Universidade de Aveiro Departamento de Biologia
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia Molecular, realizada sob a orientação científica da Profª. Doutora Odete A. B. da Cruz e Silva, Professora Auxiliar, Departamento de Biologia, Universidade de Aveiro.

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agradecimentos

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palavras-chave Dois-Híbrido, APP

resumo

Inúmeras potenciais funções foram sugeridas para a APP (Proteína Precursora de Amilóide de Alzheimer), contudo, fisiologicamente, a função precisa da APP não foi ainda desvendada. A APP tem características consistentes com a função de molécula-receptora, capaz de reconhecer sinais extracelulares. Também relevante para este trabalho, é o facto de que a sinalização através de RIP (Proteólise Intramembranar Regulada) tem consequências na expressão génica, como no caso da sinalização tipo-Notch. Tal como a proteína Notch, a APP é processada resultando num fragmento C-terminal designado por AICD (Domínio Intracelular da APP). Neste trabalho é focado o papel importante na sinalização nuclear desempenhado pelo fragmento AICD, especificamente através da interacção com proteínas adaptadoras, promovendo a transcrição. Com o objectivo de contribuir para uma melhor compreensão da base molecular da DA (Doença de Alzheimer), torna-se importante investigar as vias de localização nuclear do AICD e o seu envolvimento na transcrição génica, possivelmente afectando proteínas até agora não associadas à DA. Por estes motivos é fundamental a identificação de novas proteínas que interajam com a APP.

Um rastreio foi efectuado, utilizando o sistema Dois-Híbrido em Levedura, para identificar interacções específicas do AICD no cérebro humano e, assim, caracterizar o interactoma do AICD. Foi feito o rastreio de aproximadamente $1.1x10^8$ clones de uma biblioteca de cDNA de cérebro humano com o domínio C-terminal da APP com a mutação Y687E, que mimetiza o estado fosforilado. De experiências anteriores deste laboratório sabemos que a tirosina-687 afecta a localização subcelular da APP e é também consensual que a fosforilação é importante nos mecanismos de transdução de sinais, daí a utilização deste mutante parecer apropriada.

O rastreio originou 55 clones positivos que foram analisados para identificar proteínas que interagem com a APP. Dois clones são particularmente importantes, a RanBPM e a Transportin-SR2, visto que estão associadas ao transporte de proteínas para o núcleo e confirmam a sinalização nuclear da APP.

keywords Two-Hybrid, APP

abstract

Many putative functions for APP (Alzheimer's amyloid precursor protein) have been suggested, although the precise physiological function of APP remains to be elucidated. APP has characteristics consistent with it having a role as a receptor, capable of mediating extracellular signals. Also of relevance to the work described here is that RIP (Regulated Intramembrane Proteolysis) signalling can have consequences in gene expression, similar to Notch signalling. Like the latter, APP is processed by RIP resulting in a C-terminal fragment known as AICD. Here we test the hypothesis that the AICD fragment may play an important role in nuclear signalling, specifically by interacting with adaptor proteins potentiating transcription. Therefore, in order to contribute to our understanding of the molecular basis of AD (Alzheimer's disease) it is important to investigate the pathways of AICD nuclear targeting and its involvement in gene transcription, possibly affecting other proteins hitherto not associated with AD. Thus, it is important to identify AICD binding proteins.

A Yeast Two-Hybrid (YTH) screen was performed to identify human brain-specific AICD binding proteins, and thus characterize the AICD interactome. The screen of approximately 1.1 $x10^8$ clones from a human brain cDNA library was carried out using the AICD fragment with an Y687E mutation, which mimics phosphorylation on that residue. From previous work carried out in the laboratory we know that tyrosine-687 phosphorylation affects subcellular localization of APP, and it is also recognized that phosphorylation events are important in signal transduction mechanisms, hence the use of this mutant is appropriate.

The YTH screen yielded 55 positive clones that were analysed and several novel brain-specific APP binding proteins were identified. Two clones were particularly important, RanBPM and Transportin-SR2, being that they are associated with the nuclear transport of proteins, and support the nuclear signalling for APP.

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ABBREVIATIONS

aa	Amino acid (s)
ActD	Activation Domain
AD	Alzheimer's disease
Ade	Adenine
AICD	APP Intracellular Domain
Amp	Ampicillin
APP	Alzheimer's amyloid precursor protein
APS	Ammonium persulfate
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary deoxynucleic acid
CDS	Protein coding sequence
Chr	Chromosome
Cys	Cystein
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
DMSO	Dimethylsulfoxide
DNA	Deoxynucleic acid
DNA-BD	DNA-Binding domain
dNTP	Deoxynucleotide triphosphate
dsDNA	Double strand deoxynucleic acid
dTTP	2'-deoxythymidine-5'-triphosphate
EDTA	Ethylenodiaminotetraacetic acid
EST	Expressed sequence tag
GAL4	Gal4 transcription factor
GAL4-AD	GAL4-Activation domain
GAL4-BD	GAL4-Binding domain
His	Histidine

LB medium	Luria-Bertani Medium (Miller)
Leu	Leucine
LiAc	Lithium acetate
nt	Nucleotide
OD	Optical density
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PMSF	Phenyl methylsulfoxide
PP1	Protein phosphatase 1
QDO	Quadruple dropout
RNA	Ribonucleic acid
RT	Room Temperature
RT-PCR	Reverse transcriptase - polymerase chain reaction
SAP	Shrimp alkaline phosphatase
sAPP	Secreted APP
SD	Supplement dropout medium
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
Ser	Serine
TBS	Tris-buffered saline solution
TDO	Triple dropout
TEMED	N,N,N',N'-tetramethyletylendiamine
Thr	Threonine
Tris	Tris (hydroxymethyl)-aminoethane chloride
Trp	Tryptophan
Tyr	Tyrosine
UAS	Upstream activating sequence
UTR	mRNA Untranslated Region
UV	Ultraviolet
X-a-gal	5-bromo-4-chloro-3-indolyl-alpha-D-galactopyranoside

YPD	Yeast extract, Peptone and Dextrose medium for *S. cerevisiae*
YPDA	YPD with adenine
YTH	Yeast Two-hybrid system

I INTRODUCTION

I.1 THE YEAST TWO-HYBRID SYSTEM

Principles of the Yeast Two-Hybrid System

The Yeast Two-Hybrid system is a method that allows the identification of a large number of proteins capable of interacting with a protein of interest. A large number of clones can be simultaneously tested using a cDNA expression library selected from a particular tissue. To identify brain-specific APP binding proteins we selected the Yeast Two-Hybrid System, using the APP^{Y687E} specific intracellular domain as bait for the screening of a human brain cDNA library.

The yeast two-hybrid system (YTH), was initially developed as a simple method to probe protein-protein interactions (Fields and Song, 1989; Fields and Sternglanz, 1994). It takes advantage of the modular architecture of eukaryotic transcription activators, which comprise two functionally independent domains: a DNA-binding domain (BD) that recognizes a specific DNA sequence in the promoter region and a DNA-activation domain (ActD) that brings the transcriptional machinery to the promoter proximity, leading to expression of the gene.

The independence of DNA-BD and ActD domains was demonstrated by fusing the ActD of yeast GAL4, a yeast transcription factor involved in galactose metabolism, with the DNA binding domain (BD) of E. coli LexA (a bacterial repressor involved in the control of damage inducible genes) to create a functional transcription factor in yeast (Brent and Ptashne, 1980). Fields and Song used it to prove an interaction between two known proteins, by fusing GAL4-ActD to a protein X (prey) and GAL4-BD to a protein Y (bait) (Fields and Song, 1989). These two elements can be cointroduced into yeast strains modified with one or

more reporter genes (the use of multiple reporter genes decreases the number of false positives obtained). These reporter genes have a binding site specific to the BD on their promoter region, causing the transcription of those genes to be dependent on the interaction between prey and bait proteins. Interaction of the BD/bait fusion with the ActD/prey fusion positions the ActD in the proximity of the reporter gene, thus activating its transcription (Fig. I.1) (Causier and Davies, 2002).

