The 6th International Conference on Biochemistry and Molecular Biology

Investigation of mTOR complex 2 and tau interaction by affinity purification mass spectrometry

Narawit Pacharakullanon[1], Piriya Wongkongkathep[2], Wannapan Poolex[2], Naphat Chantaravisoot[1,2,*]

[1]Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand
[2]Center of Excellence in Systems Biology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand
*E-mail: cnaphat@gmail.com

Abstract

Tau is a group of microtubule-associated proteins consisting of six isoforms produced by alternative splicing. Tau proteins can be phosphorylated by many kinases leading to neurofibrillary tangle formation in Alzheimer's disease (AD). mTOR is a serine/threonine protein kinase present in two distinct multiprotein complexes. RAPTOR and RICTOR are specific scaffold proteins of mTOR complex 1 and 2, respectively. The mTORC1 can regulate many biological processes, such as protein synthesis and cell proliferation while mTORC2 is implicated in cytoskeleton reorganization. Previous studies found that mTORC1 can promote tau aggregation in AD brain through phosphorylation of specific epitopes. However, the relationship between mTORC2 and tau phosphorylation has not been reported. In this study, tau and RICTOR were pulled down by immunoprecipitation (IP). Then, the samples were separated on SDS-PAGE following by in-gel digestion. LC-MS/MS was performed to analyze the peptides. Fifty candidate proteins were identified from all interacting proteins intersecting between tau and RICTOR IP. These proteins are associated with ribosomes, spliceosomes, mRNA surveillance pathway, RNA transport, RNA degradation, and endocytosis. The results from this study suggested that mTORC2 might play an important role in tau formation, especially the alternative splicing of mRNA resulting in different tau isoforms. Therefore, this study is a new evidence to explore proteins that may lead to AD.

Introduction

Microtubule-associated protein tau (MAPT) or tau, a phosphoprotein, which has been discovered for 30 years by Weingarten *et al.* [1]. Six major isoforms of tau are generated by alternative mRNA splicing. The longest isoform has 441 amino acids containing 80 serine or threonine residuals and 5 tyrosine residuals. Many residuals of tau can promote microtubule assembly or disassembly depending on the phosphorylation states of specific epitopes. The hyperphosphorylated tau proteins, so called paired helical filaments (PHFs), can accumulate in the dendritic compartment of neuronal cells, and form into neurofibrillary tangles (NFTs) leading to Alzheimer's disease [2].

The mechanistic target of rapamycin (mTOR) is a member in phosphatidylinositol 3-kinase-related kinases (PI3K or PIKKs) family. It is a center of two complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2). These complexes also share mammalian lethal with Sec13 protein 8 (mLST8) and DEP domain containing mTOR-interacted protein (DEPTOR). mTORC1 is defined by two components including regulatory protein associated with mTOR (RAPTOR) and proline-rich AKT substrate 40 kDa (PRAS40). Its functions are related to many biological processes, such as protein and lipid synthesis, energy metabolism and

autophagy. Moreover, mTORC1 activities are completely inhibited by rapamycin, a known immunosuppressant agent. In contrast, mTORC2 is defined by two components, rapamycin-insensitive companion of mTOR (RICTOR), mammalian stress-activated map kinase interacting protein 1 (mSin1). Its major function is involved in cytoskeleton reorganization, but the molecular mechanism is still poorly understood [3]. There are several evidences suggesting the possibility of mTOR and tau direct interaction. First, mTOR hyperactivity may increase tau translation and its accumulation. Second, hyperactive mTOR activities may phosphorylate some residues of tau leading to tau aggregation. Third, a new growing evidence suggests that mTOR activity has been implicated in cell cycle re-entry [4]. It was shown that mTOR signaling was necessary for tauopathy and neurodegenerative disease such as Alzheimer's disease. Tang *et al.* reported that phosphorylation of tau at the flanking and repeat region on Ser-214, Thr-231 and Ser-356 by mTOR resulting in detachment of tau from microtubules and become toxic molecules that cause cell death [5]. However, they did not classify which one of the mTOR complexes can promote tau phosphorylation.

