Universidade de Aveiro
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Departamento de Biologia

ESTEFÂNIA COELHO CARVALHO

Identification of Alzheimer's Disease relevant interacting proteins using "in silico" and cellular procedures

Identificação de proteínas interactoras relevantes para a Doença de Alzheimer usando procedimentos "in silico" e celulares

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Professora Doutora Odete Abreu Beirão da Cruz e Silva, Professora Associada com Agregação do Departamento de Ciências Médicas da Universidade de Aveiro.

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Pelo amor incondicional, força e apoio, dedico esta dissertação aos meus avós José e Helena, à minha mãe e ao meu irmão.

o júri

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palavras-chave

Aβ, Complexos de proteínas, Células SH-SY5Y, GRB2, TP53BP2, Doença de Alzheimer

resumo

A APP é uma proteína de relevância para a doença de Alzheimer, pois é a precursora do péptido Aβ que é o principal constituinte das placas senis que acumulam no cérebro de pacientes com doença de Alzheimer. A desfosforilação, por fosfatases como a PP1, está entre as formas mais comuns de modificação pós-traducional em proteínas e problemas neste processo têm sido associados à doença de Alzheimer. Como tal, pode ser importante identificar proteínas que ligam à APP e que podem ser moduladas por fosfatases como a PP1. Aqui concentramo-nos em duas proteínas, TP53BP2 e GRB2. A TP53BP2 é uma proteína que desempenha um papel central na regulação da apoptose e do crescimento celular através das suas interações, tais como os membros da família p53. A GRB2 é uma proteína que fornece uma ligação entre os recetores da superfície celular que ligam fatores de crescimento e a via de sinalização Ras. Em primeiro lugar, mostramos que nenhum dos níveis de expressão destas proteínas é afetado pela exposição ao Aβ42 previamente agregado, em células SH-SY5Y indiferenciadas. Além disso, tendo em conta testes dois-híbrido em levedura anteriormente realizados pelo nosso grupo de investigação usando PP1 ou APP como isco, hipotetizamos que TP53BP2 e GRB2 poderiam ser independentemente proteínas intermediárias num tri-complexo envolvendo a APP e a PP1. Após realizar co-imunoprecipitações, validamos as interações entre a TP53BP2 e a PP1 e entre a GRB2 e APP enquanto que as interações entre a TP53BP2 e APP e entre a GRB2 e PP1 não foram detetadas.

keywords

Aβ, Protein complexes, SH-SY5Y cell line, GRB2, TP53BP2, Alzheimer's Disease

abstract

APP is a protein of relevance to Alzheimer´s disease as it is the precursor for the Aβ peptide that is the major constituent of senile plaques seen deposited in the brains of individuals with Alzheimer's Disease. Dephosphorylation, by phosphatases like PP1, is among the most common forms of post-translational modification in proteins and anomalies in this process have been linked to Alzheimer's disease. Therefore, it may be important to identify APP binding proteins which can be modulated by phosphatases such as PP1. Here we focus on two proteins, TP53BP2 and GRB2. TP53BP2 is a protein that plays a central role in regulating apoptosis and cell growth via its interactions with other proteins such as the members of the p53 family. GRB2 is an adapter protein that provides a connection between cell surface growth factor receptors and the Ras signaling pathway. Firstly, we show that neither of these proteins' expression levels are affected by exposure to previously aggregated Aβ42 in undifferentiated SH-SY5Y cells. Secondly, relying on yeast two hybrid assays previously carried out by our research group using PP1 or APP as bait, we hypothesized that TP53BP2 and GRB2 could independently be the bridging protein in a trimeric complex involving APP and PP1. After performing co-Immunoprecipitations we were able to validate the interactions between TP53BP2 and PP1 and between GRB2 and APP, while the interactions between TP53BP2 and APP and between GRB2 and PP1 were not detected.

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III. Abbreviations

AD	Alzheimer's disease
AICD	APP intracellular domain
APP	Amyloid precursor protein
APS	Ammonium persulfate
Aβ	Amyloid β peptide
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Co-IP	Co-immunoprecipitation
DAPI	4',6-diamidino-2-phenylindole
ECL	Enhanced luminescence
EDTA	Ethylenediamine tetraacetic acid
ERK1,2	Extracellular signal-regulated kinase 1 and 2
FBS	Fetal Bovine Serum
GRB2	Growth factor receptor-bound protein 2
LB	Loading buffer
LGB	Lower gel buffer
NFT	Neurofibrillary tangles
PBS	Phosphate buffered saline
PIP	PP1 Interacting protein
PMSF	Phenylmethylsulfonyl fluoride
PP1	Protein phosphatase 1
PP1α	Protein phosphatase 1 catalytic subunit α
PP1β	Protein phosphatase 1 catalytic subunit β
PP1γ	Protein phosphatase 1 catalytic subunit γ
RIPA	Radioimmunoprecipitation assay buffer
sAPPα/β	Secreted APP after α-cleavage/β-cleavage
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
Ser/Thr	Serine/threonine
SP	Senile plaques
TBS	Tris buffered saline
TBS-T	Tris buffered saline with tween
Thr	Threonine
TP53BP2	Tumor protein 53 binding protein 2
Tyr	Tyrosine
UGB	Upper gel buffer
WB	Western blot
YTH	Yeast two hybrid

1. Introduction

1.1. Alzheimer's Disease

Dementia is an umbrella term for numerous diseases, either chronic or progressive, that affect memory, other cognitive abilities and behavior, and ultimately interferes with one's ability to execute and maintain normal daily activities (World Health Organization, 2017). In 2018, 50 million people were affected with dementia worldwide and this value is expected to triple by 2050. Between 60-70% of cases are people who suffer from Alzheimer's Disease (AD) (Alzheimer's disease international, 2018).

Alzheimer's disease is a devastating neurodegenerative disorder first described in 1906 by Alois Alzheimer after studying the brain of Auguste D, a 51-year-old woman who had shown progressive cognitive impairment, delusions, and psychosocial incompetence (Maurer, Volk and Gerbaldo, 1997). It is currently a growing global health concern that heavily impacts both individuals and society (Lane, Hardy and Schott, 2018). Alzheimer's is characterized by memory loss, apathy and depression in an early stage, impaired communication, confusion and behavior changes in a later stage and, finally, difficulty in performing basic actions such as walking (Alzheimer's Association, 2019). In a general manner, the disease causes life-changing cognitive and behavioral deterioration.

The initial symptoms, however, are only noticeable after many years of neuronal loss and neuropathologic lesions and occur due to damage or destruction of neurons in parts of the brain involved in cognitive functions (Mantzavinos and Alexiou, 2017). Individuals may live with these symptoms for years before a obtaining a clinical diagnosis. Gradually, neurons in other regions of the brain are affected and certain core activities and bodily functions are compromised, eventually leaving the person bedridden and in need of constant care. Ultimately, this disease is fatal (Mantzavinos and Alexiou, 2017; Alzheimer's Association, 2019).

1.1.1. Hallmarks of Alzheimer's Disease

As aforementioned, the initial symptoms of Alzheimer's disease are only detectable after years of neuronal degradation. The two core neuropathological features

of the disease, also first reported by Alois Alzheimer, are intracellular neurofibrillary tangles (NFTs) and extracellular senile plaques (SPs).

Neurofibrillary tangles are composed of paired helical filaments of the microtubule-associated protein Tau, which is aberrantly assembled and abnormally hyperphosphorylated (Johnson and Stoothoff, 2004). In its normal state, Tau is an important protein that promotes microtubule assembly and stability, however its hyperphosphorylated state has been linked to multiple pathologies, termed tauopathies, including AD, corticobasal degeneration and progressive supranuclear palsy (Lebouvier, Pasquier and Buée, 2017).

Tau is encoded by a gene located on chromosome 17 and is spliced into six isoforms that are expressed in the central nervous system (CNS) (Johnson and Stoothoff, 2004). Besides alternative splicing, Tau is also regulated by post-translational modifications such as phosphorylation and dephosphorylation. In AD, Tau hyperphosphorylation inhibits the attachment of Tau to microtubules thus causing a breakdown of the microtubule system and facilitating the formation of tangles (Del Alonso *et al.*, 1994).

Senile plaques are the result of extracellular accumulation and deposition of abnormally folded amyloid-β peptide (Aβ) with 40 or 42 amino acids (Aβ40 or Aβ42). Both Aβ peptides are a by-product of APP metabolism, however Aβ42 is more abundantly found in SPs on account of its higher rate of fibrilization and insolubility (Lane, Hardy and Schott, 2018). The Aβ peptide will be further discussed below.

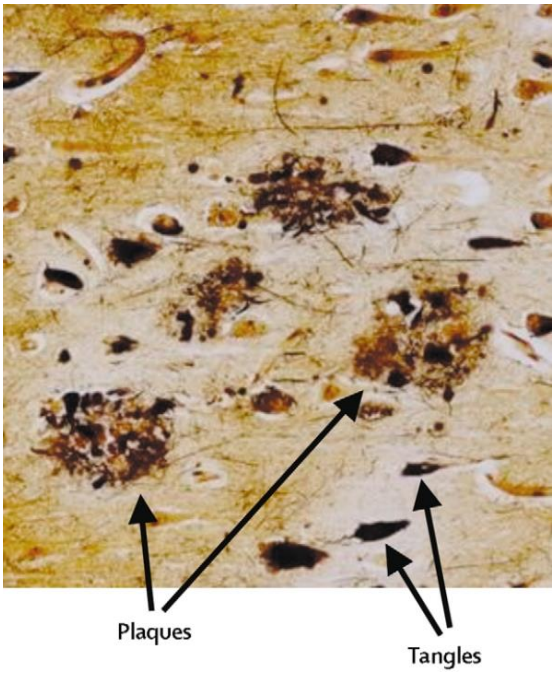


Figure 1 - Two neuropathological hallmarks of AD: Senile Plaques and Neurofibrillary Tangles. (Taken from Blennow, de Leon and Zetterberg, 2006).

1.2. The Amyloid Precursor Protein

The Amyloid Precursor Protein (APP) belongs to a highly conserved family of type-I transmembrane proteins that include homologs APP-like protein 1 (APLP1) and 2 (APLP2). Despite sharing conserved structural motifs and similar conventional processing pathways with APLP1 and APLP2, only APP contains the Aβ sequence (Wilkins and Swerdlow, 2017).

APP is encoded by a single gene located on chromosome 21 and contains 18 exons. Alternative splicing generates APP mRNAs encoding several isoforms that range from 365 to 770 amino acid residues, of which three are more common, specifically APP_{695}, APP_{751} and APP_{770} (O'brien and Wong, 2011; Wilkins and Swerdlow, 2017). APP_{751} and APP_{770}, which are ubiquitously expressed, contain a domain homologous to the Kunitz-type serine protease inhibitors (KPI) encoded by exon 7, while the APP_{695} isoform lacks the KPI domain and is predominantly expressed in neurons. However, the reason for this apparent tissue-specific alternative splicing is not yet understood (Zheng & Koo, 2006).

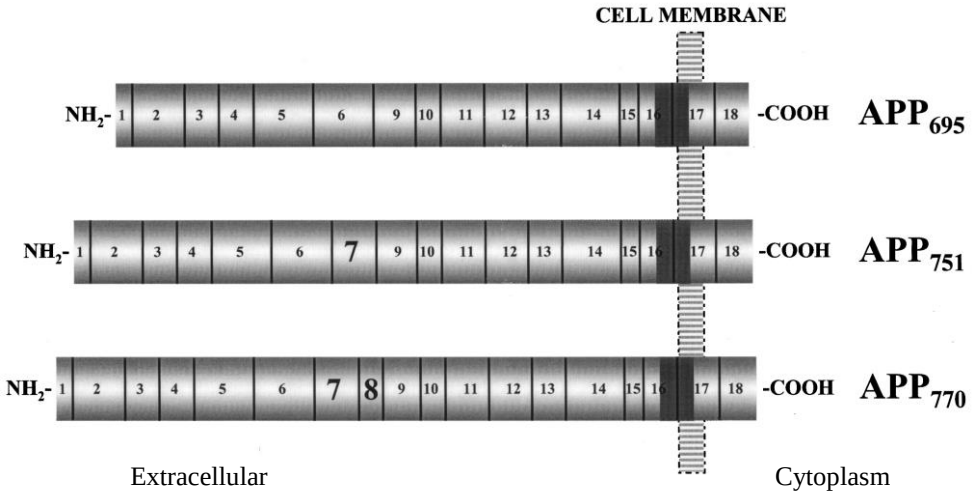


Figure 2 – Schematic representation of the three major APP isoforms in mammalian tissues. Numbers and vertical lines indicate the corresponding exons. The most abundant neuronal isoform, comprising 695 amino acids, is APP_{695}. APP_{751} and APP_{770} differ from APP_{695} in the expression of exons 7 and 8, as shown. The sequences encoded by the APP gene exons are indicated approximately to scale. The dark grey region represents the Aß peptide region, whose sequence is divided between exons 16 and 17. (Taken from Da Cruz e Silva and Da Cruz e Silva, 2003)

APP is synthesized in the endoplasmic reticulum (ER) and is transported to the cell surface via the secretory pathway. During this transportation, APP suffers post-translational modifications such as N- and O-glycosylation and tyrosine sulfation, thus becoming a mature protein. It is estimated that only 10% of mature APP localizes to the plasma membrane, where it can either be cleaved or rapidly internalized via endocytosis to be recycled back to the cell surface or degraded in lysosomes (Da Cruz e Silva and Da Cruz e Silva, 2003; Thinakaran and Koo, 2008).

Although the exact functions of APP are not completely know, members of the APP gene family are thought to play vital roles in the development of the nervous system relating to synapse structure and function, as well as in neuronal migration or adhesion (Zheng & Koo, 2006).

1.2.1. APP Processing

APP processing is dependent on secretase enzymes and is commonly divided into two pathways: non-amyloidogenic (or α-secretase) or amyloidogenic (or β-secretase).

In the non-amyloidogenic pathway, APP is initially cleaved by α-secretase at amino acid 687, within the Aβ sequence, releasing a soluble N-terminal APP fragment, sAPPα, into the extracellular space. Secondly, the remaining C-terminal fragment is cleaved by γ-secretase and originates a small extracellular peptide, p3 and the APP intracellular domain (AICD).

In the amyloidogenic pathway, APP is cleaved by β-secretase at amino acid 671 releasing extracellularly a soluble N-terminal peptide (sAPPβ), while the C-terminal fragment remains inserted in the membrane. Then, the C-terminal fragment undergoes cleavage by γ-secretase giving rise to the Aβ fragment, which is released to the extracellular space, and to the AICD. The Aβ peptide can later aggregate and form senile plaques (Nicolas & Hassan, 2014; Zheng & Koo, 2006).

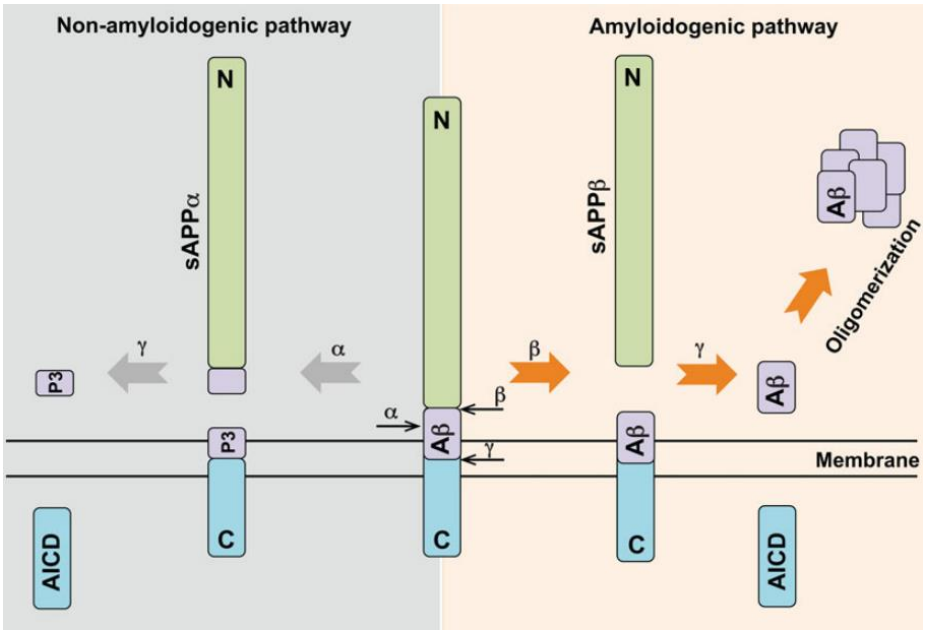


Figure 3 - Schematic representation of the proteolytic processing of APP. (Taken from Nicolas and Hassan, 2014).