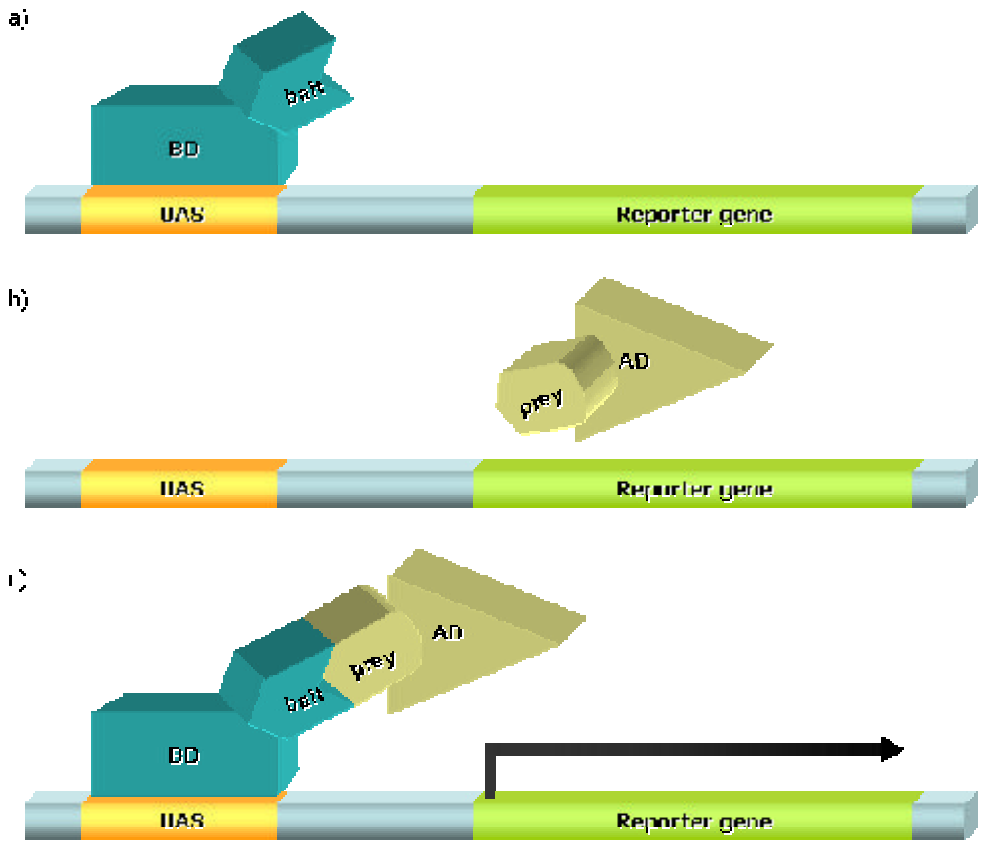

Figure I.1: *The yeast two-hybrid system. Two hybrid proteins are expressed in yeast:* ***a)*** *GAL4 DNA-binding domain (BD) fused to a bait protein and GAL4 activation domain (ActD) fused to a prey protein. The BD-bait hybrid protein can bind to upstream activation sites (UAS) but cannot activate transcription.* ***b)*** *The ActD-prey protein cannot recognize the UAS, thus, alone is not capable of initiating transcription.* ***c)*** *When the bait and the prey interact, the BD and ActD are brought together and can activate reporter gene transcription.*

The reporter genes used in this screen are the *MEL1* gene (coding for ?-galactosidase, that is secreted into the culture medium) and auxotrophic genes *HIS3* and *ADE2*, that allow these yeast strains to grow in medium lacking histidine and adenine. The nutritional selection reporter genes allow for easy recovery of interacting clones in large screening procedures, using a cDNA library, designed to identify new interacting proteins with a selected bait. Additionally, the YTH bait plasmid pAS2-1 and the library plasmid pACT2, which contain the *TRP1* and *LEU2* genes, respectively, allow for selection in medium lacking tryptophan and leucine. Hence, the high-stringency selection consists of medium lacking tryptophan, leucine, histidine and adenine, and also in the presence of X-?-Gal. The selection of positive clones with the five reporter genes *TRP1*, *LEU2*, *HIS3*, *ADE2* and *MEL1* (Matchmaker Yeast Two-Hybrid System 2, Clontech) was designed to reduce the number of false positives, thus allowing faster identification of true interactions with the bait protein.

Larger scale two-hybrid approaches typically rely on interaction mating (Serebriiskii *et al.*, 2001). In this method a yeast strain expressing the bait protein is mated with another yeast strain of opposite mating type pretransformed with the cDNA library. Interaction between two proteins can then be determined by the activation of one or more reporter genes in the diploid strain. One advantage of this approach is the possibility of using frozen aliquots of pretransformed yeast cells saving time and resources. An additional benefit of using yeast mating is that diploid cells are more tolerant to expression of toxic proteins and less false positives will appear since the diploids have reporter genes less sensitive to transcription (Kolonin *et al.*, 2000).

Over the 14 years since its introduction this system has been modified greatly expanding its biological and technological applications. The YTH has been developed first as an agent of biological discovery, second as a tool in proteomics and finally as a means towards engineering novel pharmaceutical agents. Some alternative YTH systems have been developed and in many cases resulted in remarkably elegant hybrid systems (Serebriiskii *et al.*, 2001; Tyree and Klausing, 2003; Causier, 2004).

Limitations of the YTH

Although the YTH system has been widely used both to demonstrate and to identify novel protein interactions with proteins from multiple sources, from prokaryotes to plants and mammals, this system has some intrinsic limitations that should be considered. It relies on yeast expression of two hybrid proteins and on their action as transcription factors in the yeast nucleus being dependent on their interaction. Limitations exist if one of the proteins cannot be expressed, folded or post-translationally modified in yeast cells, or if the GAL4 fusion impairs correct folding. In fact yeast doesn't possess all the post-translational machinery needed and toxicity problems may occur. Also, if any of the fusion proteins are capable of activating reporter gene transcription, false positives will arise. One also has to bear in mind that a legitimate protein-protein interaction may have no functional significance if it involves two proteins that never co-localize in physiological systems (e.g. that are expressed in different tissues). Other causes which may also lead to false results are the inability of fusion proteins to migrate to the nucleus.

The limitations of the YTH system do not exclude it from protein networks research, but reinforce the need to validate all the interactions. These should be tested in different systems, preferably, by confirming the physical association of the native proteins in the cell where the functional interactions are predicted to occur. Nevertheless, it is important to remember the usefulness of the YTH in the construction of large interaction networks, and in identifying unsuspected interactions that may latter be confirmed by a variety of independent methods.

I.2 ALZHEIMER'S AMYLOID PRECURSOR PROTEIN

I.2.1 Alzheimer's amyloid precursor protein structure

The Alzheimer's Amyloid Precursor Protein (APP) is a type I transmembrane glycoprotein consisting of a large extracellular domain, a single transmembrane domain, and a short cytoplasmic tail. Following proteolytic processing, APP gives rise to the Abeta peptide, the major component of Alzheimer's amyloid plaques (Haas *et al.*, 1995). The mammalian APP superfamily comprises three members: APP and the APP-like proteins APLP1 and APLP2 (Wasco *et al.*, 1993a; Wasco *et al.*, 1993b; Wasco *et al.*, 1993c), which are functionally and structurally related, and share similar functions (Coulson *et al.*, 2000).

The human APP gene is localized to chromosome 21 (21q21.2-3) and contains 19 exons, with the Abeta sequence as part of exons 16 and 17 (Turner *et al.*, 2003). Alternative splicing of exons 7, 8 and 15 of the APP mRNA produces eight isoforms, ranging in size from 677-770 aa (L-677, 695, L-696, 714, L-733, 751, L-752, 770) (Tanzi *et al.*, 1993). APP is ubiquitously expressed in mammalian cells with a broad tissue distribution. The three major APP isoforms are the 695, 751 and 770. APP_{695}, the exon 7-excluding isoform, is the most highly expressed in neurons (Goedert and Crowther, 1989; Tanzi and Hyman, 1991; LeBlanc *et al.*, 1997).

I.2.2 Trafficking and proteolytic processing of APP

The pathological cascade leading to clinical manifestations of AD has not been completely characterized although the "?-amyloid hypothesis" has been used to explain certain aspects of AD. According to this hypothesis, the accumulation of the A? peptide, which derives from the proteolytic processing of

APP, is the primary event that leads to all subsequent events in the pathology of AD (Hsiao *et al.*, 1996; Golde, 2002).

APP is synthesized on membrane-bound polysomes and matures as it is transported through the secretory pathway, becoming N- and O-glycosylated and tyrosyl-sulfated while moving through the trans-Golgi network. Immature APP (being N-glycosylated only) may be cleaved in the endoplasmic reticulum or the cis-Golgi but the mature APP is degraded rapidly as it is transported to or from the cell surface via either a biosynthetic or endocytic pathway (De Strooper *et al.*, 1993; Hartmann *et al.*, 1996; Selkoe, 2001).

