014303.2	Ribosome biogenesis, rRNA processing
Translation	PH_hs_0010132	1.188691035	5.05901E-05	PABPC4	NM_001135654.1	RNA processing, translation
	PH_hs_0024635	1.168850606	1.48392E-05	DHX29	NM_019030.2	Protein biosynthesis, Translation

	PH_hs_0030187	1.095459352	0.000516558	EIF5	NM_183004.3	Protein biosynthesis, Translation, GTP binding
	PH_hs_0035608	1.042437692	0.011068552	MTIF2	NM_001005369.1	Protein biosynthesis (GTP binding)
	PH_hs_0025958	1.040583003	0.000125115	EARS2	NM_001083614.1	Protein biosynthesis, RNA binding, ATP binding
	PH_hs_0009628	1.0197588	6.60575E-06	EIF5B	NM_015904.3	Protein biosynthesis, GTPase activity
Ubl conjugation pathway	PH_hs_0035285	2.072107401	0.001237875	USP9X	NM_001039590.2	Ubl conjugation pathway, cell cycle
	PH_hs_0002857	1.152481258	6.93033E-05	USP1	NM_001017416.1	Ubl conjugation pathway, DNA repair
	PH_hs_0031911	1.13463382	1.33543E-05	RAD23B	NM_002874.3	DNA repair, Ubl conjugation pathway
	PH_hs_0028596	1.101580201	0.000360835	SAE2	NM_005499.2	Ubl conjugation pathway, ligase, enzyme activator activity
	PH_hs_0035579	1.039933952	4.74425E-05	TTC3	NM_001001894.1	Ubl conjugation pathway, ubiquitin-protein ligase activity, zinc ion binding
	PH_hs_0022110	1.038937802	0.022976071	ASB3	NM_016115.3	Ubl conjugation pathway, intracellular signaling cascade
Enzymatic factors (Miscellaneous)	PH_hs_0024037	1.325172663	0.002446505	DDX18	NM_006773.3	Helicase, RNA binding
	PH_hs_0016824	1.195617454	0.013184924	NT5DC1	NM_152729.2	Hydrolase activity, metal ion binding
	PH_hs_0001621	1.192186171	3.61215E-05	WDYHV1, C8orf32	NM_018024.1	Protein modification process, glutamine amidohydrolase activity
	PH_hs_0025236	1.149509242	0.008253057	TPP2	NM_003291.2	Proteolysis, serine protease
	PH_hs_0005671	1.146838388	8.97043E-06	LOX	NM_002317.4	Oxidation reduction, copper ion binding
	PH_hs_0024267	1.126890642	1.04771E-07	MFN2	NM_001127660.1	GTPase activity, mitochondrial membrane organization
	PH_hs_0002325	1.103243716	0.013567554	GSTZ1	NM_145871.2	Transferase activity, phenylalanine catabolism, tyrosine catabolism
	PH_hs_0010293	1.07892012	0.009896506	HSDL1	NM_031463	Oxidation reduction
	PH_hs_0010383	1.05185211	0.000185021	B3GNT2	NM_006577.5	Glycosyltransferase, transferase activity, integral to membrane
	PH_hs_0004428	1.047884834	0.03043671	P4HA1	NM_000917.3	Oxidation reduction, metal ion binding

	PH_hs_0027050	1.010587531	0.016941089	PAPSS1	NM_005443.4	Nucleotidyltransferase activity, nucleotide binding
	PH_hs_0027643	1.008518999	0.000400402	TNNI3K	NM_001112808.1	Serine/threonine-protein kinase, ATP binding, metal ion binding
	PH_hs_0028077	1.003159175	0.000222788	GALNT1	NM_020474.3	Transferase, Glycosyltransferase
	PH_hs_0006772	1.002747133	0.001003217	FER	NM_005246.2	Kinase, Transferase
Cell death	PH_hs_0040978	1.267478446	0.000194703	SPG20	NM_015087.4	Cell death
	PH_hs_0035836	1.203262127	0.031404682	FEM1B	NM_015322.3	Apoptosis, Ubl conjugation pathway
	PH_hs_0005882	1.202683663	1.74693E-06	SLK	NM_014720.2	Apoptosis, nucleotide-excision repair
	PH_hs_0000560	1.063194773	0.004192624	TAX1BP1	NM_006024.5	Cell death (anti-apoptosis), zinc ion binding
	PH_hs_0024017	1.062303862	0.005549652	BIRC6	NM_016252.3	Cell death (anti-apoptosis), Ubl conjugation pathway
Stress response	PH_hs_0040139	1.415243634	3.73958E-07	HSPA8	NM_006597.3	Stress response, protein folding
	PH_hs_0031157	1.227797785	0.000647174	HSPH1	NM_006644.2	Stress response, protein folding
	PH_hs_0040944	1.158260845	0.000210827	EIF2AK1	NM_001134335.1	Stress response, translation, cell proliferation
	PH_hs_0015536	1.108161106	2.34503E-05	RBM3	NM_006743.3	Stress response, translation, RNA processing, RNA binding
Structural molecule activity	PH_hs_0016786	1.210425653	0.016814798	SYNC1	NM_030786.2	Structural molecule activity, intermediate filament
	PH_hs_0026396	1.151502531	1.10137E-05	NEFM	NM_005382.2	Structural molecule activity, intermediate filament
	PH_hs_0040765	1.118028056	2.45052E-05	MATR3	NM_199189.1	Structural molecule activity, RNA binding, metal ion binding (Zinc ion)
	PH_hs_0040083	1.008217641	0.009843552	LMNB1	NM_005573.2	Structural molecule activity, lamin filament
tRNA processing	PH_hs_0014740	1.064988674	1.04048E-05	DUS3L	NM_020175	tRNA processing, oxidation reduction, metal ion binding
	PH_hs_0005321	1.045225564	1.73631E-05	DUS4L	NM_181581.1	tRNA processing, oxidation reduction
	PH_hs_0029718	1.013466583	0.028210308	SSB	NM_003142.3	mRNA binding, Histone mRNA metabolic process, tRNA modification
Differentiation	PH_hs_0005231	1.426221577	0.000146963	TSNAX	NM_005999.2	Differentiation, spermatogenesis
	PH_hs_0003429	1.368598638	0.002547506	GLMN	NM_053274.2	Muscle cell differentiation, positive regulation of cytokine secretion