Therefore, this study aimed to find a new evidence supporting mTORC2 and tau interaction using the affinity purification of RICTOR and tau followed by LC-MS/MS technique, which is a high throughput technology for detection and identification of proteins. The study of mTORC2-tau interaction will increase the understandings about the mechanisms associated with microtubule stabilization and may identify novel candidate proteins of which deregulated interactions are able to promote the progression of important neurodegenerative diseases.

Methodology

Cell culture

SHSY-5Y (ATCC® CRL-2266™), a neuroblastoma cell line, was maintained in Eagle's Minimum Essential Medium (ATCC): Nutrient Mixture F-12 (HyClone) (EMEM/F12) 1: 1 supplemented with 10 % (v/v) fetal bovine serum (Gibco) and 1 % (v/v) Antibiotic-Antimycotic (Gibco). Cells were incubated under sterile condition in a humidified atmosphere of 5 % CO_2 at 37°C.

The study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Immunoprecipitation

Protein G and protein A magnetic beads were incubated with anti-Tau-5 and anti-RICTOR antibodies respectively for 3 hours at room temperature. SHSY-5Y cells were lysed in lysis buffer (50 mM HEPEs pH 7.4, 150 mM NaCl, 0.2 % CHAPS, 1 mM EDTA, 1 mM $NaVO_4$, 10 mM β-glycerophosphate and protease inhibitor cocktail). Cell debris was cleared by centrifugation at 16,000×g for 15 minutes at 4 °C. The supernatant was collected. Finally, cell lysate was added to protein G or protein A magnetic beads previously conjugated with antibodies overnight at 4 °C. Immunoprecipitated proteins were determined by Western blot analysis.

Western blot analysis

Ten percents of Immunoprecipitation (IP) products were separated by electrophoresis on SDS-PAGE to compare with the input (whole cell lysate). Next, proteins were transferred to a nitrocellulose membrane. The membrane was blocked in Odyssey Blocking Buffer (TBS), then probed with anti-Tau-5 or anti-RICTOR (Abcam) followed by secondary antibodies. Protein bands were detected by near-infrared fluorescence imaging system (Odyssey® CLx).

In-gel digestion and LC-MS/MS

IP products were fractionated by electrophoresis on SDS-PAGE and stained with Imperial Protein Stain (Thermo Scientific). The protein bands were cut and distained with 50 % acetonitrile (ACN) in 25 mM TEAB overnight and washed again. Supernatant was removed and dried in speed vacuum. Proteins in the gel slices were incubated with 10 mM DDT for 1 hour at 50 °C without shaking to reduce disulfide bond. Alkylation of proteins was performed by adding 55 mM iodoacetamide for 45 minutes in the dark at room temperature. ACN and 25 mM TEAB were used to wash gel slices, then speed vacuum was used to dry the gel slices. Tryptic peptides were prepared by digesting gel slices with approximately 100 ng of trypsin overnight and extracted with 50 % ACN in 1 % TFA three times. Samples were reconstituted in 0.1 % formic acid and removed salts and buffers by C18 column. Peptides were reconstituted in 0.1 % formic acid again for MS analysis with following setting modification for oxidation (M) and carbamidomethyl (C).

Ten microliters of digested peptides were injected onto a 75 µm × 15 cm, 2µm, 100Å PepMap C18 reverse-phase analytical RSLC column and separated with a linear gradient of 5 % solvent B (100 % ACN in 0.1 % FA) to 20 % solvent B over 40 minutes at constant flow rate of 300 nL/min. Top ten highest abundant precursor ions were selected to perform MS/MS with 70,000 and 17,500 resolutions at m/z 400 for MS1 and MS2 analysis. Raw data files were analyzed in Proteome Discoverer software (version 2.1.0.81; Thermo Scientific) and searched in database online as PANTHER classification system (version 13.1) and The Database for Annotation, Visualization and Integrated Discovery (DAVID version 6.8).