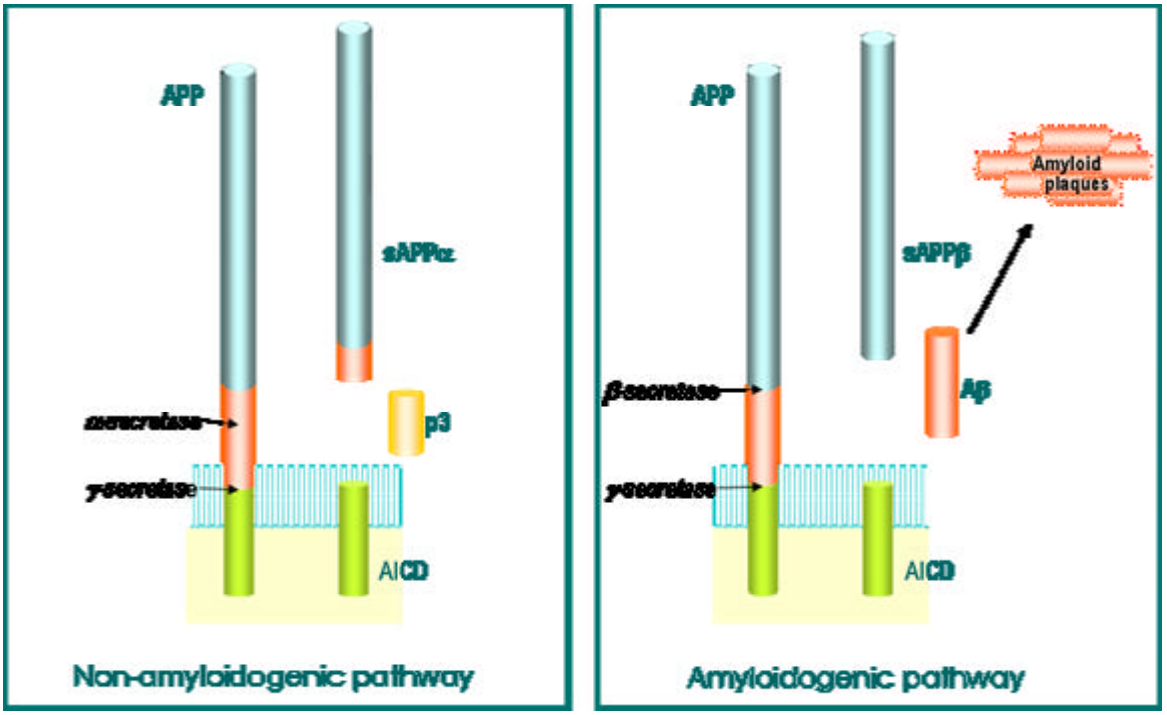

Figure I.2: *Proteolytic processing of APP.* ***Non-amyloidogenic pathway:*** *? -secretase cleaves APP within the Abeta region preventing its formation and originating the sAPP? fragment. The C-terminal fragment is then processed by ?-secretase to p3 and the AICD fragments.* ***Amyloidogenic pathway:*** *?-secretase cuts APP N-terminally to the Abeta region and produces the sAPP? fragment. The CTF is further cleaved by ?-secretase to Abeta and AICD.*

APP metabolism is complex, and can occur via several pathways, becoming an important area with respect to the generation of Abeta, which is deposited in the AD brain. APP comprises three main proteolytic sites, that correspond to ?-, ?- or ?-secretases (Esler and Wolfe, 2001), being the cleavage sites therefore denoted ?, ? and ? sites. The majority of mature APP is proteolytically cleaved via one of two competing pathways, the non-amyloidogenic and the amyloidogenic pathway (Fig. I.2), according to the ?- or ?-cleavages occurrencing (Nunan and Small, 2002). Alpha and beta-secretases cleave APP at the beginning or in the middle of the Abeta sequence and liberate a large fragment (secreted APP or sAPP) from the extracellular domain of the protein, named ?sAPP and ?sAPP, respectively. The remaining portion of APP is called the ?-carboxy-terminal fragment (?CTF or C83), or the ?-carboxy-terminal fragment (?CTF or C99).

Subsequent to the first ?- or ?-cleavage, ?-secretase cleaves APP either at the ?-site or at ?-site (Fig. I.3) and peptides of different lengths may be generated. Although both ?-site ?-site cleavages are independent, they are believed to occur nearly simultaneously. Processing of ?CTF by ?-secretase, generates a short peptide termed p3 (3kDa), which lacks the Abeta 17 N-terminal amino acids (Turner *et al.*, 2003).

When processed by ?-secretase, ?CTF generates predominantly Abeta 1-40 or 1-42 fragments (Haass *et al.*, 1992; Johnson-Wood *et al.*, 1997). Abeta species spanning from 1-39 to 1-43 amino acid long can also be produced, with Abeta fragments appearing to be more amyloidogenic with increasing length. ?CFT cleavage should release both A?40-42 and APP C-terminal fragments of 59-57 aa long, respectively. Unexpectedly, the purified and sequenced C-terminal fragment of APP, termed AICD (APP intracellular domain), is 7-9 aa shorter, consisting of 50 residues, which remains to be explained (Sastre *et al.*, 2001; Koo, 2002).

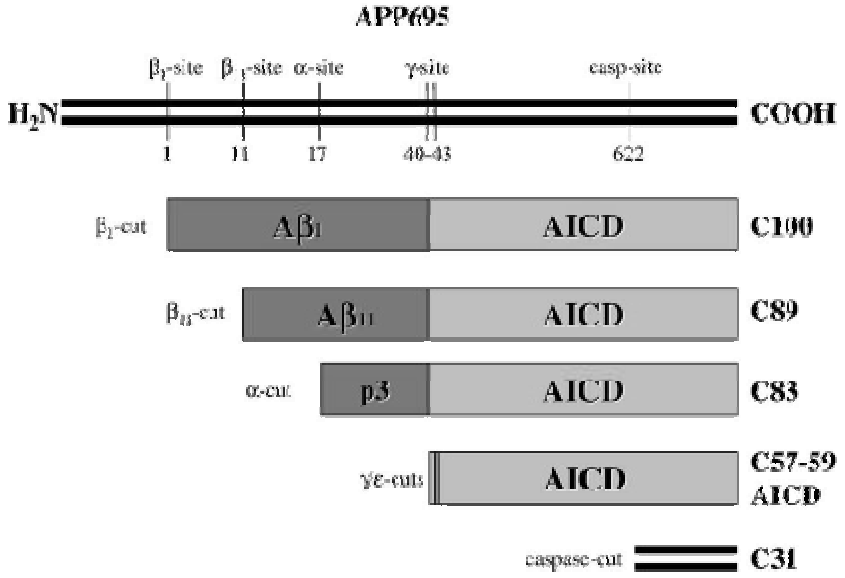

Figure I.3: *APP cleavage by ?-secretase prevents Abeta generation. In contrast, sequential cleavage of APP by ?-secretase and ?-secretase results in the production of 40 - 42 amino acid long Abeta (depending on where the ?-secretase cleavage occurs). The major cleavage sites of APP secretases are: Met-596 for ?-, and Lys-612 for ?-secretases, and Val6-36 (?-site) and Leu-645 (?-site) for ?-secretase (APP$_{695}$ numbering) (adapted from (Russo et al., 2005)).*

? **-secretase**

?-secretase cleaves APP within the Abeta region, close to the APP membrane-spanning domain (Lys^{613}-Leu^{614}, APP$_{695}$ numbering). As described above, cleavage at this site (Fig. I.3) prevents A? generation and produces a soluble protein of 612 aa, ?sAPP (Esch *et al.*, 1990). Three candidates have been identified for search for ?-secretase. The first was the tumor necrosis factor alpha converting enzyme (TACE or ADAM 17), a disintegrin and metalloprotease that releases tumor necrosis factor from its receptor form (Hooper and Turner, 1995; Esler and Wolfe, 2001; Capell *et al.*, 2002). The other candidates are also members of the metalloproteases ADAM family: ADAM 10 and MDC9. Like TACE,

they are initially expressed as inactive preproteins and their active forms can also affect ?sAPP levels (Koike *et al.*, 1999; Lammich *et al.*, 1999).

?-secretase

The site of ? cleavage (?-site) occurs between residues 596 and 597 of APP_{695}, exactly at the N-terminus of the Abeta sequence (Fig. I.3), and releases the ?sAPP of 595 aa long. Furthermore, the levels and activity of ?-secretases were found to be increased in AD (Fukumoto *et al.*, 2002). The enzymes that cleave at the ?-site have been identified as the aspartyl proteases ?-site APP cleaving enzyme 1, BACE1 (Vassar *et al.*, 1999; Hussain *et al.*, 2000), and ?-site APP cleaving enzyme 2 (BACE2) (Farzan *et al.*, 2000). BACE1 and BACE2 are both membrane-anchored enzymes that are expressed in neuronal tissues, although BACE2 expression is lower than BACE1. Like all aspartic proteinases BACE1 and BACE2 are generated as proenzymes, and while the BACE2 activation by prodomain processing is autocatalytic, BACE1 activation is mediated by furin or a furin-like enzyme (Hussain *et al.*, 2001). The third candidate identified for ?-secretase was Carboxypeptidase B, which, in contrast to BACE enzymes, lacks a transmembrane domain. It is a soluble enzyme located in the cytosol of various neurons and some microglial cells (Matsumoto, 2000).