Aβ is a 4 kDa peptide, normally secreted, as a soluble proteolytic product of the Amyloid Precursor Protein (APP). As mentioned above, Aβ is generated by the β- and γ-secretase cleavages of APP. γ-Secretase cleavages give rise to multiple Aβ species, including soluble monomeric peptides of 40 amino acids (Aβ40) and more insoluble

peptides of 42 amino acids (Aβ42). The Aβ peptide is the main component of senile plaques and occurs as clumps of the insoluble amyloid fibrils (8-10 nm) mixed with an array of non-fibrillar forms of this peptide. Although Aβ40 is the major peptide produced by APP processing, the main peptide deposited within the senile plaques is Aβ42. The Aβ42 peptide is more insoluble and therefore has an increased capacity to generate fibrils, self-aggregate and form SPs. For this reason, Aβ42 presents higher neurotoxicity (Golde, Eckman and Younkin, 2000; Murphy and Levine, 2010).

The presence of Aβ is not, however, exclusive to AD brains as Aβ is constitutively secreted by healthy cells throughout life and is found in the cerebrospinal fluid and plasma of all normal humans (Haass *et al.*, 1992; Shoji *et al.*, 1992). Aβ is, therefore, normally produced in the brain and has actually been shown to enhance long term potentiation and memory when in picomolar concentrations, however abnormally high levels of this peptide have been shown to cause the well-established synaptic and memory dysfunction characteristic of AD (Haass and Selkoe, 2007; Puzzo *et al.*, 2008).

Also relevant are studies from our research group that have shown that Aβ has the ability to modulate the activity of certain proteins, such as PP1 (Vintém *et al.*, 2009). It was determined that Aβ peptides inhibit PP1 isoforms and that fibril formation of Aβ40 and Aβ42 increased Aβ's inhibitory potency against PP1.

1.3. Protein Phosphatase 1

Protein phosphorylation is an important post-translational modification mechanism that can affect key properties of proteins including their activity, interaction with other proteins or their sub-cellular localization. The phosphorylation and dephosphorylation of proteins is carried out by opposing activities of kinases and phosphatases, respectively (Rossignol, 2006) and abnormal activity of both these enzymes has been reported in AD brains when compared to normal aged controls (Gong *et al.*, 1993; Chung, 2009).

In eukaryotic cells, phosphorylation mostly occurs on three hydroxyl-containing amino acids, serine (Ser), threonine (Thr), and tyrosine (Tyr), of which serine is the main

target. Proteomic analysis of 6600 phosphorylation sites on 2244 human proteins revealed that phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) account for 86.4%, 11.8%, and 1.8%, respectively, of the phosphorylated amino acids (Olsen *et al.*, 2006).

Protein phosphatase 1 (PP1) is a ubiquitously expressed serine/threonine (Ser/Thr) phosphatase that belongs to the phosphoprotein phosphatase superfamily along with PP2A, PP2B, PP4, PP5, PP6 and PP7. These phosphatases catalyze over 90% of all eukaryotic protein dephosphorylation reactions and among them, PP1 is the most important one in terms of substrate diversity with nearly 200 validated vertebrate interactors and many more are estimated to exist (Heroes *et al.*, 2013).

Mammalian genomes contain three PP1-encoding genes that together encode four distinct catalytic subunits: PPP1CA encodes PP1α, PPP1CB encodes PP1β (also named PP1δ) and PPP1CC encodes PP1γ1 and PP1γ2, both generated through alternative splicing. PP1α, PP1β and PP1γ1 present a ubiquitous expression, whereas the PP1γ2 isoform is mainly in the testis (Heroes *et al.*, 2013; Rebelo *et al.*, 2015).

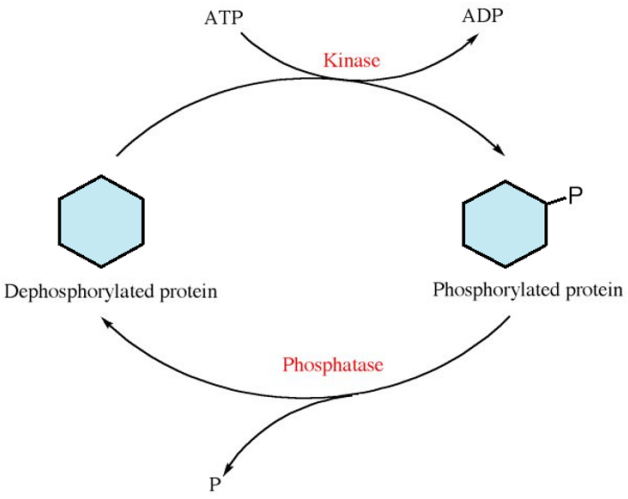


Figure 4 - Protein phosphorylation. Proteins are phosphorylated by protein kinases which add a phosphate from ATP, whereas protein phosphatases remove the phosphate group from a protein resulting in that protein's dephosphorylation.

The diversity of protein phosphatases is achieved mainly by the ability of a single catalytic subunit to associate with different regulatory subunits, also termed PP1-interacting proteins (PIPs), thus creating highly specific holoenzymes. Generally, these

regulatory subunits act as activity-modulators, such as inhibitor-1 which is able to block the activity of PP1, or enhance activity towards a specific substrate as in the case of MYPT1 (Bollen, 2001).

The catalytic subunit of PP1 has several surface grooves, which are binding sites for PIPs. The interaction with PP1 is carried out through unique short (4-6 residues) binding motifs, among them RVxF, SILK and MyPhoNE (Heroes *et al.*, 2013; Rebelo *et al.*, 2015)

The primary PP1-binding motif is the RVxF motif, which is present in approximately 70% of all PIPs. It generally conforms with the consensus sequence [K/R] [K/R] [V/I] x [F/W], where x is any residue other than Phenylalanine, Isoleucine, Methionine, Tyrosine, Aspartate or Proline. Docking of this motif doesn't affect PP1 conformation, however it rises the local concentration of the interactor and thereby promotes secondary interactions that can affect the activity or substrate specificity of PP1 (Hendrickx *et al.*, 2009; Bollen *et al.*, 2011).

Another PP1-docking sequence is the SILK motif that is present in seven of the known vertebrate PIPs. It contains the consensus sequence [G/S] I L [R/K] and is always situated N-terminal to the RVxF sequence. As in the case of the RVxF motif, the SILK motif also does not change the conformation of PP1, but rather carries out an anchoring function (Hendrickx *et al.*, 2009; Bollen *et al.*, 2011).

The myosin phosphatase N-terminal element, or MyPhoNE motif, refers to the sequence R x x Q [V/I/L] [K/R] x [Y/W], where x can be any residue. It is involved in substrate recognition and, like the SILK motif, it is present in seven PIPs and is always located N-terminal to RVxF motif (Hendrickx *et al.*, 2009; Bollen *et al.*, 2011).

1.4. Protein Complexes

Protein interactions are at the root of many biological processes. These interactions lead to the assembly of complexes formed by several proteins. The identification and characterization of protein–protein interactions are therefore essential for understanding the mechanisms and regulation of biological systems.

Below is presented a recently discovered complex that will serve as the foundation for the work presented in this thesis.

1.4.1. Amyloid-beta A4 Precursor Protein-binding Family B Member 1

Amyloid-beta A4 precursor protein-binding family B member 1 (APBB1) or FE65 is a multidomain brain-enriched adaptor protein capable of establishing multimolecular complexes. It was first discovered in a rat brain cDNA minilibrary in 1990 and named FE65 after the first author's initials and it's respective clone number (Esposito *et al.*, 1990). It was only in 1995, however, that a team of scientists established FE65 as an APP interacting protein (Fiore *et al.*, 1995). Due to this, the protein was given the alternative name "Amyloid Beta Precursor Protein-Binding, Family B, Member 1" or APBB1.

FE65 has three protein interaction domains, specifically an N-terminal WW domain and two C-terminal phosphotyrosine-binding domains (PTB). It's interaction with APP occurs via the YENPTY motif on its second PTB (Sabo *et al.*, 1999).

Several studies exist that confirm that APP and FE65 bind to each other and many functions of this complex have been identified. The APP holoprotein can anchor FE65 in the cytoplasmic membrane thus preventing the latter's translocation to the nucleus (Sabo *et al.*, 1999). The FE65/APP interaction has also been shown to modulate APP metabolism, including Aβ generation (Sabo *et al.*, 1999; Ando *et al.*, 2001).

Subsequent studies showed that FE65 and the APP intercellular domain (AICD) translocate to the nucleus where they appear to have a role in gene transactivation (Cao and Südhof, 2001; Walsh *et al.*, 2003). One report described that phosphorylation of APP was important for this role as it liberated the membrane-bound FE65 which then translocated into the nucleus, up-regulating gene transactivation mediated by AICD. This indicates that APP phosphorylation regulates AICD-mediated FE65-dependent gene transactivation in the nucleus (Nakaya and Suzuki, 2006).

In 2013, our research group showed for the first time that FE65 binds simultaneously to APP and PP1γ therefore attributing PP1 with a determinant role in APP

Thr668 dephosphorylation (Rebelo *et al.*, 2013). FE65 is the bridging protein that mediates the interaction between APP and PP1γ and although it had already been, as aforementioned, associated with APP it had never been proven to bind to PP1.

This tri-complex was first identified using a yeast tri-hybrid screen (unpublished data) and was validated through several immunoprecipitation assays using COS-7 cells, primary neuronal cultures and adult rat brain tissues.

Rebelo et al (2013) created and tested the hypothesis that FE65 promoted the interaction between APP and PP1γ and the latter was thus responsible for dephosphorylating APP. They showed that the state of phosphorylation of APP at Thr668 was relevant for the formation of the tri-complex because, as previously mentioned, APP phosphorylation liberates FE65 and therefore will liberate PP1 as well, thus, APP dephosphorylation is pivotal to maintain the APP:FE65:PP1γ complex. Also, the downregulation of FE65 did not affect the total levels of APP but did increase the phosphorylation levels of Thr668, further confirming the role of FE65 in dephosphorylating APP.

This tri-complex can also play an important role in regulating APP endocytosis as the phosphorylation of APP at Thr668 would then make APP available for subsequent events such as proteolytic cleaving.

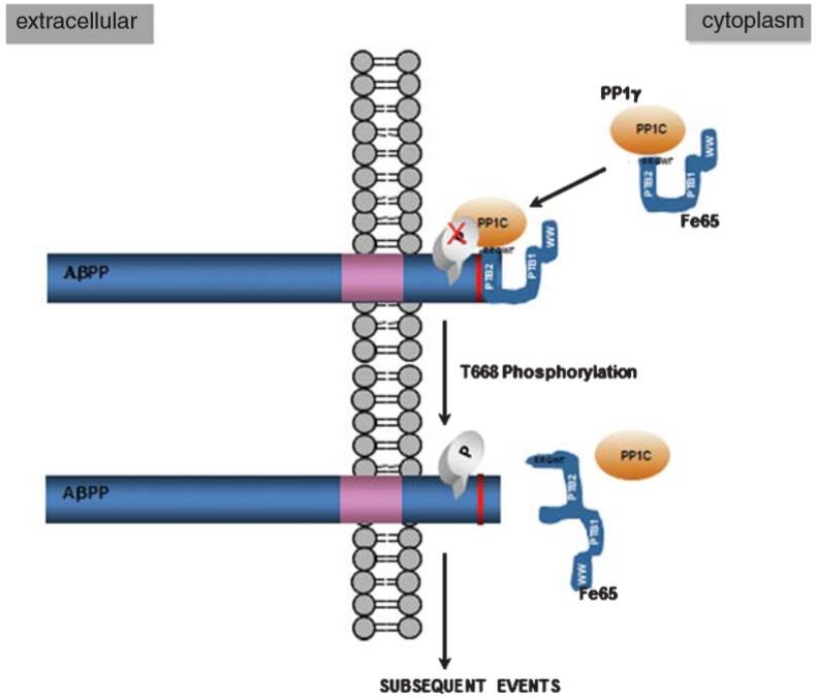


Figure 5 - Schematic representation of the putative function of the trimeric complex APP:FE65:PP1. The trimeric complex is prevalent when APP is dephosphorylated at Thr668, potentially promoted by PP1 itself. Phosphorylation at APP Thr668 promotes the release of Fe65 and PP1. (Taken from Rebelo et al., 2013)

From previous experiments carried out in the laboratory as well as data available in the literature and databases other proteins present themselves as potential candidates for tri-complexes associating with APP and PP1 simultaneously. Among these are TP53BP2 and GRB2, which are the subjects of the work here presented.

1.5. Tumor Protein 53 Binding Protein 2

Tumor Protein p53 Binding Protein 2 (TP53BP2), also known as Apoptosis-Stimulating of p53 Protein 2 (ASPP2), is a cytoplasmatic protein that belongs to a family of proteins that interact with the p53 protein (ASPP family) and is encoded by a gene located at chromosome 1.

A partial C-terminal clone of TP53BP2 was first identified in 1994 via a yeast two-hybrid screen using p53 as bait. It was named “p53 binding protein 2” (53BP2), a 528 residues protein that was able to bind to the wild type p53 core domain (Iwabuchi *et al.*, 1994). Shortly thereafter, in 1996, Naumovski and Cleary identified a second 1005

residues isoform, termed BBP (Bcl-2 binding protein), via yeast two-hybrid screen using Bcl-2 as bait. They also concluded that 53BP2 was a partial clone of BBP (Naumovski and Cleary, 1996). Later it was shown that BBP was an isoform of a larger protein called ASPP2 (Samuels-Lev *et al.*, 2001).

TP53BP2 is composed of 1128 amino acids and several domains, specifically ubiquitin-like domain (Ubl), α-helical domain, proline-rich domain (Pro), ankyrin repeats (Ank) and a Src homology 3 domain (SH3), as illustrated in figure 6 (Rotem *et al.*, 2008).

TP53BP2 is subjected to post-translational modifications such as phosphorylation, having multiple phosphorylation sites (Figure 7).

Depending on its interactions, ASPP2 has multiple functions (Table 1).

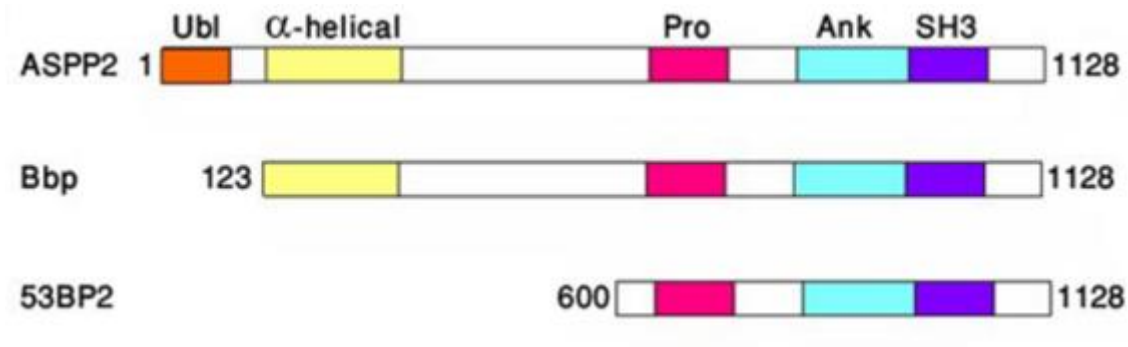


Figure 6 - ASPP2 and its truncated forms. (Taken from Rotem et al., 2008).