Results

RICTOR and tau are commonly associated with fifty candidate proteins.

To investigate the specific association between RICTOR and tau, IP and LC-MS/MS were performed to identify potential candidate proteins. Firstly, we performed Western blot analysis to confirm the IP efficiency by comparing the amount of RICTOR and tau in the whole cell lysate with IP products using the same volume for all loadings (Fig 1). The result shows that our immunoprecipitations were successful. RICTOR can be found in comparable amount between the lysate and IP products. Moreover, tau can be significanly enhanced by IP method even though the amount of tau proteins in cell lysate are very low.

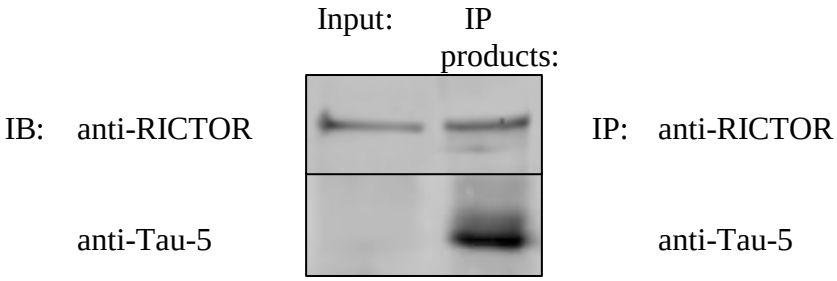

Figure 1. Efficiency of IP was performed by Western blot analysis. Anti-RICTOR and anti-Tau5 was probed to membrane.

Fifty common proteins were found in both IP products. Firstly, proteins were separated on an SDS-PAGE. Next, stained gel slices were cut and proteins were detected by LC-MS/MS. Protein identification was performed by searching candidate proteins using Proteome Discoverer software. Figure 2 shows that 591 proteins were found in RICTOR IP and 159 proteins were found in tau IP. Fifty candidate proteins were selected under the criteria of being the proteins found exclusively in IP products or the ratios of the proteins in IP products over the negative controls were over 10. However, we neither found tau in RICTOR IP nor

RICTOR in tau IP. This suggested that both proteins might not be directly bound to each other.

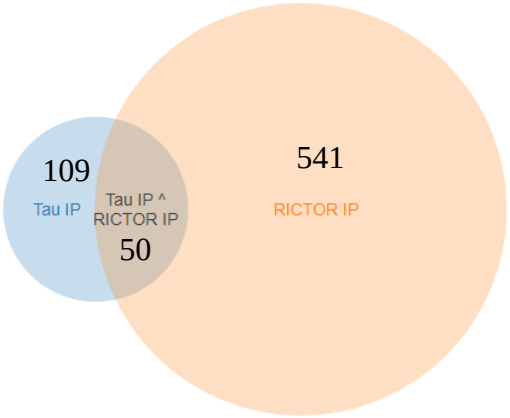

Figure 2. Venn diagram shown intersection between RICTOR IP (red circle) and tau IP (blue circle).

Table 1. Top 50 of candidate protein from intersection between RICTOR IP and tau IP