?-secretase

?-secretase, which cleaves ?CTF and ?CTF C-terminally to the A? region, within the APP transmembrane domain, is a multicomponent enzymatic complex (Wolfe *et al.*, 1999). The two prime candidates for the ?-secretase are presenilin-1 (PS1) and presenilin-2 (PS2), both initially identified by genetic linkage analysis of non-APP familial AD mutations that increased Abeta 1-42 in humans, and mapped to chromosomes 14 and 1, respectively (Clark *et al.*, 1996; Cruts *et al.*, 1998).

PS1 and PS2 are integral membrane proteins with at least eight transmembrane domains (Thinakaran *et al.*, 1998). PS2 seems to be responsible for fewer CTFs cleavages than PS1. Nonetheless, there might be multiple ?-secretase enzymes with varying degrees of selectivity for producing Abeta 1-40 and Abeta 1-42 (De Strooper *et al.*, 1999; Wolfe, 2001). Another component of ?-secretase is Nicastrin (NCT), which also affects the complex's enzymatic activity (Esler *et al.*, 2002), and interacts both with APP CTFs and PS1 and PS2. Nicastrin contains a single transmembrane domain, is extensively glycosylated and has four conserved cysteine residues at the extracellular N-terminus. Mutations within a conserved region of nicastrin lead to enhanced Abeta secretion, while deletion of the same region prevents Abeta secretion (Yu *et al.*, 2000). Two other proteins were identified as ?-secretase subunits: APH-1 or APH-2 (with APH-2 being only relevant in cerebral tissues ?-secretase activity) and PEN-2. Recent reports show that ?-secretase is indeed an aspartyl protease complex composes of the four core components APH-1, nicastrin, presenilin-1 and presenilin-2 (Capell *et al.*, 2005).

Caspase-cleveage of APP

Additional reports have revealed that APP can also be proteolytic processed at it C-terminus by caspases like caspase-3, caspase-6, caspase-8 or caspase-9 (Pellegrini *et al.*, 1999; Soriano *et al.*, 2001). The target residue for the caspase-directed APP cleavage is Asp664 (APP_{695} numbering) (Weidemann *et al.*, 1999). The resultant C-terminus C31 (Fig. I.3) peptide is a potent inducer of apoptosis, and this cleavage was shown to reduce APP internalization but to have varying effects on the Abeta secreted levels (Soriano *et al.*, 2001).

I.2.3 Putative functions of APP and its derivates

Functions of full-lengh APP

Although the precise function of APP is unknown, it is probably involved in the development of the nervous system. Transgenic mouse studies revealed that knockout of the APP gene has only mild effects on neuronal function (Seabrook *et al.*, 1999). However, triple knockout of APP, APLP-1 and APLP-2, results in abnormal layering of the cerebral cortex (Herms *et al.*, 2004), suggesting that the APP family proteins are critically involved in the development of the nervous system. The transmembrane structure of APP is consistent with a role as either a receptor or a mediator of extracellular interactions. It has also been suggested that APP may have CAM (Cell Adhesion Molecule) and SAM (Substrate Adhesion Molecule) like activities. In fact, APP possesses several domains (Fig. I.4) that promote binding to specific substrates, such as heparin and collagen, which implicate cell-surface APP in both cell-cell and cell-substrate adhesion (Breen, 1992; Multhaup, 1994). The binding of heparan sulfate proteoglycan to heparin binding domain stimulates the neurite outgrowth-promoting effects of APP (Qiu *et al.*, 1995). In fact the heparan sulphate proteoglycan glypican-1 has been identified as a putative APP ligand (Williamson *et al.*, 1996). APP may also be important for interactions between the cell surface and the extracellular matrix, since the extracellular matrix protein F-spondin also binds to the APP extracellular domain (Ho and Sudhof, 2004). The most C-terminus heparin-binding contains the ^{676}RHDS^{679} (APP_{770} numbering) sequence, which is an integrin-binding motif (Ghiso *et al.*, 1992).

The extracellular domain of APP also contains binding-sites for metals such as zinc and copper (Fig. I.4) (Maynard *et al.*, 2005). Zn(II)-binding is assumed to play a structural role, whereas APP was shown to catalyse the reduction of Cu(II) to Cu(I) (Bush *et al.*, 1994; Multhaup *et al.*, 1994).

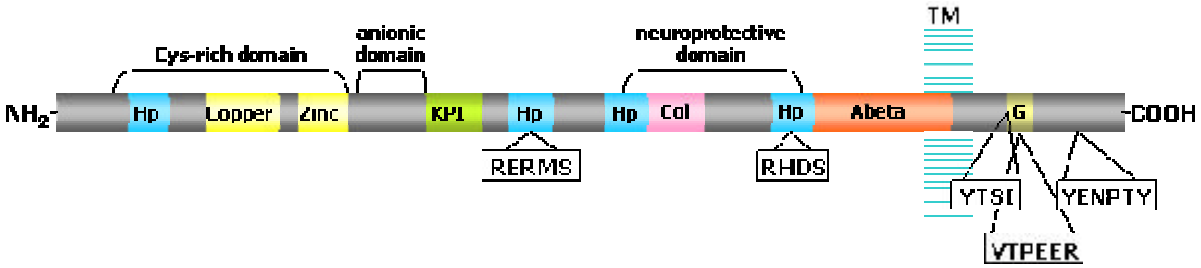

Figure I.4: *Structure of APP showing some of the major functional domains. TM: transmembrane domain; Binding domains: Heparin (Hp), Copper, Zinc, Collagen (Col), G_0 proteins (G).*

APP as an integral membrane protein

Given the APP structure as a type I integral membrane protein, which resembles a membrane-anchored receptor molecule, several studies demonstrated that full-length APP could function as a cell surface G-protein-coupled receptor (Okamoto et al., 1995). Despite being controversial, these results demonstrate that APP binds to heterotrimeric G proteins (G_0), involved in signal transduction. However, a ligand for the putative APP receptor has not yet been identified, although fibrillar forms of Abeta were reported to bind to cell-surface APP (Lorenzo et al., 2000).

Moreover, it is suggested that the APP protein family has a role in the Reelin signalling pathway, and may participate in neuronal migration and positioning during brain development (Wirths et al., 2001). The regulation of APP by cholinergic mechanisms suggests that the molecule might be multifunctional and play different roles in development and ageing (Bayer et al., 2001).

The receptor-like architecture of APP, with a conserved cytoplasmic domain, and cell-surface localization, prompted theories that addressed the processing by RIP (Regulated Intramembrane Proteolysis) (Koo, 2002; von Rotz et al., 2004). These molecules have a peptide sequence within their protein structure that is released after two consecutive cleavages on the protein (RIP

proteolysis), and can be targeted to the nucleus where it activates gene expression (Schroeter et al., 2003).

The thrilling discovery that both Notch and APP are processed by RIP led to potential implications in the basic mechanisms of brain development and degeneration. Notch is a type I transmembrane receptor involved in cell fate decisions during embryogenesis and functions through cellular RIP. Upon ligand binding, Notch is proteolytically processed, initially by a TACE- or by ADAM10, followed by ?-secretase intramembrane cleavage (De Strooper et al., 1999). In these respects APP and Notch processing may be similar (Selkoe and Kopan, 2003). In addition, the subsequent ?-secretase cleavage occurs at a site (?-site) corresponding to the S3 cleavage of Notch (Sastre et al., 2001). Both the Notch Intracellular Domain (NICD), and the APP intracellular domain (AICD) can act as a transcription factor (Cao and Sudhof, 2001; Cupers et al., 2001). The AICD fragment is extremely small when compared with the NICD and lacks motifs commonly found in transcriptional regulators, suggesting that it may function in signalling in a different way, specifically by interaction with adaptor proteins, activating transcription by an indirect mechanism (Cao and Sudhof, 2004). Significant to AD is the aspect that RIP signalling can increase gene expression. Therefore, in order to contribute to our understanding of molecular basis of AD it is important to investigate the pathways of AICD nuclear targeting and its involvement in gene transcription. Thus, it is fundamental to identify the AICD binding proteins, which are determinant for specifying protein function.