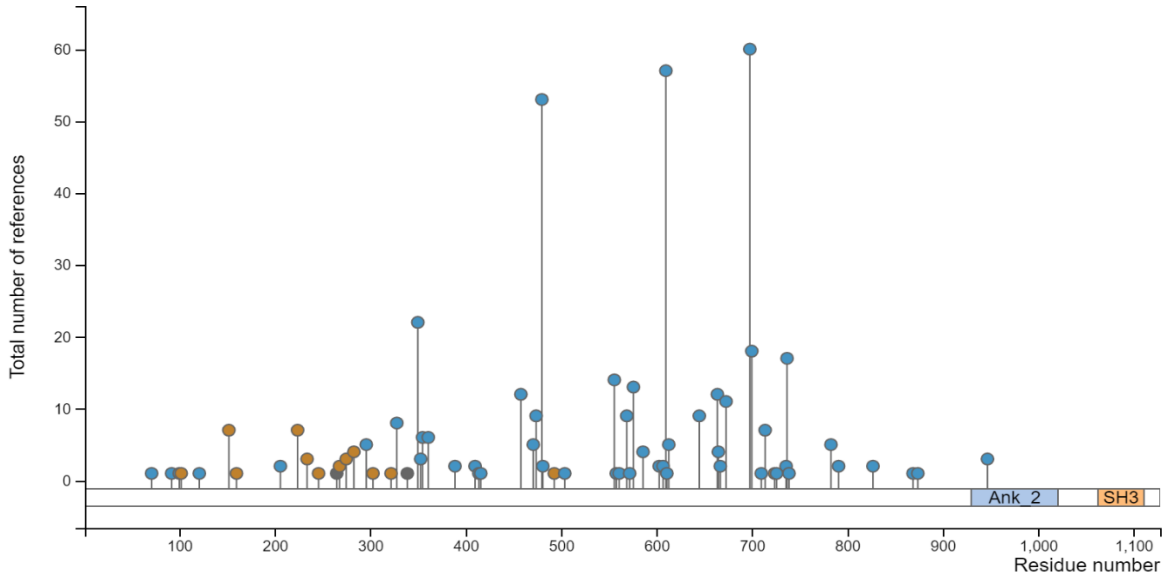


Figure 7 - Post-translational modifications in TP53BP2. Blue circles: phosphorylation sites; Orange circles: ubiquitylation sites; Grey circles: other post-translational modification sites. (Taken from Phosphosite).

Table 1 - TP53BP2-binding proteins and the putative functions of these interactions.

Interactor	*Functions*	*References*
p53 family members	Stimulates apoptotic function	Samuels-Lev *et al.*, 2001; Bergamaschi *et al.*, 2004
PP1	Modulates the specificity of PP1	Helps *et al.*, 1995
	Promotes TAZ and YAP dephosphorylation via recruitment of PP1	Liu *et al.*, 2011; Royer *et al.*, 2014
Bcl-2 family members	Impedes cell cycle progression; Induces mitochondrial-mediated apoptosis	Naumovski and Cleary, 1996; Katz *et al.*, 2008
APP-BP1	Inhibits neddylation pathway and decreases APP-BP1-induced neuronal apoptosis	Chen *et al.*, 2003
PAR-3	Regulates epithelial cell polarity	Cong *et al.*, 2010
NFκB subunit p65	Inhibits TP53BP2-mediated apoptosis	Yang *et al.*, 1999
HCV core protein	Inhibits p53-mediated apoptosis	Cao *et al.*, 2004
IRS-1	Modulates insulin signaling mediated by IRSs	Hakuno *et al.*, 2007
Ddx42p	Inhibits TP53BP2-mediated apoptosis	Uhlmann-Schiffler, Kiermayer and Stahl, 2009
DDA3	Inhibits the stimulation of p53-mediated BAX activation	Sun *et al.*, 2008
APCL	Regulates the cytoplasmic location of TP53BP2	Nakagawa *et al.*, 2000
Ras-GTP	Stimulates Ras-induced senescence	Wang *et al.*, 2013

In essence, TP53BP2 presents itself as a good candidate for a tri-complex involving both PP1 and APP. In fact, in independent interactome data it has been reported to bind to PP1 or APP. This is discussed later.

1.6. Growth Factor Receptor-bound Protein 2

GRB2 (Growth factor receptor-bound protein 2) is a small, 25 kDa, ubiquitously expressed adaptor protein originally isolated in 1992, by Lowenstein and pears, through screening for epidermal growth factor receptor (EGFR) binding proteins. It is encoded by a gene located in chromosome 17 and its entire sequence, composed of 217 amino acids, comprises a single Src homology 2 (SH2) domain flanked by two Src homology 3 (SH3) domains (Figure 8). The authors, also determined that GRB2 associates with EGFRs and platelet-derived growth factor receptors (PDGFRs) in a ligand-dependent manner and, such as other SH2 domain-containing proteins, the association between GRB2 and growth factor receptors is mediated by the SH2 domain and strictly dependent upon receptor tyrosine phosphorylation (Lowenstein *et al.*, 1992).

Its two SH3 domains bind to other proteins through proline-rich regions and its SH2 domain binds tyrosine phosphorylated sequences via YxNx motif. (Mcpherson *et al.*, 1994; Chardin *et al.*, 1995). One of the functions of GRB2 is to couple tyrosine-phosphorylated proteins, through its SH2 domain, to downstream effectors, through its SH3 domains (Mcpherson *et al.*, 1994).

GRB2's most well-known role is activating the Ras signaling pathway. This is achieved since the C-terminal part of the Ras exchange factor SOS contains several proline rich motifs that bind to GRB2 with a very high affinity. This GRB2/SOS complex mediates Ras activation in response to growth factors. Then, Ras activates Raf and starts the cascade of phosphorylations leading to MAP kinase activation (Chardin *et al.*, 1995).

As an adaptor protein, GRB2 can bind with various proteins and thus present various functions. In Table 2 some of GRB2's interactors and their functions are presented.

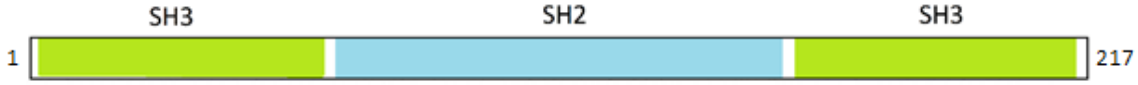


Figure 8 - Schematic representation of GRB2's domains.

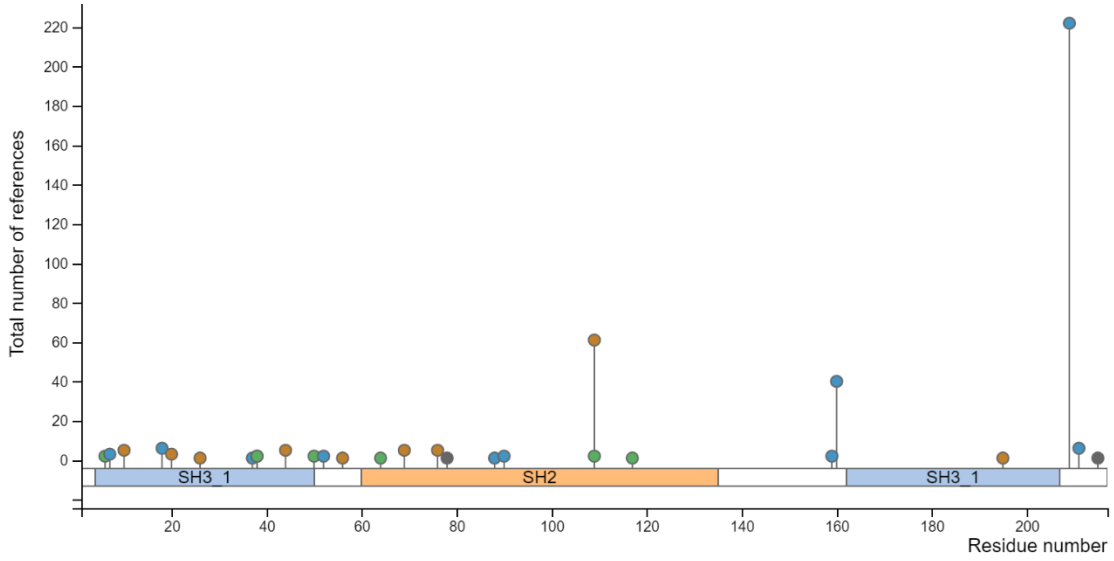


Figure 9 - Post-translational modifications in GRB2. Blue circles: phosphorylation sites; Orange circles: ubiquitylation sites; Grey circles: other post-translational modification sites. (Taken from Phosphosite).

Table 2 - GRB2-binding proteins and the putative functions of these interactions.

Interactors	*Functions*	*References*
APP	Modulates the activity of ERK1,2	(Nizzari *et al.*, 2007)
Dynamin	Stimulates dynamin GTPase activity	(Barylko *et al.*, 1998)
FAK	Mediates ERK1/2 activation through integrin signaling	(Murase, 2015)
SHC1	After insulin stimulation, SHC1 binds to GRB2 and activates Ras signaling pathway	(Skolnik *et al.*, 1993)

CBL	Mediates CBL-EGFR association	(Fukazawa *et al.*, 1996)
EGFR	Recruits SOS to the membrane in proximity to Ras, activating Ras	(Buday and Downward, 1993)

As with TP53BP2, GRB2 presents itself as a good candidate for a tri-complex involving both PP1 and APP. Similarly, in independent interactome data it has also been reported to bind to PP1 or APP. This is also discussed later.

2. Objectives of the study

Protein-protein interactions are at the root of most biological processes in both physiological and pathological conditions and are central to understanding the functional relationships between proteins. The main aim of this thesis was to validate the interaction between TP53BP2 or GRB2 with both APP and PP1, individually and simultaneously. Furthermore, the effect of Aβ on these two target proteins was also evaluated. Aβ has previously been shown to affect protein levels and to also modulate the activity of PP1.

The underlying premise is that protein complexes are critical to cellular functions and that these are modulated by protein phosphorylation; subsequently impacting APP processing and thus of relevance to Alzheimer's disease.

The objectives of this work were to:

- Use an 'in silico' approach to identify key protein potentially involved in APP and PP1 containing tri-complexes;
- Study the effect of Aβ on the expression levels of TP53BP2 and GRB2;
- Validate the interaction of the two candidate proteins with PP1 and APP independently;
- Test for the existence of the tri-complexes APP:TP53BP2:PP1 and APP:GRB2:PP1;
- Determine if the proteins under study co-localized within the cell.

3. Material and Methods

3.1. Data Mining Towards Identifying Key Interacting Proteins

In order to identify key APP and PP1 interacting proteins on which to focus the studies, an “in silico” and data mining approach was used. The aim was to identify key proteins to be subsequently addressed in this study.

Using the IntAct Molecular Interaction Database the interactome for APP, PP1CA and PP1CC were recovered. Interactomes resulting from the IntAct data mining were given the designation “IntAct”. Thus, the following interactomes were recovered: APP IntAct, PP1CA IntAct and PP1CC IntAct.

Additionally, previous data from our Neuroscience and Signalling research group on yeast two hybrid (YTH) screens was also incorporated into the search for the identification of key proteins. For APP, its interactome was denoted APP YTH and for PP1CA, PP1CA YTH. For PP1CC two data lots were used from YTH screens, specifically the two alternatively spliced isoforms that have been identified, and the interactome for each using the YTH screen were retrieved. These are PP1CC isoform 1 (PP1g1) and PP1CC isoform 2 (PP1g2).

Given the relevance of protein phosphorylation to the formation of protein complexes, data was obtained from phosphorylation state dependent YTH screens.

In essence, using phosphorylation state specific baits for the YTH screens, phosphorylation state specific interactomes were obtained. In other words, APP was mutated on residue Y687 to an F. This means that APP cannot be phosphorylated at this residue thus we have a constitutive dephosphorylated APP at residue 687. Therefore, a phosphorylation state specific interactome was obtained, APP Y687F (unpublished data).

All the interactomes obtained were pooled and analyzed via Cytoscape and are presented in the results section.

3.2. Protein Research

In efforts to have a better understanding of both target proteins, TP53BP2 and GRB2, a general research was performed. This included alternative names, chromosomal location, length, PP1-binding motifs, functions, protein domains and phosphorylation sites.

3.3. Growth and Maintenance of SH-SY5Y Cell Line

All the procedures performed in this work were carried out using the SH-SY5Y human neuroblastoma cell line (ATCC® CRL-2266™), a cell line initially derived from a metastatic bone tumor biopsy. This cell line is well-known and vastly used for research in the field of neuroscience.

Undifferentiated SH-SY5Y cells (ATCC® CRL-2266™) were (thawed from storage in liquid nitrogen) grown and maintained in Minimal Essential Medium (MEM)/F12 (1:1) supplemented with 10% fetal bovine serum (FBS), 45 mM sodium pyruvate and a cocktail of antibiotics and antimycotics (Gibco). All cultures were maintained in a humidified chamber at 37°C with 5% CO_2. Cells were divided or used as soon as they reached 80 to 90% confluency.

3.3.1. Aβ Treatment

To evaluate the effects of Aβ on our proteins of interest, TP53BP2 and GRB2, the SH-SY5Y cell line was incubated with 10µM of Aβ peptide.

Synthetic Aβ42 (GenicBio) in a quantity of 0.1 mg was dissolved in 221.6 µl of distilled water in order to prepare a 1 mM stock solution. Prior to cell exposure, an aggregation step was performed by incubating the peptides with phosphate buffered saline (PBS) 1x for 48 hours in a humidified chamber at 37°C with 5% CO_2, to a final concentration of 100 µM of the Aβ peptide.

SH-SY5Y cells were previously incubated and treated as described in section 3.3., washed twice with PSB 1x and then incubated in serum free MEM/F12 (1:1) medium, supplemented with 0.5 mM sodium pyruvate and a cocktail of antibiotics and antimycotics with 10 µM of Aβ42.

3.4. Sample Collection

Following the appropriate treatments, cells were collected in one of two buffers, according to the procedures they would subsequently be submitted to.

To analyze protein expression using western blot analysis, cells were collected with RIPA buffer. RIPA buffer contains detergents, such as sodium dodecyl sulfate (SDS), which are able to extract protein from a wide variety of cell types and membrane structures and keep them denatured. A cocktail of protease inhibitors and NaF and Na_3VO_4 as phosphatase inhibitors, were also included. This buffer guarantees efficient cell lysis and protein solubilization while also minimizing non-specific protein-binding interactions to keep background low. The lysates were posteriorly sonicated thrice for 5 seconds and then stored at -20°C.

To analyze protein interactions through co-immunoprecipitation (Co-IP), cells were collected using lysis buffer (50 mM Tris-HCL, 120 mM NaCl, 4% CHAPS) containing protease inhibitors (cOmpleteTM EDTA-free, Roche) and phosphatase inhibitors (PMSF). The inhibitors are added to prevent degradation of proteins of interest and inactivate endogenous proteolytic and phospholytic enzymes released upon cell lysis.

3.5. Protein Content Determination

For protein content quantification, the Pierce's bicinchoninic acid (BCA) protein assay kit (ThermoFisher Scientific) was used according to the manufacturer's instructions. This assay is based on the use of bicinchoninic acid (BCA) to quantify total protein. This method is based on the reduction of Cu^{2+} to Cu^{+} by cell proteins in an alkaline medium.

BCA produces a purple color in the presence of the reduced Cu^{+} ion which results from chelation of two BCA molecules with one cuprous ion. These complexes exhibit a strong absorbance that can be read at 562 nm.

The analysis was performed on a 96-well plate using standards that were prepared as described in Table 3.

Table 3 - Protein concentration standards used in the BCA assay.

Standard	BSA (µl)	1% SDS (µl)	Protein Mass (µg)
P0	0	25	0
P1	1	24	2
P2	2	23	4
P3	5	20	10
P4	10	15	20
P5	20	5	40

As for the samples, they were prepared using 5 µl of each sample plus 20 µl of 1% SDS. Subsequently, 200 µl of working reagent (50 parts of reagent A to 1 part of reagent B) was added to both samples and standards before incubating the plate for 30 minutes at 37°C. After incubation, absorbance was measured at 562 nm using a microplate reader (Infinite M200, TECAN). In order to determine protein concentration, a standard curve was calculated by plotting standard absorbance against standard BSA concentration.

3.6. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a technique that allows for the separation and characterization of proteins and peptides of a sample mixture in a polyacrylamide gel. In the presence of sodium dodecyl sulphate

(SDS), an anionic detergent, the enzyme molecules become fully unfolded and coated with the negatively charged surfactant.

This method is performed not only under denaturing conditions but also under reducing conditions as SDS is used in combination with a reducing agent (β-mercaptoethanol) and heat to dissociate proteins before the samples are loaded onto the gel. These reagents act by cleaving disulfide bonds between cysteine residues to disrupt the quaternary and tertiary structure of the proteins, creating linear chains of polypeptides.

In this procedure, 5-20% gradient SDS-PAGE was used. Each SDS-PAGE consists of two different gels: a stacking gel on top, were the samples are loaded (lower acrylamide concentration) and a resolving gel on the bottom, were the proteins from each sample are separated (higher acrylamide concentration).

Before loading the samples onto the gel, these were mixed with a loading buffer containing glycerol, SDS, β-mercaptoethanol and bromophenol blue and heated for 10 minutes to guarantee protein denaturation. Glycerol increases the samples' density which helps the sample stay in the well; SDS coats the sample proteins with a negative charge; β-mercaptoethanol breaks the disulfide bonds of the tertiary and quaternary protein structures; and the bromophenol blue is a dye used to help track the progression of the sample in the gel.

As standard molecular weight markers, Precision Plus Protein Standards Dual Color (BioRad) was used.