	PH_hs_0023417	1.155417829	0.025545215	KIAA1279, KBP	NM_015634	Differentiation, neurogenesis, mitochondrial transport
Cell adhsion	PH_hs_0003188	1.363477407	0.008953042	CD164	NM_001142404.1	Cell adhesion, immune response
	PH_hs_0024578	1.105379666	0.007457215	LMBR1	NM_022458.3	Receptor activity, integral to membrane,
	PH_hs_0004058	1.049840602	0.004336224	VAMP3	NM_004781.3	Cell junction, Exocytosis
DNA replication	PH_hs_0025726	1.043678572	0.001798291	ORC4L	NM_002552.2	DNA replication
	PH_hs_0006329	1.00689756	0.009482305	RFC3	NM_002915.3	DNA replication, ATP binding
Chromosomal protein	PH_hs_0009050	1.169444909	7.62656E-05	HIST1H1A	NM_005325.3	Chromosomal protein, spermatogenesis
	PH_hs_0028200	1.137141125	6.43232E-05	CENPI	NM_006733.2	Chromosomal protein
Proteolysis	PH_hs_0026454	1.624487759	0.000225819	MSTP9	NR_002729.2	Proteolysis
Cell growth	PH_hs_0025848	1.175109362	0.003398449	NOL8	NM_017948.5	RNA binding, cell growth
Motor protein	PH_hs_0021943	1.40010675	0.016224431	DYNLT3	NM_006520.2	Motor protein, motor activity
Muscle contaction	PH_hs_0029336	1.224864875	0.001170403	TPM3	NM_001043353.1	Muscle contraction, actin binding
Unknown	PH_hs_0004660	2.971595223	0.005844159	IGSF3	NM_001007237.1	Unknown, integral to membrane
	PH_hs_0001897	1.941844233	3.96574E-11	SEZ6	NM_001098635.1	Unknown, integral to membrane
	PH_hs_0026583	1.718987049	0.010011232	ANKRD37	NM_181726.2	Unknown
	PH_hs_0018842	1.433928025	0.013332545	SDCCAG1	NM_004713.3	Unknown
	PH_hs_0016522	1.420619397	2.62899E-05	C9orf30	NM_080655.1	Unknown
	PH_hs_0007317	1.30897845	0.034668684	GTPBP8	NM_014170.2	GTP binding, nucleotide binding
	PH_hs_0025205	1.281082097	1.14337E-07	BTF3L4	NM_001136497.1	Unknown
	PH_hs_0031534	1.209050627	0.047664478	KBTBD6	NM_152903.4	Unknown
	PH_hs_0003189	1.206872203	0.005594049	C13orf24	NM_006346.2	Unknown

PH_hs_0013656	1.188898639	0.011847478	C9orf40	NM_017998.2	Unknown
PH_hs_0025216	1.18646529	0.003399445	HSPA14	NM_016299.2	Unknown, ATP binding
PH_hs_0019273	1.184023227	1.44599E-05	MAPK1IP1L	NM_144578.3	Unknown
PH_hs_0022096	1.181216348	0.000101295	LTV1	NM_032860.3	Unknown
PH_hs_0017851	1.171957759	0.000587906	C9orf57	NM_001128618.1	Unknown, integral to membrane
PH_hs_0032836	1.155292296	0.000158433	SPTY2D1	NM_194285.2	Unknown
PH_hs_0003610	1.143776381	0.002022816	GPATCH1	NM_018025.2	Unknown, nucleic acid binding
PH_hs_0025719	1.135211195	0.00047741	C13orf7	NM_024546.3	Metal ion binding (Zinc ion)
PH_hs_0005694	1.092575845	0.001662991	ARMC8	NM_014154.2	Unknown
PH_hs_0003551	1.082767105	0.001234646	GPATCH2	NM_018040	Unknown, nucleic acid binding
PH_hs_0024479	1.054833331	0.020717621	ZCCHC7	NM_032226.2	Metal ion binding, nuclear acid binding
PH_hs_0031513	1.036286651	0.002682272	C7orf20	NM_015949.2	Unknown
PH_hs_0023337	1.035404054	0.001401895	C17orf45	NM_152350	Unknown
PH_hs_0021519	1.021421524	0.013782745	RSBN1L	NM_198467.2	Unknown
PH_hs_0026246	1.018210718	1.26634E-05	BRD9	NM_001009877	Unknown
PH_hs_0007959	1.017558501	0.001753333	C2orf34	NM_024766.3	Unknown
PH_hs_0023519	1.015343944	0.006970776	ANKRD16	NM_001009943.2	Unknown