Functions of sAPP

The secreted APP fragments have been shown to be involved in neuronal cell survival, adhesive interactions, neurite outgrowth, synaptogenesis, and synaptic plasticity (Furukawa et al., 1996; Turner et al., 2003). sAPP effects on ion fluxes and various signalling pathways might underlie its reported physiological roles (Mattson, 1997). sAPP? also induces activation of NF-?B and alter various

second messengers and effectors including cyclic GMP and protein kinase G (Barger et al., 1995), phospholipase C/protein kinase C, extracellular signal-regulated protein kinase (ERK) and inositol tri-phosphate (Greenberg et al., 1994; Greenberg et al., 1995).

Secreted APP (sAPP) containing the KPI domain (57 aa stretch with homologies to the Kunitz-type protease inhibitors) was initially found to be identical to protease nexin II, a growth regulatory molecule produced by fibroblasts that is an inhibitor of extracellular serine proteinases (Schilling et al., 1991; Hook et al., 1999). A growth-promoting effect of sAPP has been shown for fibroblasts and cultured neurons, and this activity was attributed to the 403RERMS407 sequence (APP_{770} numbering), which is within the heparin-binding domain (Fig. I.4) (Ninomiya et al., 1994). Secreted APP has also been reported to be neuroprotective and sAPP is capable of protecting cells against Abeta or glutamate-mediated neuronal damage (Furukawa et al., 1996). The sAPP? fragment was reported to be 100-fold more effective than sAPP? in protecting hippocampal neurons against Abeta mediated toxicity. This may be due to the VHHQK heparin-binding domain (aa 12-16 of Abeta) which is present on sAPP? but not sAPP? (Ohsawa et al., 1997).

Functions of Abeta

Biological activities of Abeta seem to be critically dependent on its state of aggregation, with its dimeric or oligomeric (protofibrilar) forms possessing cytotoxic properties (Roher *et al.*, 2000; Walsh *et al.*, 2000). Interestingly, the neurotoxic properties of injected Abeta on primate brains only appeared in aged but not in young animals, suggesting that aged brains may be more vulnerable to Abeta-mediated toxicity (Geula *et al.*, 1998). However further complexity is added to the system being that weak neurotrophic properties were also attributed to the Abeta soluble form (Atwood *et al.*, 2003).

Several studies demonstrated that Abeta affects neuronal signalling. For instance, physiological concentrations of Abeta increased tyrosine phosphorylation and phosphatidylinositol 3-kinase activity in cultured cortical neurons (Luo *et al.*, 1995). Abeta1-42 activated phospholipase A2 (PLA2) (Lehtonen *et al.*, 1996) and Abeta1-40 maximally activated NF-?B (an immediate-early transcriptional regulator of numerous proinflamatory genes) in primary neurons (Kaltschmidt *et al.*, 1997). Other studies report Abeta induced rapid changes in intracellular Ca^{2+} levels and ecto-PKC (Wolozin *et al.*, 1995).

I.3 THE CYTOPLASMIC DOMAIN OF APP

The functions attributed to AICD are related either to its phosphorylation or to interactions with other proteins. The carboxy-terminus of APP contains three functional motifs corresponding to phosphorylation sites, critical for interaction with binding proteins that are thought to regulate the rate of APP secretion, endocytosis, and Abeta production (da Cruz e Silva *et al.*, 2004). The ^{653}YTSI^{656} (APP_{695} isoform numbering) sequence matches YXXI tyrosine-based internalization and/or basolateral sorting signal. The second is the motif ^{667}VTPEER^{672}, containing the Thr-668, which is important for controlling interactions with APP binding proteins. The third domain ^{682}YENPTY^{687}, which is absolutely conserved across APP homologues and across species (Fig. I.5), contains a NPXY motif, which is found in several cell surface proteins, including growth factor receptors, transporters and adhesion molecule receptors.

The NPXY sequence was first identified as the sequence required for internalization of LDL receptors. In some cases, NPXY motifs are required for anchoring of receptors in clathrin-coated pits (Chen *et al.*, 1990). NPXY motifs are also important for the function of some molecules, e.g. in insulin-like growth factor receptors. The NPXY motif is required for efficient ligand-mediated internalization and biological signalling (Hsu et al., 1994). In the cytoplasmic tail

of integrin ?3 the NPXY sequence is essential for post-ligand-binding events involved in cell migration (Filardo *et al.*, 1995). Furthermore, the NPXY motifs found in many integrin ? subunits regulate the affinity for their ligands. Interestingly, an NPXY sequence in the integrin ?1 cytoplasmic domain is required for localization to focal adhesions (Reszka *et al.*, 1992), which in turn is necessary for integrins accurate functioning. In the EGF receptor, tyrosine phosphorylation of NPXY is required for recognition by Shc and subsequent signalling (Russo *et al.*, 2002).

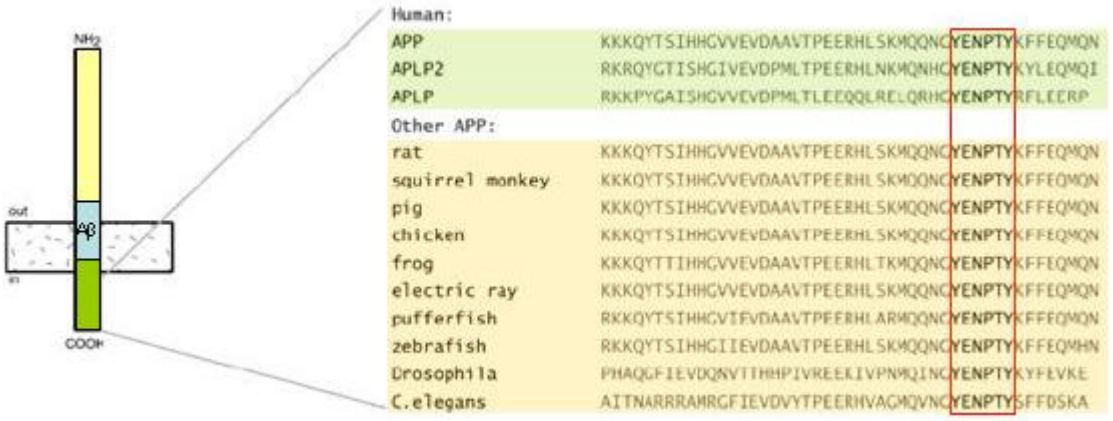

Figure I.5: *The short cytoplasmic tail of APP and its homologues contains the YENPTY sequence, which is phylogenetically conserved.*

The YENPTY sequence was also demonstrated to be important in the regulation of APP processing and trafficking (Lai *et al.*, 1995)). APP deletions within the YENPTY sequence results in increased secretion of sAPP and decreased secretion of A? (Koo and Squazzo, 1994). The effects of these deletions are thought to be the result of altered internalization of APP from the cell surface. Mutation of the second tyrosine in the YENPTY sequence to alanine also increases sAPP secretion but has no effect on A? secretion (Jacobsen *et al.*, 1994), suggesting that secretion of A? and sAPP may be regulated independently by signals in the cytoplasmic tail of APP. These observations bring together the AICD YENPTY-dependent regulation and the AICD role in AD.

I.4 AICD-BINDING PROTEINS

Given the characteristics of the APP cytoplasmic tail, the complex network of protein-protein interactions that centred around it became an exciting new target for therapeutic intervention (Russo *et al.*, 1998; Annaert and De Strooper, 2002). Many proteins, indeed, interact with this C-terminal domain of APP, most of them possessing multiple protein-protein interacting domains, which in turn form complexes with other proteins. This suggests that these proteins function as adaptor proteins bridging APP to specific molecular pathways.

Several laboratories have used the AICD as ''bait'' in the yeast hybrid systems, identifying two major families of APP binding proteins, the FE65 proteins and the X11/Mint proteins (Fiore *et al.*, 1995; Bressler *et al.*, 1996; Guenette *et al.*, 1996; McLoughlin and Miller, 1996; Borg *et al.*, 1998b; Lau *et al.*, 2000a; Lau *et al.*, 2000b; Mueller *et al.*, 2000; Minopoli *et al.*, 2001; Zambrano *et al.*, 2001).

Examples of APP binding proteins involved in signal transduction are: Abl, Disabled-1 protein (mDab-1), Fe65 and Fe65-like proteins, X11 (or MINT-1) and X11-like proteins (or MINT-2 and 3), Numb, G0 and the UV-damaged DNA-binding protein (Fig.I.6) (Turner *et al.*, 2003).