No.	Description	Accession No.
1	Thyroid hormone receptor-associated protein 3 [OS=Homo sapiens]	Q9Y2W1
2	Isoform 3 of Polyadenylate-binding protein 4 [OS=Homo sapiens]	Q13310
3	Keratin, type II cytoskeletal 1 - Homo sapiens (Human).	P04264
4	Carboxypeptidase A4 [OS=Homo sapiens]	Q9UI42
5	probable ATP-dependent RNA helicase DDX5 [OS=Homo sapiens]	P17844
6	Alpha-2-macroglobulin-like protein 1 [OS=Homo sapiens]	A8K2U0
7	Isoform 2 of Extended synaptotagmin-1 [OS=Homo sapiens]	Q9BSJ8
8	ATP-dependent RNA helicase DDX3X [OS=Homo sapiens]	O00571
9	plectin [OS=Homo sapiens]	Q15149
10	eukaryotic translation initiation factor 6 [OS=Homo sapiens]	P56537
11	Keratin, type I cytoskeletal 18 - Homo sapiens (Human).	P05783
12	alpha-internexin [OS=Homo sapiens]	Q16352
13	Eukaryotic initiation factor 4A-III [OS=Homo sapiens]	P38919
14	Complement C4-A [OS=Homo sapiens]	P0C0L4
15	Keratin type II cuticular Hb5 - Homo sapiens (Human).	P78386
16	Ras-related protein Rab-4A [OS=Homo sapiens]	P20338
17	Neurosecretory protein VGF [OS=Homo sapiens]	O15240
18	Fragile X mental retardation syndrome-related protein 2 [OS=Homo sapiens]	P51116
19	Isoform 2 of 40S ribosomal protein S20 [OS=Homo sapiens]	P60866
20	Isoform 2 of Histone H2B type 2-F [OS=Homo sapiens]	Q5QNW6
21	Calpain-1 catalytic subunit [OS=Homo sapiens]	P07384
22	keratin, type I cuticular Ha4 [OS=Homo sapiens]	O76011
23	ATP synthase subunit beta, mitochondrial [OS=Homo sapiens]	P06576
24	Probable ATP-dependent RNA helicase DDX6 [OS=Homo sapiens]	P26196
25	Protein CASC3 [OS=Homo sapiens]	O15234
26	ATP-dependent 6-phosphofructokinase, liver type [OS=Homo sapiens]	P17858
27	RNA-binding protein 33 [OS=Homo sapiens]	Q96EV2

28	Mannosyl-oligosaccharide glucosidase [OS=Homo sapiens]	Q13724
29	Polyadenylate-binding protein 1 [OS=Homo sapiens]	P11940
30	Insulin-like growth factor 2 mRNA-binding protein 3 [OS=Homo sapiens]	O00425
31	DNA mismatch repair protein MSH2 [OS=Homo sapiens]	P43246
32	60S ribosomal protein L27 [OS=Homo sapiens]	P61353
33	40S ribosomal protein S15a [OS=Homo sapiens]	P62244
34	Serine/threonine-protein kinase D2 [OS=Homo sapiens]	Q9BZL6
35	Ras GTPase-activating protein-binding protein 2 [OS=Homo sapiens]	Q9UN86
36	Regulator of nonsense transcripts 1 [OS=Homo sapiens]	Q92900
37	40S ribosomal protein S27 [OS=Homo sapiens]	P42677
38	Cytoplasmic dynein 1 heavy chain 1 [OS=Homo sapiens]	Q14204
39	Probable ATP-dependent RNA helicase DDX17 [OS=Homo sapiens]	Q92841
40	Heat shock cognate 71 kDa protein [OS=Homo sapiens]	P11142
41	60S ribosomal protein L12 [OS=Homo sapiens]	P30050
42	Heterogeneous nuclear ribonucleoprotein M [OS=Homo sapiens]	P52272
43	Isoform 2 of AP-2 complex subunit beta [OS=Homo sapiens]	P63010
44	Pre-mRNA-processing-splicing factor 8 [OS=Homo sapiens]	Q6P2Q9
45	Lamin-B2 [OS=Homo sapiens]	Q03252
46	60S ribosomal protein L7 [OS=Homo sapiens]	P18124
47	Gelsolin [OS=Homo sapiens]	P06396
48	AP-2 complex subunit alpha-1 [OS=Homo sapiens]	O95782
49	Transferrin receptor protein 1 [OS=Homo sapiens]	P02786
50	Atypical kinase ADCK3, mitochondrial [OS=Homo sapiens]	Q8NI60