After loading the samples, electric current was applied to the system for approximately 3 to 4 hours at 90 mA, enabling protein separation. Following separation, the proteins were immobilized by being transferred, or "blotted", onto a solid support matrix, a nitrocellulose membrane, for subsequent antibody detection.

3.7. Western Blot

Western blotting, also termed immunoblotting, is a technique whereby proteins previously separated by electrophoresis are transferred and immobilized to a solid matrix

(nitrocellulose membrane) and subsequently selectively detected using specific antibodies. This procedure is regularly used in laboratories to identify a specific protein from complex biological samples.

3.7.1. Transfer

After separating proteins by SDS-PAGE, these were transferred from the gel to a nitrocellulose membrane. The gel was placed in contact with the membrane and both were sandwiched between two sheets of filter paper and two thin porous sponges and were kept in place by a plastic support. This ensemble was subsequently placed in a specialized electrophoresis tank filled with transfer buffer and electric current was applied at 200 mA for 16-18 hours.

3.7.2. Detection

Once the transfer was complete, membranes were incubated with Ponceau S staining solution for 5 minutes and then washed with distilled water to make the bands clearer. This solution rapidly stains protein bands on membranes and is used to ensure equally loaded samples and transfer efficiency. The staining solution was removed by washing the membranes with Tris Buffered Saline Tween (TBS-T) 1x. Subsequently, membranes were blocked by being incubated with 5% BSA in TBS-T 1x for 2 hours at room temperature thus blocking possible non-specific binding-sites.

After the blocking stage, membranes were washed 6 times for 10 minutes each with TBS-T 1x and incubated with a specific primary antibody overnight. Following primary antibody removal, the membranes were washed 6 times with TBS-T 1x for 10 minutes each, incubated with the respective secondary antibody for 2 hours and then subjected to further washing.

For protein detection, the membrane was then incubated with the enhanced chemiluminescence (ECL) reagent (GE Healthcare Life Sciences) and scanned in Chemidoc

XR (BioRad). The ECL reagent method relies on the oxidation of cyclic diacylhydrazides, such as luminol, by horseradish peroxidase (present in the secondary antibody) in the presence of hydrogen peroxide (H_2O_2). Immediately following the oxidation, luminol is in an excited state, which decays to the ground state by emitting light.

3.7.3. Antibodies

Antibodies were fundamental to most experiments performed in this work. They were used for western blot, co-immunoprecipitation and immunocytochemistry.

Some antibodies were produced "in house" but some were obtained commercially. All antibodies used, and their basic information, are presented in Table 4.

Table 4 - Antibodies used in Western Blots (WB) and Co-Immunoprecipitations (Co-IP).

Primary Antibody	Type and dilution	Secondary Antibody
Anti-APP (6E10) (Sigma)	Mouse, monoclonal **Co-IP**: 5 µL/1000 µg	-
Anti-APP (22C11) (Chemicon)	Mouse, monoclonal **WB**: 1 µL:250 µL **Co-IP**: 5 µL/1000 µg	Anti-mouse IgG, Horseradish Peroxidase-linked Antibody (Cell Signaling) 1 µL:5000 µL
Anti-GRB2 (BD Transduction laboratories)	Mouse, monoclonal **WB**: 1 µL:5000 µL	Anti-mouse IgG, Horseradish Peroxidase-linked Antibody (Cell Signaling) 1 µL:5000 µL
Anti-TP53BP2 (ThermoFisher Scientific)	Rabbit, **WB**: 1 µL:1000 µL	Anti-rabbit IgG, Horseradish Peroxidase-linked Antibody (Cell Signaling) 1 µL:5000 µL
Anti-PP1 (In house)	Rabbit, polyclonal **WB**: 1 µL:5000 µL **Co-IP**: 5 µL/1000 µg	Anti-rabbit IgG, Horseradish Peroxidase-linked Antibody (Cell Signaling) 1 µL:5000 µL
Anti-PP1γ (In house)	Rabbit, polyclonal **Co-IP**: 5 µL/1000 µg	-

3.8. Co-Immunoprecipitation

Co-Immunoprecipitation (Co-IP) is a technique that was developed from the immunoprecipitation technique with which it shares the basic principle of specific antigen-antibody reaction.

Immunoprecipitation (IP) is used to isolate individual proteins. By using an antibody that is specific for a certain protein, this protein could be isolated from a complex biological sample. As for Co-IP, this method is used to immunoprecipitate protein complexes out of a sample therefore making it possible to identify unknown protein-protein interactions. This is achieved by immunoprecipitating a target protein using a specific antibody which also co-precipitates any bound interacting proteins within the sample. This complex is subsequently detected by Western blot using a second antibody targeted against one of the bound interacting proteins.

The Co-IP was performed using Dynabeads Protein G (Invitrogen) as the solid support where the antibodies, and therefore the complexes, will be bound.

The cells used in this procedure were maintained as described in section 3.3., washed once with PBS 1x and collected using 1 ml of lysis buffer (50 mM Tris-HCL, 120 mM NaCl, 4% CHAPS) containing a cocktail of protease inhibitors and phosphatase inhibitors (PMSF).

To prepare the Dynabeads, these were transferred to a microtube, placed in the Dynal magnet to remove its conservation solution and washed thrice with washing solution (3% BSA/PBS 1x) for 10 minutes each at 4°C with agitation.

The lysates were subjected to BCA protein quantification (as described in section 3.5.) and normalized lysates were then pre-cleared by being incubated with 10 µl of Dynabeads for 1 hour at 4°C with agitation. This step is important as it allows for the removal of proteins to which the Dynabeads bind in a non-specific manner.

To bind the antibody to the Dynabeads, 40 µl of beads and the specific quantity of antibody were added to 400 µl of washing solution and incubated for 2-3 hours at 4°C with agitation. Next, the microtube was placed in the Dynal magnet to remove the

supernatant and the lysates subjected to pre-clearance were added to the antibody-bound beads and left to incubate overnight at 4°C with agitation.

The next day, the supernatant was removed and kept for posterior SDS-PAGE analysis as the presence of our target protein can indicate a non-successful Co-IP. The beads were then washed once with washing solution, thrice with PBS 1x (for 10 minutes each at 4°C with agitation) and transferred to a new microtube where they were resuspended in Loading Buffer 1x (LB 1x) and heated at 90°C for 10 minutes to separate the beads from the protein complex.

Finally, the microtubes were placed in the Dynal magnet and the supernatant, containing the protein complex, was kept for subsequent SDS-PAGE analysis.

3.9. Immunocytochemistry

Immunocytochemistry is a scientific method that relies on an antibody's power to specifically bind to a certain protein to microscopically locate and identify proteins in cells and tissues. The antibody (generally termed primary antibody) binds to a specific protein and is then visualized by being linked to a reporter, which is usually a fluorophore or an enzyme (generally termed secondary antibody). This reporter generates a signal that can be detected using an appropriate microscope.

In this work, immunocytochemistry was performed to visualize the location of TP53BP2, GRB2, APP and PP1 in the cell in order to support results obtained from the co-Immunoprecipitations.

The cells were grown and maintained as described in section 3.3. and then plated onto glass coverslips for 48 hours. They were then fixed using a solution of 4% paraformaldehyde in PBS 1x (pH 7.4) for 10 minutes at room temperature and subsequently washed thrice with cold PBS 1x.

As our target proteins are intracellular, the cells had to be permeabilized. In order to do so, the cells were incubated for 10 minutes with PBS 1x containing 0.1% Triton, a detergent that improves the penetration of the antibody, and washed three times with cold PBS 1x.

For the blocking stage, the cells were incubated with 3% BSA in PBS 1x for 30 minutes and incubated with the primary antibody (antibody in 3% BSA in PBS 1x) for 1 hour at room temperature before being washed thrice with PBS 1x for 5 minutes each.

Next, the samples were incubated with the secondary antibody (antibody in 3% BSA in PBS 1x) for 1 hour at room temperature in the dark and washed thrice with PBS 1x for 5 minutes each, also in the dark.

Lastly, the coverslips were mounted using a drop of mounting medium (Vectashield Mounting Medium with DAPI) and sealed using a regular nail polish.

Table 5 - Antibodies used in immunocytochemistry.

Target	Primary Antibody	Secondary Antibody
APP	Anti-APP (22C11) (Chemicon), mouse, monoclonal 1 µL:200 µL	Alexa Fluor™ 488 1 µL:300 µL
PP1	Anti-PP1 (in house), rabbit, polyclonal 1 µL:200 µL	Texas Red 1 µL:300 µL
TP53BP2	Anti-TP53BP2 (ThermoFisher Scientific), rabbit, polyclonal 1 µL:200 µL	Texas Red 1 µL:300 µL
GRB2	Anti-GRB2 (BD Transduction laboratories), mouse, monoclonal 1 µL:200 µL	Alexa Fluor™ 488 1 µL:300 µL

3.10. Statistical Analysis

The results from the Aβ treatment were analyzed on GraphPad using Student's t-test with Welch's correction.

4. Results and Discussion

4.1. APP and PP1 interactome networks

"Omics" analysis was carried out in order to identify key interacting proteins that bind APP and PP1. The approach used was as described in the methods and the results obtained are presented below.

The number of interacting proteins for all the data mining exercise are shown in Table 6. Of note, PP1 has far more interacting proteins than APP. This reflects its involvement in a wide array of functions.

Table 6 - List of number of interacting proteins.

Screen	Number of Protein Interactors	Network
APP IntAct	88	Figure 10A
APP YTH	21	
APP Y687F	10	
PP1CA IntAct	351	Figure 10B
PP1CA YTH	246	
PP1CC IntAct	143	Figure 10C
PP1g1 YTH	52	
PP1g2 YTH	142	
All above pooled	811	Figure 11

The interactomes (APP IntAct, PP1CA IntAct and PP1CC IntAct) from the IntAct database were merged with the YTH data for each of the proteins individually (Figure 10A, 10B and 10C). This data, as mentioned in section 3.1., was acquired in our laboratory

using the YTH system and were designated APP YTH, PP1CA YTH, PP1g1 YTH and PP1g2 YTH, as well as the phosphorylation state specific network for APP, APP Y687F.

Of note for APP (Figure 10A), APBB1 (FE65) was the only interactor common to both the IntAct database and the YTH for APP Y687F (blue node in figure 10A).

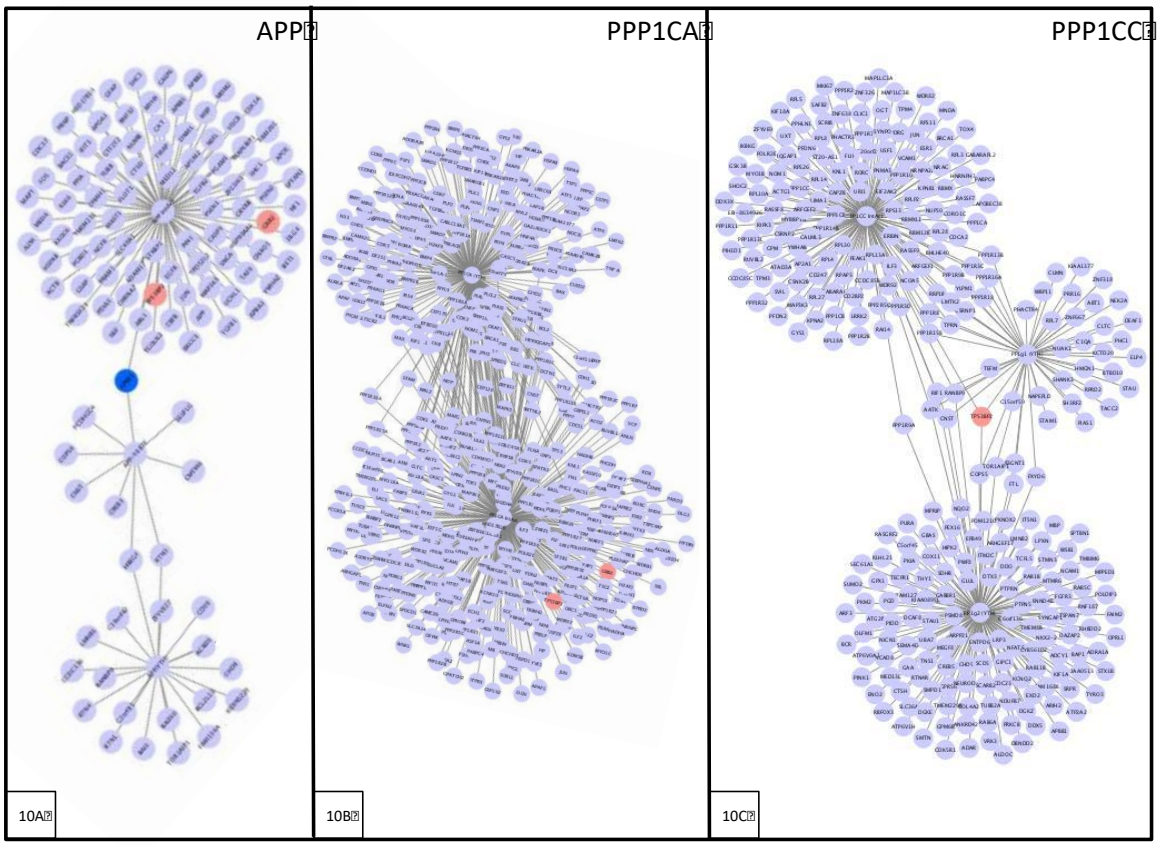


Figure 10 - Networks of pooled interactomes for APP, PPP1CA and PPP1CC. A: Data were pooled from APP IntAct, APP YTH and APP Y687F; B: Data were pooled from PPP1CA IntAct and PPP1CA YTH; C: Data were pooled from PPP1CC IntAct, PP1g1 YTH and PP1g2 YTH. The dark blue node corresponds to APBB1 (FE65) and the red nodes correspond to TP53BP2 and GRB2.

The red nodes in Figure 10 denote the two proteins subsequently studied, TP53BP2 and GRB2. According to the data mining exercise both proteins bind to APP and PP1CA but for PPP1CC only TP53BP2 is reported to bind.

Subsequently all the networks in Figure 10 were merged and are presented in Figure 11.

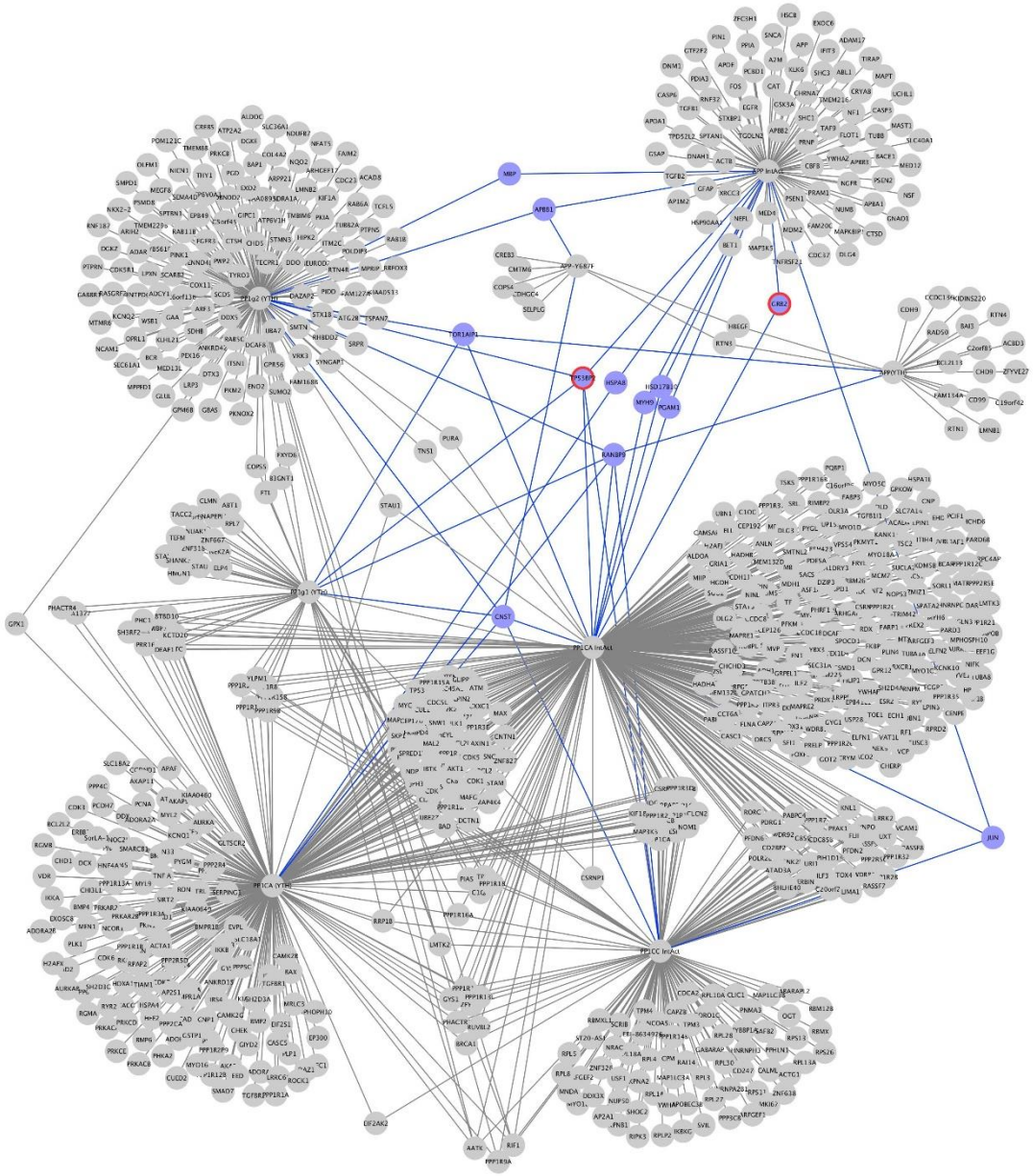


Figure 11 - Merged networks of pooled interactomes of APP, PP1CA and PP1CC. The 12 nodes of interest (see below) are shown in blue, TP53BP2 and GRB2 are identified with a red circumference. Edges of the 12 nodes are also marked in blue to facilitate the identification of interacting proteins/genes.