Several APP binding proteins are involved in APP subcellular localization including PAT1, Kinesin and JNK/JIP-1 (Jun N-terminal kinase (JNK) interacting protein 1) and the X11/MINT family proteins (Fig.I.6), the latter with a role in microtubule association, and recently found putative functions as an APP vesicle coat-protein (Okamoto and Sudhof, 1997; da Cruz e Silva *et al.*, 2004). The already mentioned binding to G_0, which links APP to G-protein signalling, is likely to play a role in APP targeting, since G_0 and other related heterotrimeric G-proteins were found to be located at subcellular membranes domains that are specialized in the sorting of trafficking proteins (Turner *et al.*, 2003). The function of all the APP binding proteins has yet to be completely elucidated, but considerable contributions have already been made.

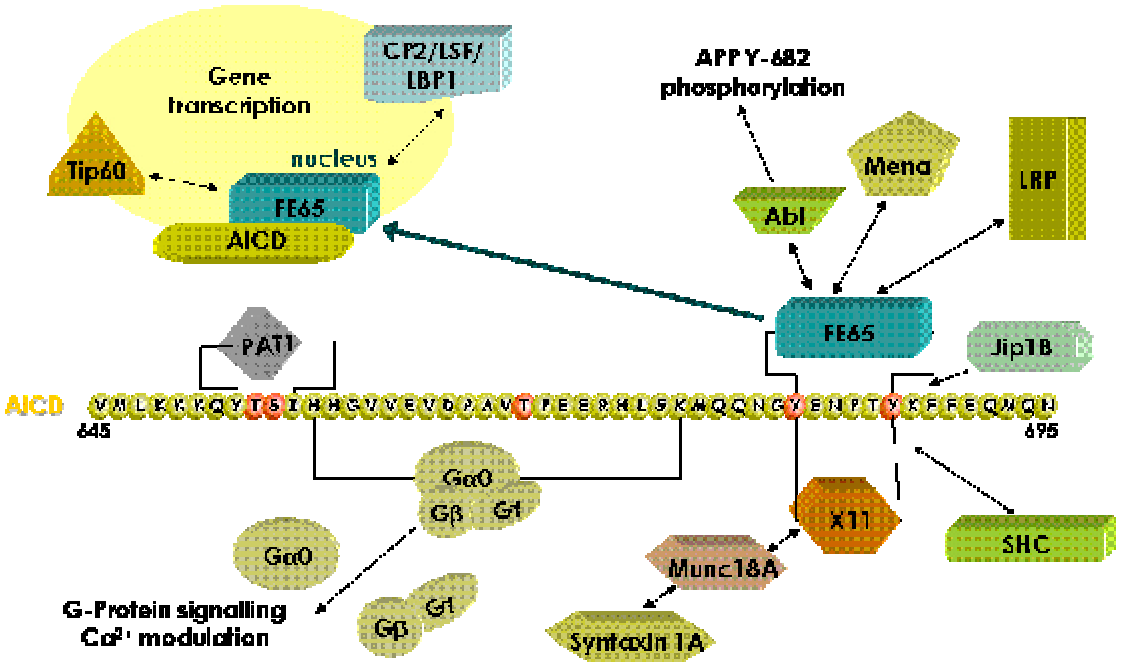

Figure I.6: *Protein network around the cytoplasmic domain of APP (adapted from (Turner et al., 2003)).*

FE65 protein family

FE65 is a multimodular adaptor protein, possessing three protein-protein interacting domains: a WW domain (a protein module with two conserved triptophans) and two tandem phosphotyrosine binding domains – PTB1 and PTB2 (or phosphotyrosine interaction domains – PID1 and PID2) (Bressler et al., 1996; McLoughlin and Miller, 1996). The Fe65 family comprises three members: FE65, FE65L1 and FE65L2, being all reported to interact with APP (Fiore et al., 1995). Whereas, the FE65L1 and FE65L2 are ubiquitously expressed the FE65 is neuronally enriched and a splice variant of FE65 (E9) is neuronal specific (Fiore et al., 1995; Duilio et al., 1998).

The most extreme C-terminal phosphotyrosine binding domain (PTB2) of FE65 is responsible for the interaction with the Alzheimer's Amyloid Precursor Protein (APP) intracellular domain, through the latter's YENPTY motif. The

pathways for APP processing are particularly important with respect to the generation of the Abeta peptide, which is deposited in the Alzheimer's disease (AD) brain. The amyloidogenic processing, with the subsequent Abeta production is affected by the interaction of APP with FE65 (Sabo et al., 1999). Likewise FE65 also appears to bind to APP like proteins (APLP1 and APLP2) (Duilio et al., 1998).

Interaction of the FE65 PTB2 domain with APP requires the YENPTY motif as well as Thr-668, 14 residues N-terminal to the internalization sequence. Phosphorylation of APP Thr-668 impairs FE65 interaction suggesting that adaptor protein interactions with APP are differentially regulated by phosphorylation states. APP has been demonstrated to act as a cytosolic anchor for FE65, able to regulate its nuclear translocation (Ando et al., 2001).

Besides APP-binding, FE65 in turn forms complexes with other proteins, suggesting that it bridges APP to specific molecular pathways (Fig. I.7). For instance, FE65 has been found to be associated with Neurofibrillary Tangles (NFTs) in AD. The main constituent of NFTs is Tau, a protein involved in neurite morphogenesis, axonal growth and axonal transport (Shahani and Brandt, 2002; Stamer et al., 2002). In AD Tau is hyperphosphorylated, which favours expansion of NFTs. Barbato et al. demonstrated the interaction of FE65 PTB1 domain with N-terminal domain of Tau in vivo and in vitro. The physical interaction between the adaptor protein FE65 and Tau is dependent on microtubule network integrity and is regulated by Tau phosphorylation, apparently via the proline-directed kinases GSK3ß and Cdk5, since they are reported to be complexed with phosphorylated Tau (Hamdane et al., 2003; Barbato et al., 2005). This interaction suggests that FE65 bridges Tau to APP, representing a functional link between the two hallmarks of AD, namely NFTs and amyloid plaques.

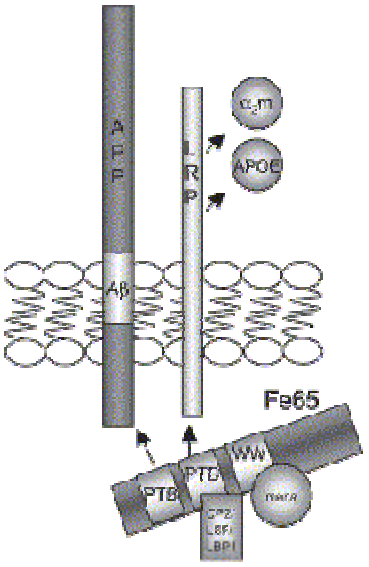

Figure I.7: *FE65 contains multiple protein interaction domains: the PTB1 domain interacts with the transcription factor complex CP2/LSF/LBP1 and with LRP, linking FE65 to ?2M and ApoE; the PTB2 domain interacts with APP; the WW interacts with Mena, thus linking FE65 and APP to actin (adapted from (King and Scott Turner, 2004).*

The PTB1 domain of FE65 binds the low density lipoprotein receptor-related protein (LRP), a transmembrane glycoprotein which mediates the internalization and degradation of extracellular ligands, including a-2-macroglobulin, apolipoprotein E and KPI-containing isoforms of APP (Trommsdorff et al., 1998; Herz and Strickland, 2001). LRP interacts with APP also by an extracellular ligand-receptor interaction. FE65 in turn interacts with the cytoplasmic tails of APP and LRP, acting as a bridging protein. Pietrzik et al. demonstrated that FE65 can also be a functional linker between APP and LRP and the APP-FE65-LRP complex formation is critical for APP processing (Pietrzik et al., 2004).

The WW domain of FE65 binds proline-containing motifs (PPXY or PPLP) of Mena (mammalian homologue of enabled), a protein involved in the regulation of actin dynamics (Ermekova et al., 1997). Mena belongs to the Ena/VASP family of proteins, which concentrate in focal adhesions and stress fibers and are found in dynamic actin remodeling areas, e.g. lamellipodia and axonal growth cones

(Ermekova et al., 1997). FE65 and APP colocalize with Mena in lamellipodia, which associates the FE65-APP complex to cytoskeletal dynamics and cellular motility and morphology (Sabo et al., 2001). Integrins are concentrated in lamellipodial adhesion sites, where they co-localize with FE65 and APP. FE65 PTB1 binds the ß1-integrin cytoplasmic domain, at one of the two NPXY motifs of the latter. Integrins are a family of cell adhesion receptors that mediate cell–matrix interactions, playing a role in cell proliferation, differentiation, and migration (Sabo et al., 2001). Another ligand for FE65 WW domain is the c-Abl tyrosine kinase, which phosphorylates FE65 on Tyr-547, in the PTB2 domain. Tyrosine kinase c-Abl is localized within the nucleus and also phosphorylates APP, on Tyr-682 (Zambrano *et al.*, 1997; Zambrano *et al.*, 2001; Perkinton *et al.*, 2004).