As briefly introduced above, the main function of mTORC2 is related to cell actin cytoskeleton organization and other functions have not been completely elucidated. Therefore, we would like to explore this complex more thoroughly, including the association with other types of cytoskeleton. The answer will lead to the explanation of how mTORC2 and tau interact. In order to understand more information about mTORC2 and tau relationship, uniprot accession numbers of 50 candidate proteins were analyzed by PANTHER classification system (version 13.1) to further study about molecular functions, biological processes and cellular components. The criteria for selected classification must be more than 10 % from total protein classification.

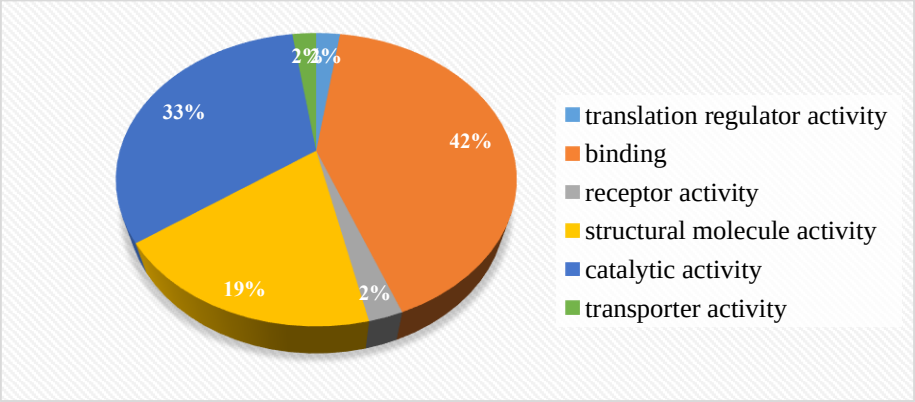

Figure 3. Molecular Function classification of 50 candidate proteins

First of all, binding activity (GO:0005488), catalytic activity (GO:0003824), and structural molecule activity (GO:0005198) were represented in Molecular Function (MF) at 41.9 % 32.6 % and 18.6 % respectively. Protein binding (GO:0005515) as a subset of binding activity represented 40.0 % of all binding activities. Interestingly, gelsolin (P06396) and plectin (Q15149) were shown in cytoskeletal protein binding (GO:0008092) and structural constituent of cytoskeleton (GO:0005200). Both candidate proteins have been implicated in protein interaction especially with actin filaments and may associate with microtubule function [6].