Considering all the data presented above, 12 proteins came up in the various screens (Table 7) and these can be considered as potential key bridging proteins between APP and PP1. Among them are the proteins that were the focus of this work, TP53BP2 and GRB2, which were chosen among the 12 proteins due to being known proteins for

which some information is already available. The first three proteins, APBB1 (FE65), RANBP9 and TOR1AIP1 have already been studied by the Neuroscience and Signalling group and the data has already been published (Rebelo *et al.*, 2013; Santos *et al.*, 2013; Domingues *et al.*, 2014).

Table 7 - List of the 12 proteins of interest.

	APP IntAct	APP YTH	APP Y687F	PP1CA IntAct	PP1CC IntAct	PP1CA YTH	PP1g1 YTH	PP1g2 YTH
APBB1	✔		✔					✔
RANBP9		✔		✔	✔	✔	✔	✔
TOR1AIP1		✔		✔			✔	✔
CNST			✔	✔	✔	✔	✔	✔
HSD17B10	✔			✔				
HSPA8	✔					✔		
JUN	✔			✔	✔			
MBP	✔							✔
MYH9	✔			✔				
PGAM1	✔			✔				
GRB2	✔			✔				
TP53BP2	✔			✔	✔		✔	✔

4.2. Protein Research

As an initial step in this work, in order to better understand the two target proteins, a basic protein research was conducted and information attained is presented below (Tables 8 and 9).

Most of the information was collected from UniProt and the NCBI gene portal, with the exception of the phosphorylation sites, which were gathered from PhosphoSitePlus (www.phosphosite.org), an open, curated and interactive resource for studying experimentally observed post-translational modifications.

Table 8 - Basic information about TP53BP2.

Protein name	Apoptosis-stimulating of p53 protein 2 / Tumor protein p53 binding protein 2
Official symbol	ASPP2 / TP53BP2
UniProt accession number	Q13625
Alternative names	Bcl-binding protein (BBP)
Chromosomal location	1q41
Exon count	20
Gene type	Protein coding
Length	1128 amino acids
Protein Domains (N- to C-terminal)	Ubiquitin-like domain (Ubl) α-helical domain Proline-rich domain (Pro) Ankyrin repeats (Ank) Src homology 3 domain (SH3)
Main function	Regulator that plays a central role in regulation of apoptosis and cell growth via its interactions
PP1-binding motif	RVKF (from amino acid 921 to 924)
Protein phosphorylation sites	**Serines**: 71, 92, 100, 121, 206, 296, 328, 353, 361, 414, 416, 458, 471, 480, 481, 556, 558, 561, 569, 572, 576, 607, 611, 665, 667, 673, 698, 714, 726, 736, 737, 783, 827 **Threonines**: 355, 389, 474, 504, 586, 603, 700, 739 **Tyrosines**: 350, 610, 613, 645, 664, 710, 791, 869, 874, 947

Table 9 - Basic information about GRB2

Protein name	Growth factor receptor-bound protein 2
Official symbol	GRB2
UniProt accession number	P62993
Alternative names	Adapter protein GRB2 Protein Ash ASH SH2/SH3 adapter GRB2
Chromosomal location	17q25.1
Exon count	6
Gene type	Protein coding
Length	217 amino acids
Protein Domains (N- to C-terminal)	Src Homology 3 Domain (SH3) Src Homology 2 Domain (SH2) Src Homology 3 Domain (SH3)
Main function	Adapter protein that provides a link between cell surface growth factor receptors and the Ras signaling pathway
PP1-binding motif	LSVKF (from amino acid 97 to 101)
Protein phosphorylation sites	**Serines**: 18, 88, 90 **Threonines**: 159, 211 **Tyrosines**: 7, 37, 52, 160, 209

4.3. Aβ effect

Aβ is a peptide that, in normal physiological conditions, can be beneficial by enhancing long term potentiation and memory, however, in pathological conditions, can

deploy its toxic effects and contribute significantly to neuronal damage, as that seen in AD (Carrillo-Mora, Luna and Colín-Barenque, 2014).

Both proteins, TP53BP2 and GRB2, are expressed in the brain and since there are no studies on the Aβ effect on their expression levels, this was addressed in this work. SH-SY5Y cells were treated with previously aggregated Aβ at a concentration of 10µM for 24 hours in order to assess whether this neuropeptide influenced their expression level. After treatment, cells were lysed and analyzed by western blot using the appropriate antibodies.

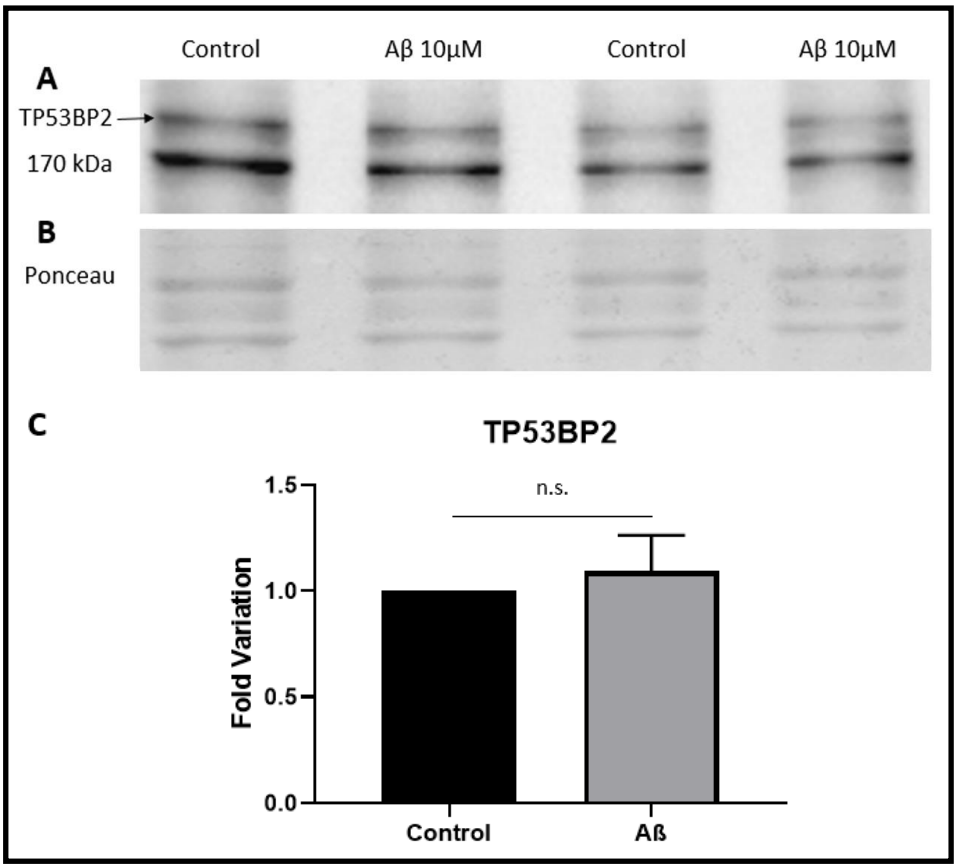


Figure 12 - TP53BP2 expression in SH-SY5Y cells treated with Aβ42. A. Western blot analysis of SH-SY5Y lysates treated with Aβ42. B. Loading control, Ponceau Staining. C. Comparison of TP53BP2 expression levels in cells. All data was normalized to Ponceau levels prior to analysis. Data was obtained from triplicate experiments (n=3). n.s., no significant difference, P>0,05.

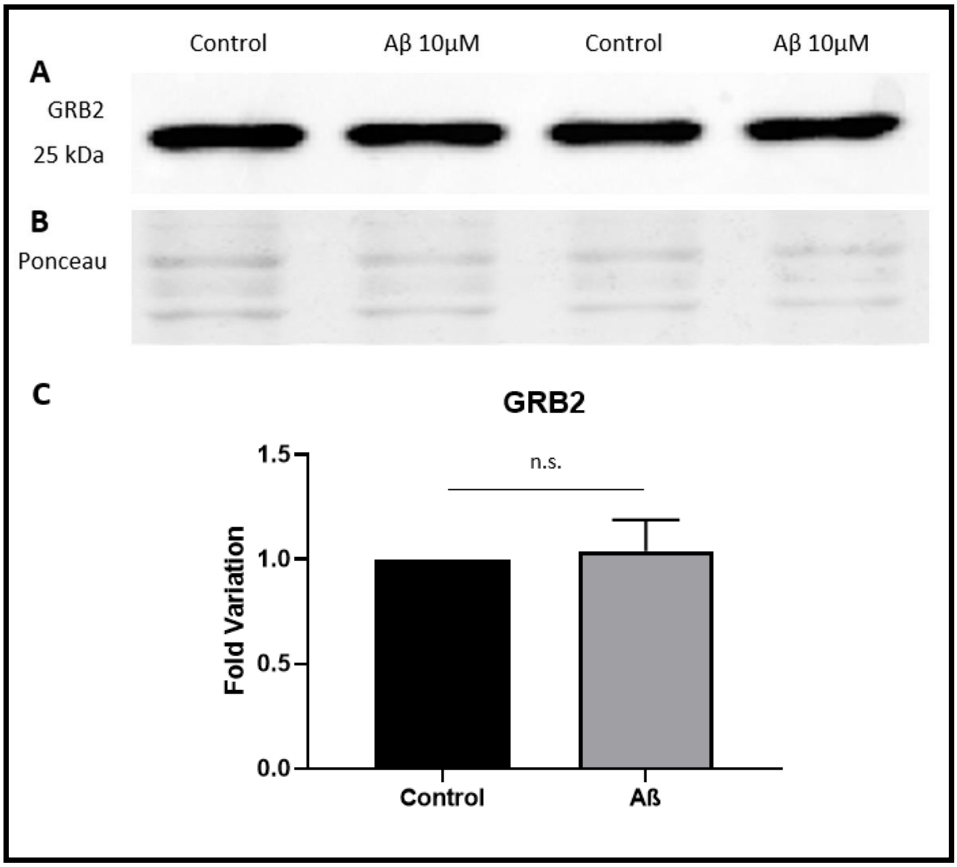


Figure 13 - GRB2 expression in SH-SY5Y cells treated with Aβ42. A. Western blot analysis of SH-SY5Y lysates treated with Aβ42. B. Loading control, Ponceau Staining. C. Comparison of GRB2 expression levels in cells. All data was normalized to Ponceau levels prior to analysis. Data was obtained from triplicate experiments (n=3). n.s., no significant difference, P>0,05.

The treatment of cells with previously aggregated Aβ is used to mimic the pathological conditions observed in the brains of patients with AD. Cell exposure to Aβ as an AD model system is not a novel practice and has been previously carried out in other studies (Zheng *et al.*, 2014; Oliveira *et al.*, 2015).

Concerning the results obtained for both proteins, Figures 12 and 13 represent triplicate experiments and their data was analyzed on GraphPad using Student's t-test with Welch's correction. After analyzing the results, it appears that no significant difference was observed between the control cells and those treated with Aβ, meaning the neuropeptide seems to not have an influence on our target proteins' expression levels.

4.4. Formation of complexes

As mentioned above, protein interactions drive cellular functions. Thus, in this part of the work it is important to validate the interactions reported in the literature.

Thus, a relevant step in this work was to assess the existence of the tri-complexes APP:TP53BP2:PP1 and APP:GRB2:PP1 via co-Immunoprecipitations. Since neither TP53BP2 nor GRB2 appeared to be affected by the treatment with Aβ, all cells used for the co-Immunoprecipitation procedures were maintained under basal conditions with no specific Aβ treatment.

4.4.1. TP53BP2

As aforementioned, the TP53BP2 protein showed up as a PP1-binding protein and APP-binding protein in the IntAct database, as well as a PP1-binding protein using the YTH system 'in house'. Thus, it was possible to hypothesize that TP52BP2 is a potential bridging protein, and that the APP:TP53BP2:PP1 tri-complex could be formed.

In order to verify if TP53BP2 interacts directly with both APP and PP1 we performed two different sets of co-Immunoprecipitations (Co-IPs), one where APP immunoprecipitated and another immunoprecipitating PP1 (PP1 and PP1γ). It was then tested if TP53BP2 did in fact co-immunoprecipitate in both cases. Only if these two Co-IPs were positive would further procedures be carried out to validate the tri-complex.

Firstly, to validate the interactions with PP1, a co-Immunoprecipitation was conducted by using anti-PP1 and anti-PP1γ antibodies to immunoprecipitate PP1. Results are presented in Figure 14. It is important to refer that the antibodies used for this experiment were, as mentioned, the PP1 and PP1γ antibodies. The PP1 antibody binds to both PP1α and PP1γ, therefore it represents the expression of total PP1 levels. The PP1γ antibody is specific for that isoform.

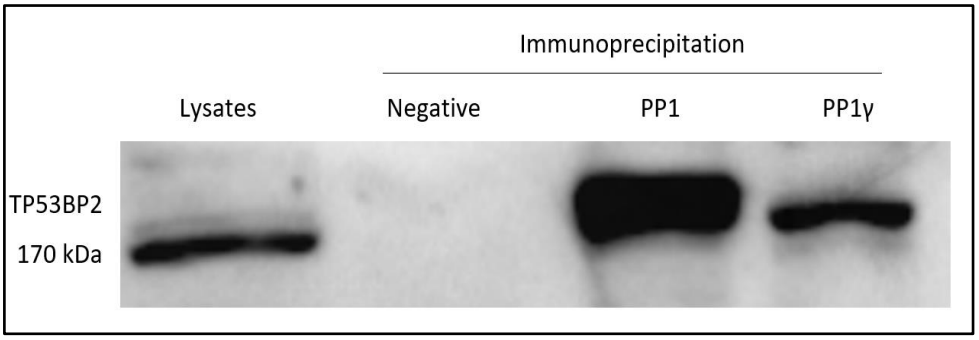


Figure 14 - Immunoprecipitation of PP1 and PP1γ to monitor if TP53BP2 is a binding protein. PP1 and PP1γ were immunoprecipitated and binding proteins were detected by western blot analysis, using anti-TP53BP2 antibody. Lysates, cell lysates not subjected to immunoprecipitation; Negative, Dynabeads control without either PP1 antibodies; PP1, cells immunoprecipitated with anti-PP1 antibody; PP1γ, cells immunoprecipitated with anti-PP1γ antibody. This result illustrates duplicate experiments (n=2).

The results obtained from the PP1 Co-Immunoprecipitation show that TP53BP2 binds to both PP1α and PP1γ (Figure 14). This is relevant because it shows that there is no preference for the different PP1 isoforms used in this study.

Helps et al. (1995), in an attempt to identify important novel regulatory subunits of PP1, were the first to report this interaction. By performing a yeast two hybrid screen using the catalytic subunit of PP1γ as bait, they managed to identify TP53BP2 as a PP1-binding protein. They also reported that TP53BP2 modulated PP1's specificity by inhibiting it's activity towards certain substrates (Helps *et al.*, 1995). Shortly afterwards it was shown that the TP53BP2:PP1 interaction occurred via TP53BP2's RVXF motif (Egloff *et al.*, 1997). Since then several other studies have linked TP53BP2 and PP1 (Liu *et al.*, 2011; Esteves *et al.*, 2013).