The FE65 N-terminal PTB1 domain binds the transcription factor CP2/LSF/LBP1, involved in the regulation of several genes. The FE65-CP2/LSF/LBP1 complex was found both in nuclear and non-nuclear fractions (Zambrano et al., 1998); however the ternary complex AICD–FE65–CP2/LSF/LBP1 can assemble in the nucleus, inducing GSK-3ß expression which can potentially increase Tau phosphorylation, contributing to AD (Kim et al., 2003). Other transcriptional factors may also be formed, for example the PTB1 FE65 domain functionally interacts with the histone acetyltransferase Tip60, forming the complex AICD–FE65–Tip60 which may regulate gene transcription (Cao and Sudhof, 2001). Alternatively, APP may anchor FE65 in the cytoplasm impairing its nuclear translocation (Minopoli et al., 2001).

X11/mint protein family

The X11/mint protein family comprises three members: X11? , ? , and ? or mint-1, -2, and -3 or X11, X11-L and X11-L2 (McLoughlin and Miller, 1996; Tanahashi and Tabira, 1999a, 1999b). X11 family members contain divergent N-terminal sequences but highly conserved C-termini consisting of a PTB domain and two PDZ domains. The “Mint” designation arose from interaction of X11 and

X11-L, but not X11-L2, with munc 18-1, a protein essential for synaptic vesicle docking and exocytosis (Biederer et al., 2002). While X11-L2 expression is ubiquitous, X11 and X11-L are expressed only in the brain (McLoughlin et al., 1999; Hase et al., 2002).

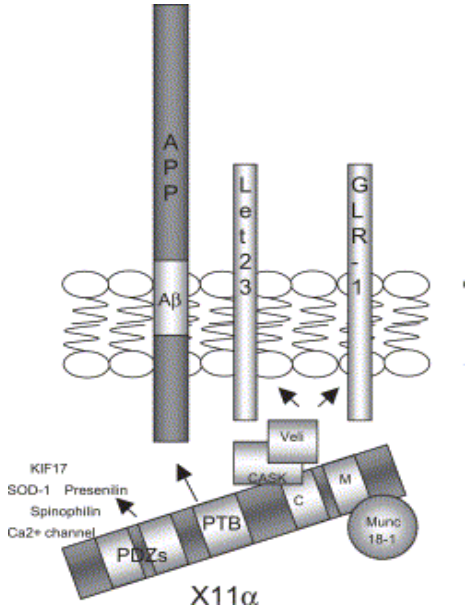

Figure I.8: *X11 contains multiple protein interaction domains: the PTB domain interacts with the APP, the munc-interacting domain (M) interacts with munc 18-1; the CASK-interacting domain (C) interacts with CASK-Veli complex. The two PDZ domain interact with several proteins (adapted from (King and Scott Turner, 2004).*

Besides binding to the YENPTY of APP, through its PTB domain, several other proteins have been reported to interact with X11 (Fig. I.8), for instance it interacts with CASK–Veli to form a heterotrimeric complex that may target transmembrane receptor proteins in polarized cells (Borg et al., 1998a).

A yeast two-hybrid screen of a brain cDNA library using the X11 PTB domain as bait reveals a specific interaction with APP, APLP-1, and APLP-2 (Borg et al., 1996; McLoughlin and Miller, 1996; Tomita et al., 1999). In contrast, the PDZ domains of X11 may interact with several proteins (Fig. I.8) including presenilin-1 (Lau et al., 2000a), spinophilin–neurabin II, the copper chaperone of SOD1, the dendritic kinesin KIF-17 and, via X11-CASK-Veli, to the N-methyl-D-

aspartate receptor NR2B subunit (King and Scott Turner, 2004). X11 was also reported to potentially interacting with itself by PDZ domain dimerization (Walhout et al., 2000). The X11/MINT protein family was implicated in APP vesicle coat-protein (Okamoto and Sudhof, 1997; Hill et al., 2003). Several X11 binding proteins mediate synaptic functions, implying an adaptor role for X11 in the pre- and postsynaptic complex.

I.5 PROTEIN PHOSPHORYLATION IN AD

Protein phosphorylation is a major control mechanism of eukaryotic organisms, allowing for rapid and reversible regulation of multiple protein activities in response to diverse environmental and developmental changes. Protein phosphorylation is a reversible process in which a protein kinase transfers a phosphate group from ATP to a substract, altering the conformation and function of the latter. A protein phosphatase removes the phosphate and the protein reverts to its dephosphorylated state. The phosphorylation state of a single protein depends on the balance between the highly regulated cellular activities of multiple protein kinases and protein phosphatases.

As knowledge on the molecular basis of AD expands, interest on protein phosphorylation continues to increase, as misregulation of normal phosphorylation/dephosphorylation control mechanisms is found to underlie an increasing number of pathologies. Since so many diseases have at their core a deficiency in cellular signalling involving protein phosphorylation, kinases have for some time been considered viable targets for the design of novel therapeutics, contrary to phosphatases, that only recently have started to be considered targets for the development of therapeutic strategies (da Cruz e Silva et al., 2004).

Brain ageing is characterized by a progressive decline in cognitive functions and memory loss. Protein phosphorylation may be one of the fundamental processes associated with memory and brain function, playing a role in the processing of neuronal signals and in the short-term or long-term modulation of

synaptic transmission. In neurodegenerative disorders such as AD there is evidence for abnormal regulation of protein phosphorylation, which appears to contribute to the disease condition (Wagey and Krieger, 1998).

The high levels of protein kinases and phosphatases in the brain suggest that phosphorylation is critically important in brain function. Misregulation of the cellular phosphorylation system has been reported to occur in AD. These include abnormalities in both expression and activity levels of kinases, and/or phosphatases, thus leading to alterations in the processing of APP and Abeta production (Gandy et al., 1993; da Cruz e Silva et al., 1995). For instance, altered activities of protein kinase C (PKC), decreased activity of phosphatases PP1 and PP2A, overexpression of calcineurin mRNA levels, protein Tau and Beta-tubulin hyperphosphorylated state have all been associated with AD (Gong et al., 1993; Matsushima et al., 1996; Bennecib et al., 2000; Vijayan et al., 2001; da Cruz e Silva and da Cruz e Silva, 2003). Concomitantly, many proteins that are relevant to AD, including APP, Tau, PS-1 and PS-2, or BACE, are phosphoproteins. It is also worth noting that alterations on the phosphorylation state of AICD were recently reported in the brains of AD patients (Lee et al., 2003).

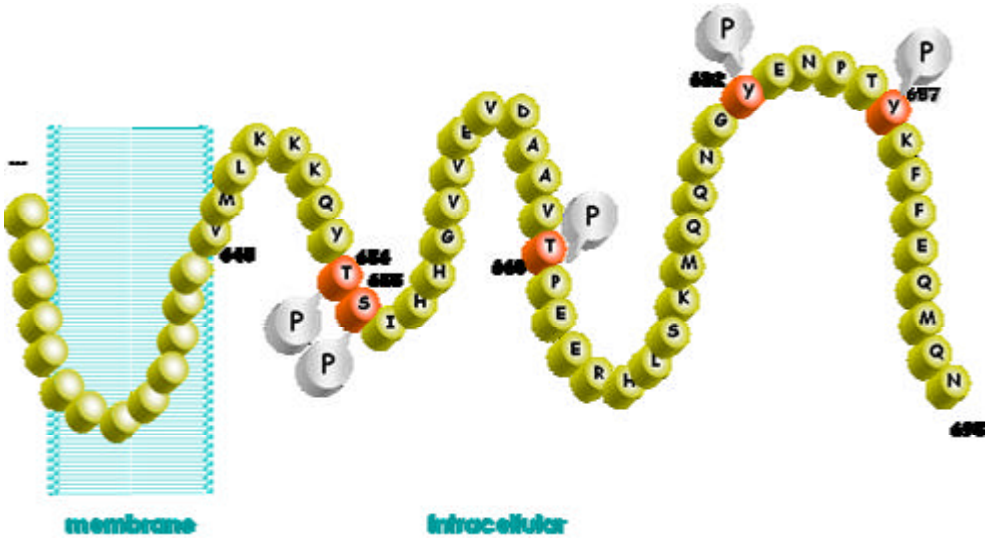

Figure I.9: *AICD phosphorylatable residues: Thr654, Ser655, Thr668 and Tyr682 and Tyr687.*

AICD has five potentially phosphorylatable residues , which belong to three APP functional motifs previously mentioned in section I.3: ^{653}YTSI^{656}, ^{667}VTPEER^{672}, and ^{682}YENPTY^{687} (Fig. I.9).