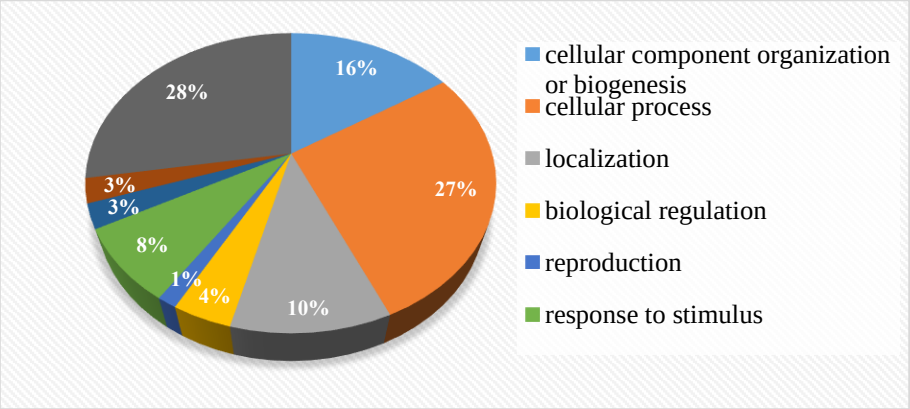

Figure 4. Biological Process classification of 50 candidate proteins

Next, Biological Process analysis (BP) was explored to describe many events in cell culture model. Cellular process (GO:0009987), metabolic process (GO:0008152) represented 27.6 %. Cellular component organization or biogenesis (GO:0071840) and localization (GO:0051179) represented 15.8 % and 10.5 % respectively. Cytoplasmic dynein 1 heavy chain 1 (Q14204) and Fragile X mental retardation syndrome-related protein 2 (P51116) were discovered in a subset of cellular component movement (GO:0006928) in cellular process term (GO:0009987). In cellular component morphogenesis (GO:0032989), gelsolin (P06396), fragile X mental retardation syndrome-related protein 2 (P51116) and lamin-B2 (Q03252) were found, suggesting that these proteins are necessary for dynamic process in cell reoganization.

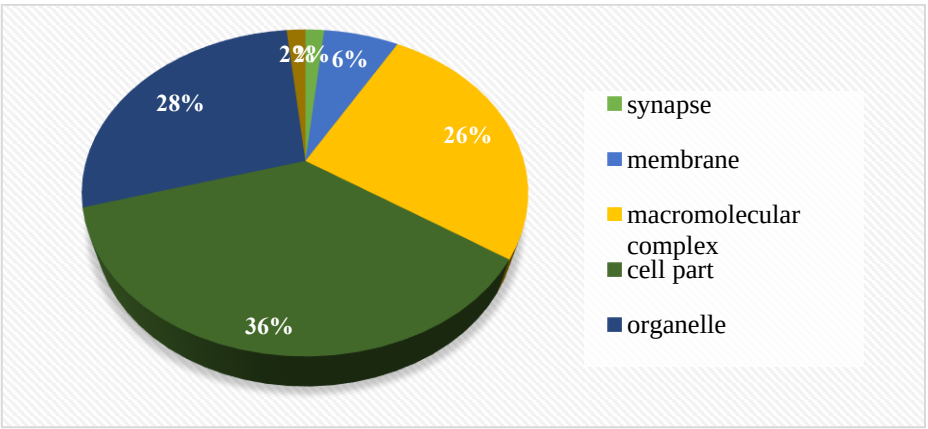

Figure 5. Cellular Component classification of 50 candidate proteins

Lastly, cell part (GO:0044464), organelle (GO:0043226), macromolecular complex (GO:0032991) represented 36.1 %, 27.9 % and 26.2 % of all candidate proteins, respectively. Gelsolin, cytoplasmic dynein 1 heavy chain 1 and Fragile X mental retardation syndrome-related protein 2 were found under the sub-term cytoskeleton (GO:0005856) under organelle (GO:0043226).

The implication of gelsolin and plectin as cytoskeletal proteins binders between RICTOR and tau

From analyzed data above, we found that gelsolin and plectin which are proteins capable of binding cytoskeletal proteins were associated with both RICTOR and tau. Gelsolin is an actin-binding protein that is a key regulator of actin filament assembly and disassembly. It is found in cytosol, mitochondria and blood plasma[7]. From an *in vitro* study, gelsolin was reported as a protein that positively promotes actin filaments (F-actin). Gelsolin can react with globular actin (G-actin) faster than F-actin only when ADP amount is more than ATP in the cell [8]. Gelsolin and tau can bind to phosphatidylinositol bisphosphate (PIP2) and affect the formation of PIP2 aggregation [9]. PIP2 is an upstream regulator of mTORC1 that control cell proliferation, but S6K directly mTORC1 downstream, can inhibit mTORC2 [10]. Therefore, from the analyzed data, gelsolin may be associated with the signaling cascades for cell movement and cell communication by interacting with both tau and mTORC2. mTORC2 may be positively controlled by gelsolin and tau by binding with PIP2 leading to aggregated PIP2 for inactivated mTORC1.