The procedure was likewise carried out to verify the interaction with APP by using an anti-APP antibody to immunoprecipitate APP. Results are shown in Figure 15.

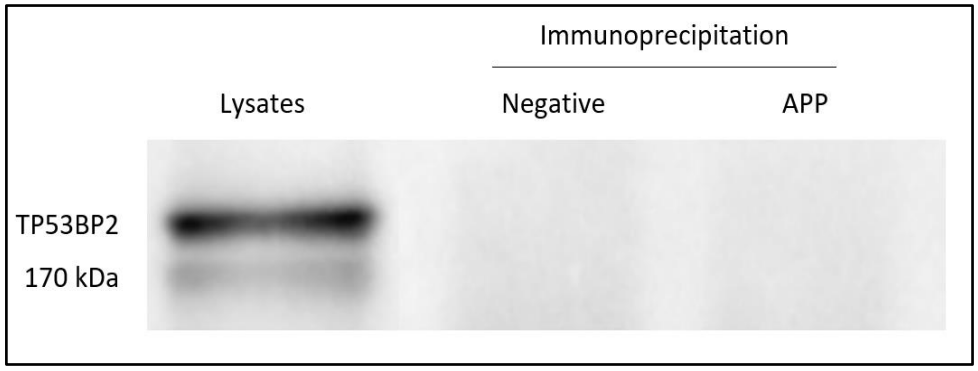


Figure 15 - Immunoprecipitation APP and detection for TP53BP2. APP binding proteins were not detected by western blot analysis, using anti-TP53BP2 antibody. Lysates, cell lysates not subjected to immunoprecipitation; Negative, Dynabeads control without APP antibody; APP, cells immunoprecipitated with anti-APP antibody. This result illustrates duplicate experiments (n=2).

Considering the results obtained from the APP co-Immunoprecipitation, no interaction between APP and TP53BP2 is detected (Figure 15).

As above-mentioned, an interaction between APP and TP53BP2 showed up in the IntAct database, yet this interaction relied solely on one publication. This paper describes the identification of a novel Aβ-binding protein, termed BBP (β-amyloid Binding Protein) (Kajkowski *et al.*, 2001). However, after extensive reading, it is of my understanding that the protein identified, and named BBP, isn't in fact TP53BP2. Perhaps some confusion occurred due to the fact that TP53BP2 can also be called BBP after it's identification as a Bcl-2 Binding Protein.

To date, no other studies have been published that explore the direct interaction between TP53BP2 and APP. However, studies have shown that TP53BP2 interacts directly with the APP-binding protein 1 (APP-BP1) to modulate the neddylation pathway and decrease APP-BP1-mediated apoptosis (Chen, Liu, Naumovski, *et al.*, 2003). It would be interesting to further investigate whether TP53BP2, through its connection with APP-BP1, interacts with APP.

4.4.2. GRB2

Similarly to TP53BP2, GRB2 was also considered as a potential bridging protein in the tri-complex involving APP and PP1, after considering results from the YTH assays and the search of the IntAct database.

To assess the existence of the APP:GRB2:PP1 tri-complex, the same protocol as the one used for TP53BP2 was performed. Thus, two separate co-Immunoprecipitations were carried out and analyzed.

In the first place, in an effort to validate the GRB2:PP1 interaction, a co-Immunoprecipitation was performed by using anti-PP1 and anti-PP1γ antibodies to immunoprecipitate PP1 (Figure 16). As mentioned previously, the PP1 antibody binds to both PP1α and PP1γ, as such it represents the expression of total PP1 levels.

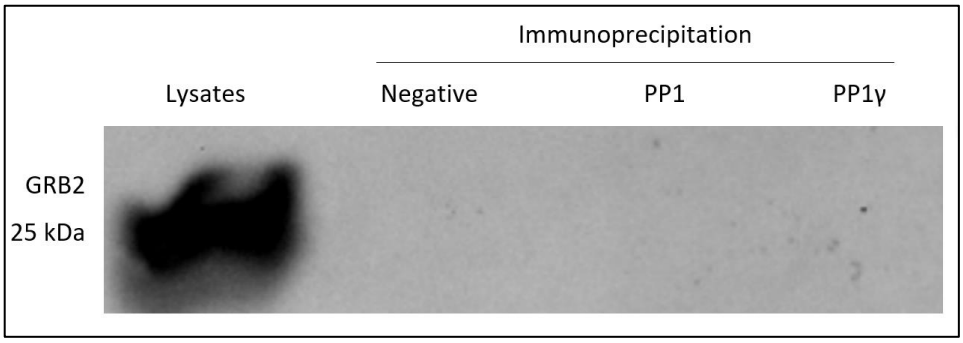


Figure 16 - Immunoprecipitation of PP1 and PP1γ and detection of GRB2 as a binding protein. PP1 and PP1γ binding proteins were not detected by western blot analysis, using anti-GRB2 antibody. Lysates, cell lysates not subjected to immunoprecipitation; Negative, Dynabeads control without either PP1 antibodies; PP1, cells immunoprecipitated with anti-PP1 antibody; PP1γ, cells immunoprecipitated with anti-PP1γ antibody. This result illustrates duplicate experiments (n=2).

As displayed in Figure 16, the co-Immunoprecipitation of PP1 and PP1γ did not reveal that GRB2 was co-immunoprecipitated, thus one is led to conclude that there is no interaction between GRB2 and PP1.

After extensive search of the published literature, one article referring a possible interaction between GRB2 and PP1 was found (Bisson *et al.*, 2011). In this article, the authors performed an affinity purification assay to isolate GRB2 interactors and mass spectrometry to identify said proteins. They managed to identify 108 GRB2-binding proteins including proteins already known to interact with GRB2 and some proteins that

were not previously identified as associating with GRB2. These novel GRB2-binding proteins included both PP1α and PP1γ with a coverage of 6 and 11%, respectively. The authors, however, did not further investigate this interaction and therefore no further information is available. One can hypothesize however, that both PP1 proteins found may not result from direct interaction with GRB2 but may have been detected because they were bound to GRB2-binding proteins.

Despite the result in the above-mentioned publication, the experiments with SH-SY5Y cells carried out during this work failed to confirm a PP1:GRB2 interaction, even if indirect.

A second set of co-Immunoprecipitations was performed in order to assess the GRB2:APP interaction by using an anti-APP antibody to immunoprecipitate APP. These results are displayed in Figure 17.

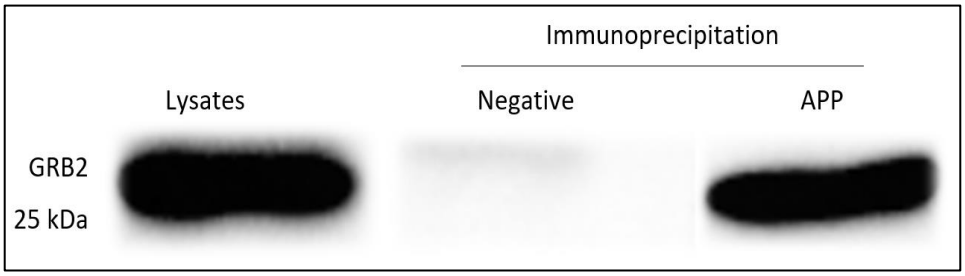


Figure 17 - Immunoprecipitation of APP and detection of GRB2 as a binding protein. APP binding proteins were detected by western blot analysis, using anti-GRB2 antibody. APP, cells immunoprecipitated with anti-APP antibody; Negative, Dynabeads control without APP antibody; Lysates, cell lysates not subjected to immunoprecipitation. This result illustrates duplicate experiments (n=2).

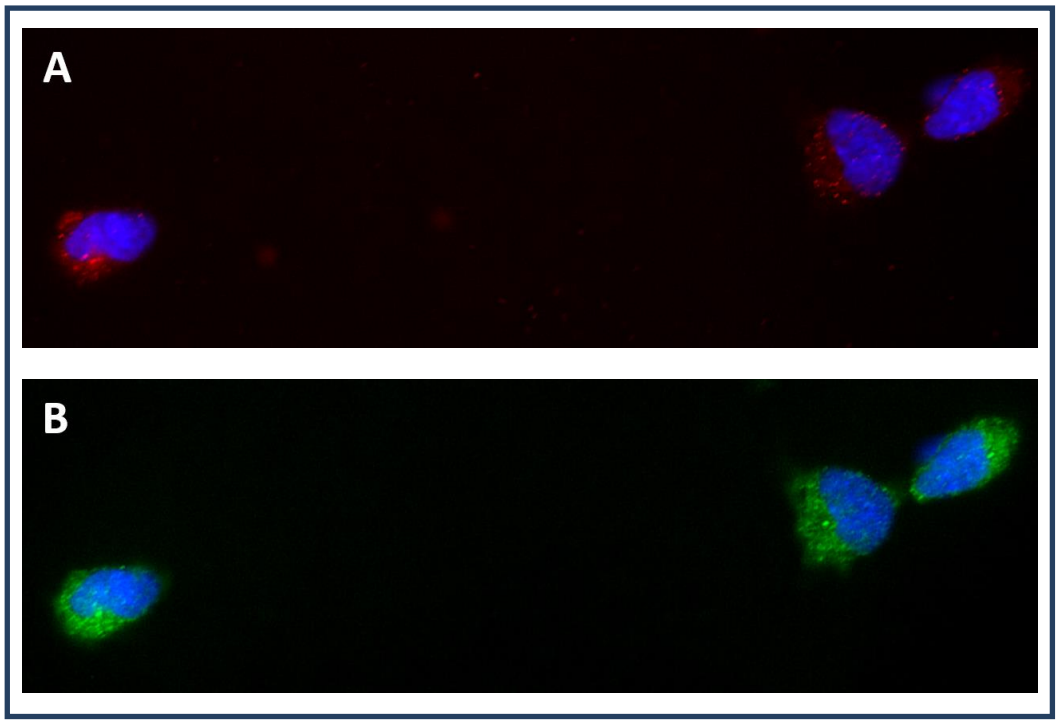
Concerning these results (Figure 17), it is clear that a direct interaction between APP and GRB2 exists. This interaction however has previously been described in the literature.

In 2004, Zhou et al. (2004) identified, through immunoprecipitations, that GRB2 directly interacts with APP and that the interaction requires phosphorylation of APP's Tyr^{682}. They also showed that GRB2 binds to the APP $^{682}Y^{P}ENPTY^{687}$ motif via its SH2 domain (Zhou *et al.*, 2004).

Later, in 2007, Nizzari et al. reported that APP bound to GRB2 in the centrosome and the interaction enhanced ERK1,2 phosphorylation (Nizzari *et al.*, 2007).

4.5. Immunocytochemistry

Regarding the co-Immunoprecipitation results, two interactions TP53BP2:PP1 and GRB2:APP were clearly evident, however two were not observed, namely the TP53BP2:APP and GRB2:PP1 interactions. In an effort to further support these findings, immunocytochemistry assays were performed on SH-SY5Y cells in order to observe if the proteins co-localized or not. Consequently, two separate immunocytochemistry assays were performed, one to simultaneously assess the localization of TP53BP2 and APP, and another to simultaneously assess the localization of GRB2 and PP1 within the cell (Figures 18 and 19). If co-localization was not apparent in either assay, this would give strength to our findings that the interactions do not occur in the tested cell line.



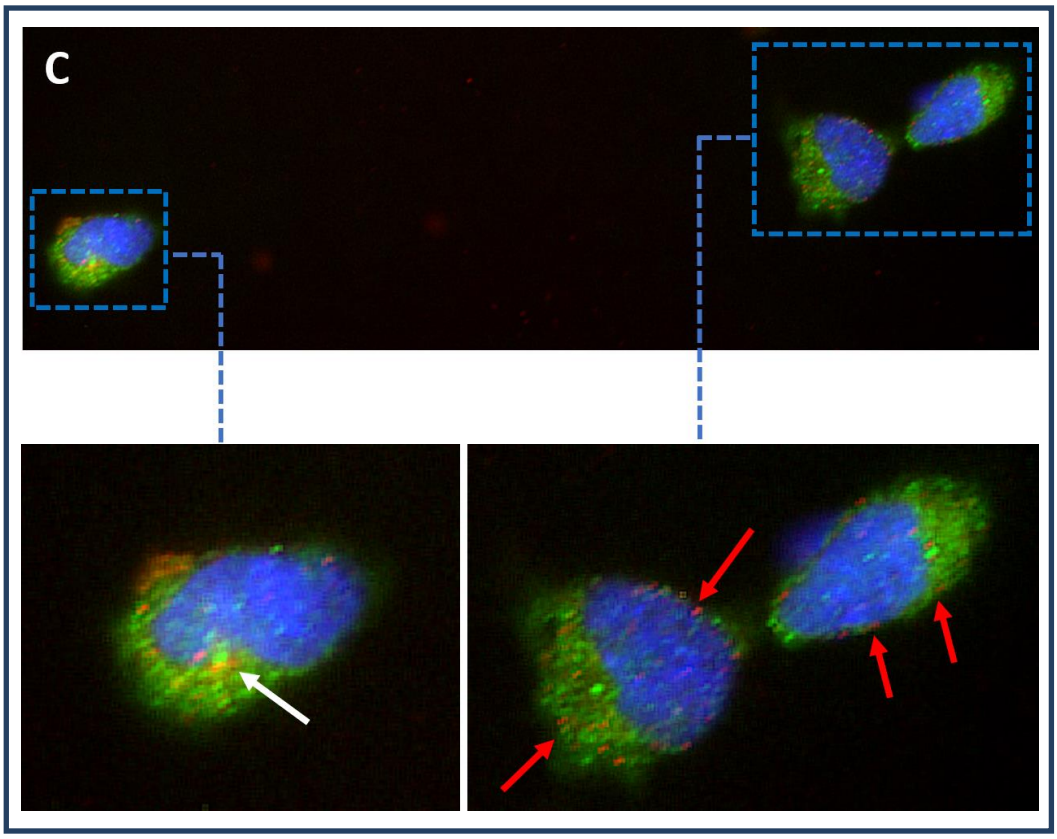


Figure 18 - Immunocytochemistry of SH-SY5Y cells. Cells stained with anti-TP53BP2 antibody (red) and anti-APP antibody (green), mounted with Vectashield Mounting Media with DAPI (blue). A. Red Staining for TP53BP2, blue staining for the nucleus; B. Green staining for APP; C. Composite image of red and green staining. White arrow represents area of potential overlap. Red Arrows represent areas staining for TP53BP2.

In order to assess relative localization of TP53BP2 and APP, control SH-SY5Y cells were incubated with anti-TP53BP2 antibody (red), anti-APP antibody (green) and mounted with Vectashield Mounting Media with DAPI (blue) (Figure 18). APP typically stains throughout the cell and stains heavily around the Golgi area. This was evident with the green staining presented in Figure 18B. From previous reports TP53BP2 appears to stain in a nuclear and perinuclear fashion. This is consistent with the work here presented, albeit the staining is not particularly intense. In global terms the red appears not to overlap with the APP staining, however some areas of yellow coloring, and potentially some degree of overlap could be detected. Whether or not this represents some co-localization deserves further studies in the future.