On the other hand, plectin is a large protein which acts as a link between the three main components of the cytoskeleton: actin microfilaments, microtubules and intermediate filaments. Moreover, it can bind to transmembrane receptors, components of the nuclear envelope, and many kinase proteins that play roles in cell migration, proliferation, and energy metabolism. Alternative mRNA splicing can cause them have several plectin isoforms, and its full form is around 500 kDa. Plectin binds to MAPs to control a cycle of microtubule assembly and disassembly [11]. Plectin deficiency causes actin stress fiber formation and reduces microtubule dynamics rather than actin stabilization and tubulin assembly [12]. Previous studies have shown that plectin is involved in cell cytoskeleton dynamic process but there has been no report about plectin and mTORC2 or plectin and tau. This is the first evidence that identifies mTORC2/tau/plectin interaction which can be further investigated for the importance of this protein in mTORC2-tau interaction.

mRNA alternative splicing might be a process connecting mTORC2 and tau.

We found many proteins involved in mRNA splicing process in the 50 candidate proteins, including DEAD-box helicase 5 (DDX5), pre-mRNA processing factor 8 (PRPF8), heat shock protein family A (Hsp70) member 8 (HSPA8), eukaryotic translation initiation factor 4A3 (EIF4A3) and heterogeneous nuclear ribonucleoprotein M (HNRNPM) as shown in figure 6. Even though more studies must be performed to confirm the evidence, our results suggested a highly possible association between mTORC2 and mRNA alternative splicing regulators of tau. They may contribute to alternative splicing of six-isoforms tau. Until now, there is no evidence to support the interaction between mTORC2/tau and these five candidate proteins. The study of mTORC2/tau interaction with five candidate proteins should be performed to investigate the functions and correlations in the future.