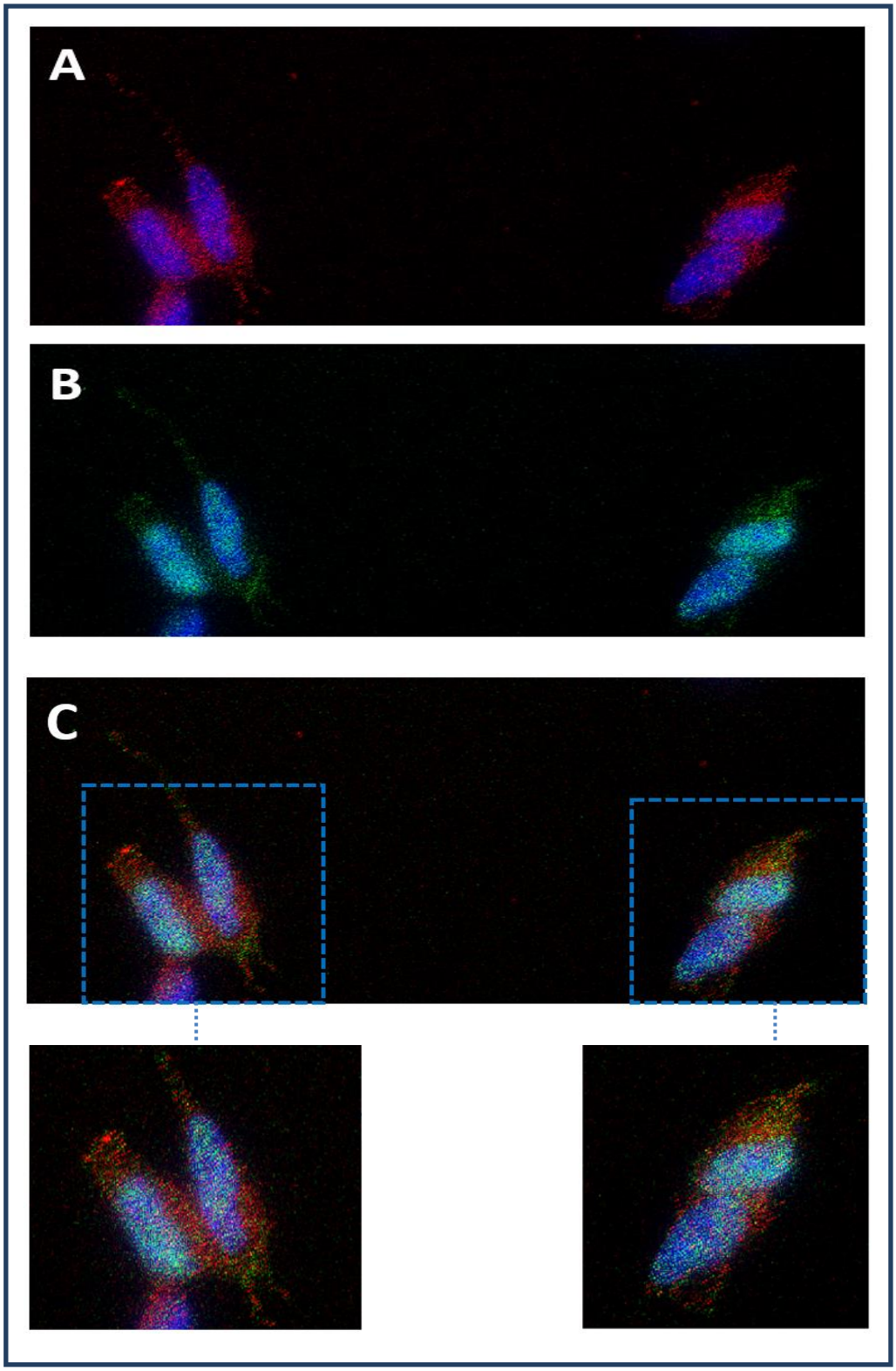


Figure 19 - Immunocytochemistry of SH-SY5Y cells. Cells stained with anti-PP1 antibody (red) and anti-GRB2 antibody (green), mounted with Vectashield Mounting Media with DAPI (blue). A. Red Staining for PP1, blue staining for the nucleus; B. Green staining for GRB2; C. Composite image of red and green staining.

To verify the if GRB2 and PP1 could potentially co-localize, control SH-SY5Y cells were incubated with anti-PP1 antibody (red), anti-GRB2 antibody (green) and mounted with Vectashield Mounting Media with DAPI (blue) (Figure 19).

Both PP1 and GRB2 have been reported to stain diffusely throughout the cell. It would appear that this pattern is also observed in the data here presented. Given the close proximity of these proteins it is difficult to assess with certainty whether co-localization exists (Figure 19C). Therefore, in order to confirm or deny co-localization, further studies and analysis are needed.

Overall it appears that the immunocytochemistry and immunoprecipitation data are consistent, although this should be further confirmed.

5. Conclusion

The ‘in silico’ approach here presented was successful in identifying key proteins that warrant further study from a signal transduction perspective. Of the 12 target proteins shortlisted, several have already been shown to form critical tri-complexes of relevance to Alzheimer’s disease, in particular FE65 (Rebelo *et al.*, 2013) and RANBP9 (Domingues *et al.*, 2014). The two proteins selected for this study were TP53BP2 and GRB2. The two were chosen given their relevance to AD and associations with protein phosphorylation systems. Of note in AD, Aβ can affect the expression and processing of certain proteins (Vintém *et al.*, 2009). Thus, the effect of this peptide on the two proteins under study was tested but neither TP53BP2’s nor GRB2’s expression levels where significantly affected by the treatment with Aβ42.

For the immunoprecipitation experiments, previously reported or suggested interactions were validated. That is, we were able to show that APP binds GRB2 and that PP1 binds TP53BP2. Although not novel, it is important to validate these findings. However, when co-Immunoprecipitations were tested for, we concluded that TP53BP2 did not appear to bind to APP. Likewise, GRB2 binds to APP, but does not appear to interact with PP1. It could be that these interactions are extremely transient and further methods must be developed to identify them. However, our conclusion at the moment with the available technology ‘in house’ is that the aforementioned complexes are not formed/detected.

As already mentioned, with respect to TP53BP2 it is evident that it clearly binds to PP1 but does not appear to bind to APP. However, one cannot dismiss that there may be an interaction via another additional bridging protein. As mentioned in the results section TP53BP2 binds APP-binding protein 1 (APP-BP1) to modulate the neddylation pathway and decrease APP-BP1-mediated apoptosis (Chen, Liu, Naumovski, *et al.*, 2003). APP-BP1, in turn binds APP. This could result in a functionally relevant complex and could explain the yellow overlapping staining observed in Figure 18C. Therefore, we hypothesize that a hitherto not described complex may exist and be important in functional terms. The

proposed complex would include the following proteins APP:APP-BP1:TP53BP2 (Figure 20).

APP-BP1 is a cell cycle protein that functions as one half of the bipartite that activates protein NEDD8. Expression of APP-BP1 in dividing cells drives the cell cycle through the S-M checkpoint and this function is mediated by the neddylation pathway (Chen *et al.*, 2000). The interaction between APP-BP1 and APP activates the neddylation pathway and induces cell cycle entry, which in neurons causes apoptosis rather than cell cycle progression (Chen, Liu, Mcphie, *et al.*, 2003). On the other hand, TP53BP2 interacts with APP-BP1 and negatively regulates the neddylation pathway which in turn protects neurons from APP-BP1-induced apoptosis (Chen, Liu, Naumovski, *et al.*, 2003). Finally, given that TP53BP2 can bind to PP1 as clearly shown in Figure 14 this could provide a means to regulate the formation of this complex via protein phosphorylation.

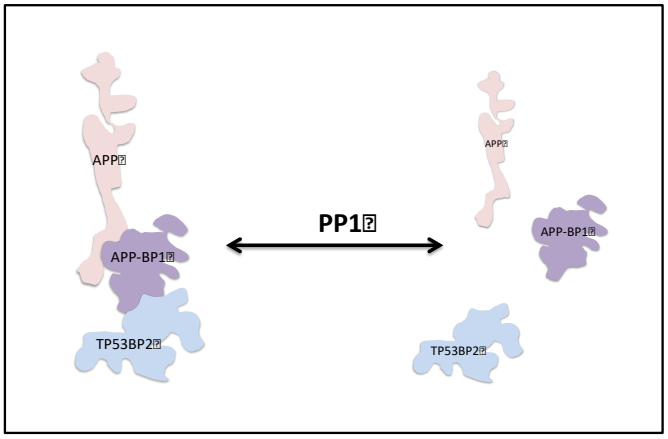


Figure 20 – Proposed complex bridging APP to TP53BP2 (APP:APP-BP1:TP53BP2). Given that TP53BP2 binds PP1, this could potentially represent a model for regulating complex formation via protein phosphorylation.

6. References

Del Alonso, A. C. *et al.* (1994) *Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease*, *National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.91.12.5562.

Alzheimer's Association (2019) *2019 Alzheimer's disease facts and figures*, *Alzheimer's & Dementia*. doi: 10.1016/j.jalz.2019.01.010.

Alzheimer's disease international (2018) *World Alzheimer Report 2018 - The state of the art of dementia research: New frontiers; World Alzheimer Report 2018 - The state of the art of dementia research: New frontiers*.

Ando, K. *et al.* (2001) 'Phosphorylation-dependent Regulation of the Interaction of Amyloid Precursor Protein with Fe65 Affects the Production of β-Amyloid', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology Inc., 276(43), pp. 40353–40361. doi: 10.1074/jbc.M104059200.

Barylko, B. *et al.* (1998) *Synergistic Activation of Dynamin GTPase by Grb2 and Phosphoinositides**. Available at: http://www.jbc.org/.

Bergamaschi, D. *et al.* (2004) 'ASPP1 and ASPP2: Common Activators of p53 Family Members', *Molecular and Cellular Biology*. American Society for Microbiology, 24(3), pp. 1341–1350. doi: 10.1128/mcb.24.3.1341-1350.2004.

Bisson, N. *et al.* (2011) 'Selected reaction monitoring mass spectrometry reveals the dynamics of signaling through the GRB2 adaptor', *Nature Biotechnology*, 29(7), pp. 653–658. doi: 10.1038/nbt.1905.

Blennow, K., de Leon, M. J. and Zetterberg, H. (2006) 'Alzheimer's disease', *The Lancet*, 368(9533), pp. 387–403. doi: 10.1016/S0140-6736(06)69113-7.

Bollen, M. (2001) 'Combinatorial control of protein phosphatase-1', *Trends in Biochemical Sciences*, pp. 426–431. doi: 10.1016/S0968-0004(01)01836-9.

Bollen, M. *et al.* (2011) 'The extended PP1 toolkit : designed to create specificity', 35(8), pp. 450–458. doi: 10.1016/j.tibs.2010.03.002.The.

Buday, L. and Downward, J. (1993) 'Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange

factor', *Cell*, 73(3), pp. 611–620. doi: 10.1016/0092-8674(93)90146-H.

Cao, X. and Südhof, T. C. (2001) 'A transcriptivety active complex of APP with Fe65 and histone acetyltransferase Tip60', *Science*, 293(5527), pp. 115–120. doi: 10.1126/science.1058783.

Cao, Y. *et al.* (2004) 'Hepatitis C virus core protein interacts with p53-binding protein, 53BP2/Bbp/ASPP2, and inhibits p53-mediated apoptosis.', *Biochemical and biophysical research communications*, 315(4), pp. 788–95. doi: 10.1016/j.bbrc.2004.01.124.

Carrillo-Mora, P., Luna, R. and Colín-Barenque, L. (2014) 'Amyloid beta: Multiple mechanisms of toxicity and only some protective effects?', *Oxidative Medicine and Cellular Longevity*, 2014. doi: 10.1155/2014/795375.

Chardin, P. *et al.* (1995) 'The Grb2 adaptor', *FEBS Letters*, 369(1), pp. 47–51. doi: 10.1016/0014-5793(95)00578-W.

Chen, Y. *et al.* (2000) *The Amyloid Precursor Protein-binding Protein APP-BP1 Drives the Cell Cycle through the S-M Checkpoint and Causes Apoptosis in Neurons*. Available at: http://www.jbc.org/ (Accessed: 7 December 2019).

Chen, Y., Liu, W., Mcphie, D. L., *et al.* (2003) 'APP-BP1 mediates APP-induced apoptosis and DNA synthesis and is increased in Alzheimer's disease brain', *The Journal of Cell Biology JCB The Journal of Cell Biology*, 163(1), pp. 27–33. doi: 10.1083/jcb.200304003.

Chen, Y., Liu, W., Naumovski, L., *et al.* (2003) 'ASPP2 inhibits APP-BP1-mediated NEDD8 conjugation to cullin-1 and decreases APP-BP1-induced cell proliferation and neuronal apoptosis', *Journal of Neurochemistry*. Blackwell Publishing Ltd., 85(3), pp. 801–809. doi: 10.1046/j.1471-4159.2003.01727.x.

Chung, S.-H. (2009) 'Aberrant phosphorylation in the pathogenesis of Alzheimer's disease', *BMB Reports*. Korean Society of Biochemistry and Molecular Biology, 42(8), pp. 467–474.

Cong, W. *et al.* (2010) 'ASPP2 Regulates Epithelial Cell Polarity through the PAR Complex', *Current Biology*, 20, pp. 1408–1414. doi: 10.1016/j.cub.2010.06.024.

Da Cruz e Silva, E. F. and Da Cruz e Silva, O. A. B. (2003) 'Protein phosphorylation and APP

metabolism', *Neurochemical Research*, pp. 1553–1561. doi: 10.1023/A:1025630627319.

Domingues, S. C. *et al.* (2014) 'RanBP9 modulates AICD localization and transcriptional activity via direct interaction with Tip60', *Journal of Alzheimer's Disease*. IOS Press, 42(4), pp. 1415–1433. doi: 10.3233/JAD-132495.

Egloff, M. P. *et al.* (1997) 'Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1', *EMBO Journal*, 16(8), pp. 1876–1887. doi: 10.1093/emboj/16.8.1876.

Esposito, F. *et al.* (1990) 'Isolation of cDNA Fragments Hybridizing to Rat Brain-Specific mRNAs', *Developmental Neuroscience*, 12(6), pp. 373–381. doi: 10.1159/000111865.

Esteves, S. L. C. *et al.* (2013) 'Protein phosphatase 1γ isoforms linked interactions in the brain.', *Journal of molecular neuroscience : MN*, 50(1), pp. 179–97. doi: 10.1007/s12031-012-9902-6.

Fiore, F. *et al.* (1995) 'The Regions of the Fe65 Protein Homologous to the Phosphotyrosine Interaction/Phosphotyrosine Binding Domain of Shc Bind the Intracellular Domain of the Alzheimer's Amyloid Precursor Protein', *Journal of Biological Chemistry*, 270(52), pp. 30853–30856. doi: 10.1074/jbc.270.52.30853.

Fukazawa, T. *et al.* (1996) 'Tyrosine phosphorylation of Cbl upon epidermal growth factor (EGF) stimulation and its association with EGF receptor and downstream signaling proteins', *Journal of Biological Chemistry*, 271(24), pp. 14554–14559. doi: 10.1074/jbc.271.24.14554.

Golde, T. E., Eckman, C. B. and Younkin, S. G. (2000) 'Biochemical detection of Aβ isoforms: Implications for pathogenesis, diagnosis, and treatment of Alzheimer's disease', *Biochimica et Biophysica Acta - Molecular Basis of Disease*, pp. 172–187. doi: 10.1016/S0925-4439(00)00043-0.

Gong, C. -X *et al.* (1993) 'Phosphoprotein Phosphatase Activities in Alzheimer Disease Brain', *Journal of Neurochemistry*, 61(3), pp. 921–927. doi: 10.1111/j.1471-4159.1993.tb03603.x.

Haass, C. *et al.* (1992) 'Amyloid β-peptide is produced by cultured cells during normal

metabolism', *Nature*, 359(6393), pp. 322–325. doi: 10.1038/359322a0.

Haass, C. and Selkoe, D. J. (2007) 'Soluble protein oligomers in neurodegeneration: Lessons from the Alzheimer's amyloid β-peptide', *Nature Reviews Molecular Cell Biology*, pp. 101–112. doi: 10.1038/nrm2101.

Hakuno, F. *et al.* (2007) '53BP2S, interacting with insulin receptor substrates, modulates insulin signaling', *Journal of Biological Chemistry*, 282(52), pp. 37747–37758. doi: 10.1074/jbc.M702472200.

Helps, N. R. *et al.* (1995) 'Protein phosphatase 1 interacts with p53BP2, a protein which binds to the tumour suppressor p53', *FEBS Letters*, 377(3), pp. 295–300. doi: 10.1016/0014-5793(95)01347-4.

Hendrickx, A. *et al.* (2009) 'Docking Motif-Guided Mapping of the Interactome of Protein Phosphatase-1', *Chemistry and Biology*. Elsevier Ltd, 16(4), pp. 365–371. doi: 10.1016/j.chembiol.2009.02.012.

Heroes, E. *et al.* (2013) 'The PP1 binding code: A molecular-lego strategy that governs specificity', *FEBS Journal*, 280(2), pp. 584–595. doi: 10.1111/j.1742-4658.2012.08547.x.

Iwabuchi, K. *et al.* (1994) 'Two cellular proteins that bind to wild-type but not mutant p53', *Proceedings of the National Academy of Sciences of the United States of America*, 91(13), pp. 6098–6102. doi: 10.1073/pnas.91.13.6098.

Johnson, G. V. W. and Stoothoff, W. H. (2004) 'Tau phosphorylation in neuronal cell function and dysfunction', *Journal of Cell Science*, pp. 5721–5729. doi: 10.1242/jcs.01558.

Kajkowski, E. M. *et al.* (2001) 'β-Amyloid Peptide-induced Apoptosis Regulated by a Novel Protein Containing a G Protein Activation Module', *Journal of Biological Chemistry*, 276(22), pp. 18748–18756. doi: 10.1074/jbc.M011161200.