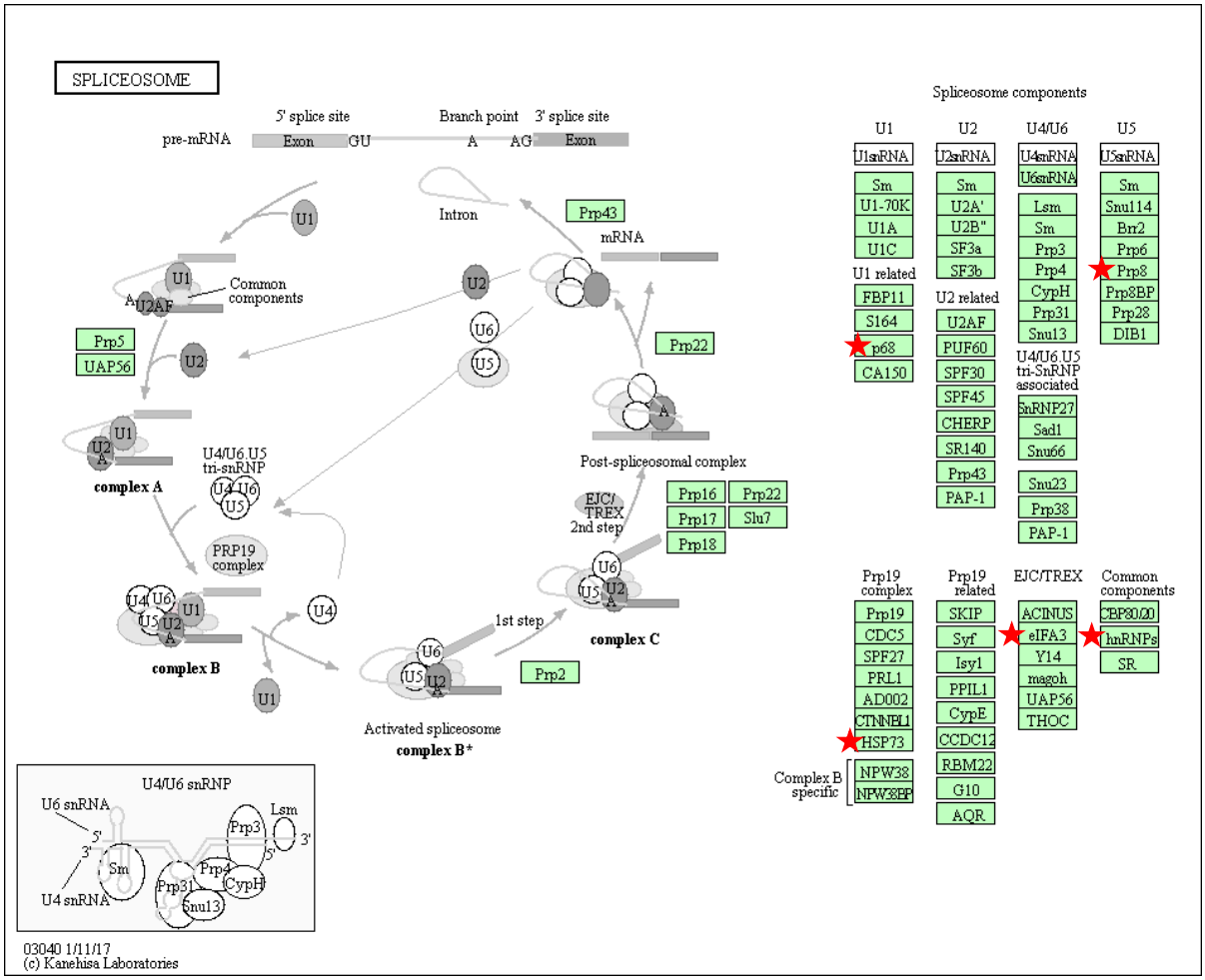

Figure 6. Common interactors of RICTOR and tau are components of spliceosome. Data were analyzed by DAVID version 6.8.

Conclusion

The results from this study demonstrated that mTORC2 might play an important role in tau formation, especially the alternative splicing of mRNA resulting in different tau isoforms. Interestingly, plectin and gelsolin might be implicated in protein-protein interaction between mTORC2 and tau. Therefore, this study is a new evidence to explore potential players and molecular mechanisms regulating the proteins that may lead to AD. Biological functions and significance of identified candidate proteins would be investigated in further studies.

References

1. Weingarten MD, Lockwood AH, Hwo S-Y,Kirschner MW, Proceedings of the National Academy of Sciences. 1975;72:1858-1862
2. Liu F,Gong C-X, Molecular neurodegeneration. 2008;3:8
3. Laplante M,Sabatini DM, Cell. 2012;149:274-293
4. Oddo S, Frontiers in bioscience (Scholar edition). 2012;4:941
5. Tang Z, Bereczki E, Zhang H, Wang S, Li C, Ji X, Branca RM, Lehtiö J, Guan Z,Filipcik P, Journal of Biological Chemistry. 2013;288:15556-15570
6. Sun HQ, Yamamoto M, Mejillano M,Yin HL, Journal of Biological Chemistry. 1999;274:33179-33182
7. Ghoshdastider U, Popp D, Burtnick LD,Robinson RC, Cytoskeleton. 2013;70:775-795
8. Laham LE, Lamb JA, Allen PG,Janmey PA, Journal of Biological Chemistry. 1993;268:14202-14207

9. Flanagan LA, Cunningham CC, Chen J, Prestwich GD, Kosik KS,Janmey PA, Biophysical journal. 1997;73:1440-1447
10. Hay N,Sonenberg N, Genes & development. 2004;18:1926-1945
11. Castanón MJ, Walko G, Winter L,Wiche G, Histochemistry and cell biology. 2013;140:33-53
12. Wiche G,Winter L, Bioarchitecture. 2011;1:14-20

Acknowledgements

This work was supported by the Scholarship from the Graduate School, Chulalongkorn University to commemorate The Celebrations on the Auspicious Occasion of Her Royal Highness Princess Maha Chakri Sirindhorn's 5th Cycle (60th) Birthday.