Katz, C. *et al.* (2008) 'Molecular basis of the interaction between the antiapoptotic Bcl-2 family proteins and the proapoptotic protein ASPP2', *Proceedings of the National Academy of Sciences of the United States of America*, 105(34), pp. 12277–12282. doi: 10.1073/pnas.0711269105.

Lane, C. A., Hardy, J. and Schott, J. M. (2018) 'Alzheimer's disease.', *European journal of*

neurology, 25(1), pp. 59–70. doi: 10.1111/ene.13439.

Lebouvier, T., Pasquier, F. and Buée, L. (2017) 'Update on tauopathies', *Current Opinion in Neurology*. Lippincott Williams and Wilkins, pp. 589–598. doi: 10.1097/WCO.0000000000000502.

Liu, C. Y. *et al.* (2011) 'PP1 cooperates with ASPP2 to dephosphorylate and activate TAZ', *Journal of Biological Chemistry*, 286(7), pp. 5558–5566. doi: 10.1074/jbc.M110.194019.

Lowenstein, E. J. *et al.* (1992) 'The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling', *Cell*, 70(3), pp. 431–442. doi: 10.1016/0092-8674(92)90167-B.

Mantzavinos, V. and Alexiou, A. (2017) 'Biomarkers for Alzheimer's Disease Diagnosis', *Current Alzheimer Research*, 14(11). doi: 10.2174/1567205014666170203125942.

Maurer, K., Volk, S. and Gerbaldo, H. (1997) 'Auguste D and Alzheimer's disease', *THE LANCET*, 349(9064), pp. 1546–1549. doi: 10.1016/S0140-6736(96)10203-8.

Mcpherson, P. S. *et al.* (1994) 'Interaction of Grb2 via its Src homology 3 domains with synaptic proteins including synapsin I', *Proceedings of the National Academy of Sciences of the United States of America*, 91, pp. 6486–6490. doi: 10.1073/pnas.91.14.6486.

Murase, S. (2015) 'Impaired focal adhesion Kinase-Grb2 interaction during elevated activity in hippocampal neurons', *International Journal of Molecular Sciences*. MDPI AG, 16(7), pp. 15659–15669. doi: 10.3390/ijms160715659.

Murphy, M. P. and Levine, H. (2010) 'Alzheimer's disease and the amyloid-β peptide', *Journal of Alzheimer's Disease*. IOS Press, pp. 311–323. doi: 10.3233/JAD-2010-1221.

Nakagawa, H. *et al.* (2000) 'APCL, a Central Nervous System-specific Homologue of Adenomatous Polyposis Coli Tumor Suppressor, Binds to p53-binding Protein 2 and Translocates it to the Perinucleus 1', *American Association for Cancer Research*, 60(1), pp. 101–105.

Nakaya, T. and Suzuki, T. (2006) 'Role of APP phosphorylation in FE65-dependent gene transactivation mediated by AICD', *Genes to Cells*, 11(6), pp. 633–645. doi: 10.1111/j.1365-2443.2006.00968.x.

Naumovski, L. and Cleary, M. L. (1996) 'The p53-Binding Protein 53BP2 Also Interacts with Bcl2 and Impedes Cell Cycle Progression at G 2 /M', *Molecular and Cellular Biology*, 16(7), pp. 3884–3892. doi: 10.1128/mcb.16.7.3884.

Nicolas, M. and Hassan, B. A. (2014) 'Amyloid precursor protein and neural development', *Development (Cambridge)*. Company of Biologists Ltd, 141(13), pp. 2543–2548. doi: 10.1242/dev.108712.

Nizzari, M. *et al.* (2007) 'Amyloid precursor protein and presenilin1 interact with the adaptor GRB2 and modulate ERK1,2 signaling', *Journal of Biological Chemistry*, 282(18), pp. 13833–13844. doi: 10.1074/jbc.M610146200.

O'brien, R. J. and Wong, P. C. (2011) 'Amyloid Precursor Protein Processing and Alzheimer's Disease'. doi: 10.1146/annurev-neuro-061010-113613.

Oliveira, J. M. *et al.* (2015) 'Amyloid-β Modulates Both AβPP and Tau Phosphorylation', *Journal of Alzheimer's Disease*. IOS Press, 45(2), pp. 495–507. doi: 10.3233/JAD-142664.

Olsen, J. V. *et al.* (2006) 'Global, In Vivo, and Site-Specific Phosphorylation Dynamics in Signaling Networks', *Cell*, 127(3), pp. 635–648. doi: 10.1016/j.cell.2006.09.026.

Puzzo, D. *et al.* (2008) 'Picomolar amyloid-β positively modulates synaptic plasticity and memory in hippocampus', *Journal of Neuroscience*, 28(53), pp. 14537–14545. doi: 10.1523/JNEUROSCI.2692-08.2008.

Rebelo, S. *et al.* (2013) 'Identification of a novel complex AβPP:Fe65:PP1 that regulates AβPP Thr668phosphorylation levels', *Journal of Alzheimer's Disease*, 35(4), pp. 761–775. doi: 10.3233/JAD-130095.

Rebelo, S. *et al.* (2015) 'Protein phosphatase 1 is a key player in nuclear events', *Cellular Signalling*. Elsevier Inc., pp. 2589–2598. doi: 10.1016/j.cellsig.2015.08.007.

Rossignol, M. (2006) 'Proteomic analysis of phosphorylated proteins', *Current Opinion in Plant Biology*, pp. 538–543. doi: 10.1016/j.pbi.2006.07.004.

Rotem, S. *et al.* (2008) 'The Structure and Interactions of the Proline-rich Domain of ASPP2', *Journal of Biological Chemistry*, 283(27), pp. 18990 –18999. doi: 10.1074/jbc.M708717200.

Royer, C. *et al.* (2014) 'ASPP2 Links the Apical Lateral Polarity Complex to the Regulation of YAP Activity in Epithelial Cells'. doi: 10.1371/journal.pone.0111384.

Sabo, S. L. *et al.* (1999) 'Regulation of-Amyloid Secretion by FE65, an Amyloid Protein Precursor-binding Protein', *Journal of Biological Chemistry*, 274(12), pp. 7952–7957. doi: 10.1074/jbc.274.12.7952.

Samuels-Lev, Y. *et al.* (2001) 'ASPP proteins specifically stimulate the apoptotic function of p53', *Molecular Cell*, 8(4), pp. 781–794. doi: 10.1016/S1097-2765(01)00367-7.

Santos, M. *et al.* (2013) 'The Nuclear Envelope Protein, LAP1B, Is a Novel Protein Phosphatase 1 Substrate', *PLoS ONE*, 8(10). doi: 10.1371/journal.pone.0076788.

Shoji, M. *et al.* (1992) 'Production of the Alzheimer amyloid β protein by normal proteolytic processing', *Science*, 258(5079), pp. 126–129. doi: 10.1126/science.1439760.

Skolnik, E. Y. *et al.* (1993) 'The function of GRB2 in linking the insulin receptor to Ras signaling pathways', *Science*, 260(5116), pp. 1953–1955. doi: 10.1126/science.8316835.

Sun, W. T. *et al.* (2008) 'p53 Target DDA3 binds ASPP2 and inhibits its stimulation on p53-mediated BAX activation', *Biochemical and Biophysical Research Communications*, 376(2), pp. 395–398. doi: 10.1016/j.bbrc.2008.08.168.

Thinakaran, G. and Koo, E. H. (2008) 'Amyloid precursor protein trafficking, processing, and function', *Journal of Biological Chemistry*, pp. 29615–29619. doi: 10.1074/jbc.R800019200.

Uhlmann-Schiffler, H., Kiermayer, S. and Stahl, H. (2009) 'The DEAD box protein Ddx42p modulates the function of ASPP2, a stimulator of apoptosis.', *Oncogene*, 28(20), pp. 2065–73. doi: 10.1038/onc.2009.75.

Vintém, A. P. B. *et al.* (2009) 'PP1 inhibition by Aβ peptide as a potential pathological mechanism in Alzheimer's disease', *Neurotoxicology and Teratology*, 31(2), pp. 85–88. doi: 10.1016/j.ntt.2008.11.001.

Walsh, D. M. *et al.* (2003) 'γ-secretase cleavage and binding to FE65 regulate the nuclear translocation of the intracellular C-terminal domain (ICD) of the APP family of proteins', *Biochemistry*, 42(22), pp. 6664–6673. doi: 10.1021/bi027375c.

Wang, Z. *et al.* (2013) 'N terminus of ASPP2 binds to Ras and enhances Ras/Raf/MEK/ERK activation to promote oncogene-induced senescence', *Proceedings of the National Academy of Sciences of the United States of America*, 110(1), pp. 312–317. doi: 10.1073/pnas.1201514110.

Wilkins, H. M. and Swerdlow, R. H. (2017) 'Amyloid Precursor Protein Processing and Bioenergetics', *Brain Research Bulletin*, 133, pp. 71–79. doi: 10.1016/j.brainresbull.2016.08.009.

World Health Organization (2017) *Global action plan on the public health response to dementia 2017 - 2025*. Geneva.

Yang, J. P. *et al.* (1999) 'NF-κB subunit p65 binds to 53BP2 and inhibits cell death induced by 53BP2', *Oncogene*, 18(37), pp. 5177–5186. doi: 10.1038/sj.onc.1202904.

Zheng, H. and Koo, E. H. (2006) 'The amyloid precursor protein: Beyond amyloid', *Molecular Neurodegeneration*, 1(1). doi: 10.1186/1750-1326-1-5.

Zheng, X. L. *et al.* (2014) 'Methyllycaconitine alleviates amyloid-β peptides-induced cytotoxicity in SH-SY5Y cells', *PLoS ONE*. Public Library of Science, 9(10). doi: 10.1371/journal.pone.0111536.

Zhou, D. *et al.* (2004) 'Growth factor receptor-bound protein 2 interaction with the tyrosine-phosphorylated tail of amyloid beta precursor protein is mediated by its Src homology 2 domain.', *The Journal of biological chemistry*, 279(24), pp. 25374–80. doi: 10.1074/jbc.M400488200.

7. Appendix

1. Cell Culture Reagents and Equipment

Equipment

I. Hera cell CO2 incubator (Heraeus)
II. Safety cabinet Hera safe (Heraeus)
III. Inverted optical microscope (LEICA)
IV. Hemacytometer (Sigma)
V. Sonicator (U200S (IKA))
VI. Bath SBB6 (Grant)

Reagents/Solutions

I. SH-SY5Y Complete Medium (Eagle's Minimum Essential Medium/F12 Medium 1:1)

For a final volume of 500 ml:
- 2.4 g MEM
- 2.655 g F12
- 0.025 g Sodium Phosphate
- 0.85 g Sodium Bicarbonate (Sigma)
- 1.25 mL L-Glutamine (200 mM)
- 5 mL AA (1%)

Adjust to pH 7.2-7.4
- 50 mL FBS (10%)

Sterilize by filtering through a 0.2 μm filter and store at 4^{o}C.

II. PBS (1x)

For a final volume of 500 ml, dissolve one pack of BupH Modified Dulbecco's Phosphate Buffered Saline Pack (Pierce) in distilled H_2O. Final composition:
- 8 mM Sodium Phosphate
- 2 mM Potassium Phosphate
- 140 mM Sodium Chloride
- 10 mM Potassim Chloride

Sterilize by filtering through a 0.2 μm filter and store at 4°C.

III. Trypsin-EDTA 0,05% (ThermoFisher Scientific)

IV. Trypan Blue solution cell culture tested (Sigma)

V. RIPA buffer

Per 980 μl of RIPA buffer (Sigma-Aldrich) add:
- 20 μl Protease inhibitor cocktail (Roche)

VI. Beta-Amyloid 1-42, TFA (GenicBio)

2. Protein Content Determination

Equipment

I. Tecan 5000

Reagents/Solutions

I. BCA assay kit (Pierce, Rockfort, IL)
II. Bovine Serum Albumin (BSA) (Pierce)
III. Working Reagent (WB) (50 Reagent A : 1 Reagent B)
- Reagent A: sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0,2N sodium hydroxide
- Reagent B: 4% cupric sulfate

3. SDS-PAGE

Equipment

I. Electrophoresis system (Hoefer SE600 vertical unit)
II. PowerPac 3000 Electrophoresis Power Supply (BioRad)

Reagents/Solutions

I. Acrylamide: Bis-Acrylamide 29:1 solution 40%, Dnase, Rnase free (Fisher)

II. 10% APS (ammonium persulfate)

In 10 ml of distilled H_2O dissolve 1 g of APS. Note: prepare fresh before use.

III. 10% SDS (sodium dodecyl sulfate)

In 10 ml of distilled H_2O dissolve 1 g of SDS.

IV. LGB (Lower gel buffer) (4x)

Per 900 ml of distilled H_2O add:
- 181.65 g Tris
- 4 g SDS

Mix until the solutes have dissolved. Adjust the pH to 8.9 and adjust the volume to 1L with distilled H_2O.

V. UGB (Upper gel buffer) (5x)

Per 900 ml of distilled H_2O add:

- 75.69 g Tris

Mix until the solute has dissolved. Adjust the pH to 6.8 and adjust the volume to 1 L with distilled H_2O.

VI. Loading Gel Buffer (LB) (4x)

- 2.5 mL 1 M Tris solution (pH 6.8) (250 mM)
- 0.8 g SDS (8%)
- 4 ml Glicerol (40%)
- 2 ml Beta-Mercaptoetanol (2%)
- 1 mg Bromofenol blue (0.01%)

Adjust the volume to 10 ml with distilled H_2O. Store in darkness at room temperature.

VII. 1 M Tris (pH 6.8)

Per 150 ml of distilled H_2O add:

- 30.3 g Tris base

Adjust the pH to 6.8 and adjust the final volume to 250 ml.

VIII. 10x Running Buffer

- 30.3 g Tris (250 mM)
- 144.2 g Glycine (2.5 M)
- 10 g SDS (1%)

Dissolve in distilled H_2O, adjust the pH to 8.3 and adjust the volume to 1 L.

IX. Stacking (upper) and Resolving (lower) gel solution

Per system:

	Stacking Gel	Resolving Gel	
	3.5%	5%	20%
- H_2O	13.83 ml	18.59 ml	7.34 ml
- 29:1 Bis-Acrylamide	1.75 ml	3.75 ml	15 ml
- LGB (4x)	-	7.5 ml	7.5 ml
- UGB (5x)	4.0 ml	-	-
- 10% APS	200 µl	150 µl	150 µl
- 10% SDS	200 µl	-	-
- TEMED	20 µl	15 µl	15 µl
	20 ml	60 ml	

4. Western-Blot

Equipment

I. Transfer Electrophoresis unit (Hoefer^{TM} TE 42)
II. Electrophoresis power supply EPS 1000 (Amersham Pharmacie Biotec)
III. ChemiDoc Touch Imaging System (BioRad)

Reagents/Solutions

I. 1x Transfer Buffer

- 3.03 g Tris (25 mM)
- 14.41 g Glycine (192 mM)

Mix until solutes dissolution. Adjust the pH to 8.3 with HCl and adjust the volume to 800 ml with distilled H_2O. Just prior to use add 200 ml of methanol (20%).

II. 10x TBS (Tris buffered saline)

- 12.11 g Tris (10 mM)
- 87.66 g NaCl (150 mM)

Adjust the pH to 8.0 with HCl and adjust the volume to 1L with distilled H_2O.

III. 10x TBS-T (TBS+Tween)

- 12.11 g Tris (10 mM)
- 87.66 g NaCl (150 mM)
- 5 ml Tween20 (0.05%)

Adjust the pH to 8.0 with HCl and adjust the volume to 1L with distilled H_2O.

IV. Blocking solution

5% of non-fat dried milk or BSA (Bovine Serum Albumine, Sigma) in 1x TBS-T.

V. Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare Life Sciences)

5. Immunoprecipitation

I. Dynabeads® Protein G (ThermoFisher Scientific)

Reagents/Solutions

II. Blocking solution

0.6g of BSA (Bovine Serum Albumine, Sigma) per 20 mL of PBS 1x.

6. Immunocytochemistry

Equipment

I. Zeiss Axio Imager Z1

Reagents/Solutions

I. Blocking solution

Per 10 mL of PBS 1x add 0.3 g of Bovine Serum Albumine (BSA).

II. 4% Paraformaldehyde

For a final volume of 100 mL, add 4 g of paraformaldehyde to 25 mL distilled H_2O. Dissolve by heating the mixture at 58°C while stirring. Add 1-2 drops of 1 M NaOH to clarify the solution and filter through a 0.2 µm filter. Add 50 mL of PBS 2x and adjust the volume to 100 ml with distilled H_2O.