In the internalization signal domain ^{682}YENPTY^{687}, Tyr^{682} phosphorylation is a consensual site, in contrast with Tyr^{687} phosphorylation (Haass et al, 1993; Koo et al 1994; Minopoli et al, 2001). However Tyr^{687}, which is within the consensus sequence "NPTY", was recently reported to be phosphorylated *in vivo* (Lee *et al.*, 2003), concomitantly with results from our group (unpublished).

I.6 AIMS OF THE THESIS

In AD, phosphorylation/dephosphorylation of consensus sites in the intracellular domain of APP is believed to affect its subcellular localization and subsequent APP metabolism and Abeta production, probably through regulating the interactions with other proteins. We known from work in the laboratory (S. Rebelo *et al*, personal communication), that tyrosine-687 phosphorylation specifically affects subcellular localization of APP. The identification of neuronal specific binding proteins will contribute to elucidate some of the biochemical mechanisms controlling AICD function, specifically from a signal transduction perspective. Furthermore human AICD binding proteins represent potential targets for novel therapeutic approaches in AD.

The use of Yeast Two-Hybrid method for the identification of interacting proteins allows for the selection, among a large number of clones, from a human brain cDNA library, of proteins capable of interacting with a bait protein. Therefore the aim of this work was to identify brain proteins capable of interacting with the APP intracellular domain harbouring a mutation that mimics the phosphorylation state of tyrosine-687. To achieve this a YTH screen was carried out using as bait the AICD^{Y687E}. Therefore, the specific aims were as follows:

1. Construct the bait plasmid with AICD^{Y687E} cDNA in PAS2-1 vector, in frame with GAL4-BD, and transform it into Y187 yeast strain.

2. Verify the expression of the fusion protein GAL4-BD/AICD^{Y687E} by immunoblotting.

3. Perform the Two-Hybrid Screen using large-scale yeast mating with a pretransformed brain library.

4. Identify the positive clones.

5. Confirm the interactions between selected positive clones and AICD^{Y687E}, and also with the counterpart peptides, wild-type AICD and AICD^{Y687F} (also inserted in pAS2-1 vector).

II YEAST-TWO HYBRID SCREEN

II.1 INTRODUCTION

In order to find new interactions between APP and other proteins a screen using the MATCHMAKER GAL4 Two-Hybrid System was carried out for a human brain library obtained from Clontech. Several steps were required to prepare the YTH screen, namely, bait plasmid construction and transformation into Y187 yeast strain. The transformants were assayed for *HIS3*, *ADE2* and *MEL1* reporter genes' activation and the bait protein expression was verified by immunoblotting, following preparation of the yeast protein extracts. The YTH screen was then performed by yeast mating.

The vector used to insert the bait cDNA encoding $AICD^{Y687E}$, was Clontech's GAL4 binding domain expression vector pAS2-1 (Appendix IV). pAS2-1 has several characteristics that make it suitable for YTH. It has the GAL4-BD, two independent yeast and bacteria replication origins, it confers ampicillin and cycloheximide resistance and allows the yeast to grow without tryptophan in the culture media. It also has a multiple cloning site which used to insert the bait cDNA.

The cDNA for human $AICD^{Y687E}$ was amplified by PCR from a vector in the laboratory containing this APP mutant cDNA (pAV10).

II.2 CONSTRUCTION OF THE BAIT PLASMID

II.2.1 Materials and Methods

For the complete composition of all reagents, media and solutions used, see Appendix I. All the reagents were cell culture grade or ultrapure.

II.2.1.1 Isolation of pAS2-1 plasmid from bacteria - PROMEGA "Megaprep"

A 1L cell culture was pelleted by centrifugation at 1,500g for 20 min at RT. The cell pellet was resuspended in 30ml of Cell Resuspension Solution by manually disrupting the pellet with a pipette. 30ml of Cell Lysis Solution were added to the cells and the solution mixed gently by inverting until it became clear and viscous. Then, 30ml of Neutralization Solution were added and immediately mixed by inverting the tube. After centrifugation at 14,000g for 15 min at RT the clear supernatant was transferred by filtering through gauze swabs to a new tube and the volume of supernatant was measured. At this stage 0.5 volumes of RT isopropanol were added and the solution mixed by inversion. This solution was centrifuged at 14,000g for 15 min at RT, the supernatant was discarded and the pellet resuspended in 4ml of TE buffer. 20ml of Wizard™ Megapreps DNA purification resin were added to the DNA and mixed by swirling. A Wizard ™ Megacolumn was inserted into the vacuum manifold port and the DNA/resin mix was transferred into the Megacolumn. Vacuum was applied to pull the mix through the Megacolumn. Two washes with 25ml of Column Wash Solution were performed and the resin was rinsed with 10ml of 80% ethanol. The Megacolumn was inserted into a 50ml screw cap tube and centrifuged at 2,500 rpm for 5 min using a swinging bucket rotor centrifuge. The Megacolumn was placed in a clean tube and 3ml of pre-heated nuclease-free water (70ºC) were added to the

column. After waiting 1 min the DNA was eluted by centrifugation at 2,500 rpm for 5 min. The DNA was stored at –20ºC.

This procedure was used to prepare large amount of plasmid DNA for storage and subsequent cloning, yeast transformation and other purposes.

II.2.1.2 Plasmid DNA digestion with restriction enzymes

Plasmid DNA was sequentially digested with *Sma I* and *Nco I* with the DNA being precipitated between the digests.

For a typical DNA digestion the manufacturer's instructions were followed. In a microtube the following components were added:

- 100µg/ml DNA
- 1X reaction buffer (specific for each restriction enzyme)
- 1U/µg DNA of restriction enzyme

The mixture was incubated at the appropriate temperature (30ºC for *Sma I*; 37ºC for *Nco I*) for a few hours (or overnight if convenient).

II.2.1.3 Plasmid DNA purification with ethanol

This method was used to concentrate nucleic acids as well as to purify them. Approximately 1/10 volume of 3M sodium acetate (pH 5.2) was added to the DNA solution to adjust the salt concentration, followed by 2 volumes of ice-cold ethanol. The solution was well mixed and stored at –20ºC for 30 min to allow the DNA precipitate to form. DNA was recovered by centrifugation at 4ºC for 15 min at 14,000 rpm. The supernatant was carefully removed without disturbing the pellet. The microtube was half filled with ice-cold 70% ethanol and recentrifuged at 14,000 rpm for 5 min. The supernatant was again removed and the pellet allowed to dry before being resuspended in sterile water.

II.2.1.4 AICD^{Y687E} cDNA amplification by PCR

The gene fragment was generated by PCR with the *Nco I* and *Sma I* restriction sites incorporated into the primers *NAAPC* and *APPCTERMIII*, respectively. The reaction was carried out in a 0.5ml tube where the following components were added:

- 10ng template DNA
- 10pmol *NAPPC* primer
- 10pmol *APPCTERMIII* primer
- 2µl dNTP's 10mM
- 1x reaction Taq buffer
- 2 Units of Taq
- H_2O to a final volume of 50µl

The PCR was then performed using the following conditions:

95ºC 4 min		
95ºC 30 sec	}	5 cycles
55ºC 30 sec		
72ºC 1 min		
95ºC 30 sec	}	25 cycles
60ºC 30 sec		
72ºC 1 min		

II.2.1.5 Insert digestion with restriction enzymes

The PCR product was sequentially digested with *Sma I* and *Nco I*, with a purification step between the two reactions, as described in sections II.2.1.2 and II.2.1.3.

II.2.1.6 Insert DNA purification – *QIAGEN DNA Purification kit*

The QIAGEN DNA Purification kit was used to purify DNA fragments from PCR and other enzymatic reactions. It allowed purification from primers, nucleotides, polymerases and salts by using QIAquick spin columns. Briefly, 5 volumes of buffer PB were added to 1 volume of the solution to be purified and mixed. The spin column was placed in a collection microtube and the sample was applied to the column and centrifuged for 1 min at 14,000 rpm to bind the DNA. The flow-through was discarded and the column was washed with 0.75ml of buffer PE, centrifuged for 1 min at 14,000 rpm and the flow-through discarded. The column was placed back in the same microtube and centrifuged again to remove traces of washing buffer. Then, the column was placed in a clean microtube, 50µl of H_2O were added and allowed to stand for 1 min. To elute the DNA, the column was centrifuged for 1 min at 14,000 rpm.

II.2.1.7 DNA ligation

Alkaline phosphatase treatment

In order to prevent self ligation of vector molecules, the digested plasmid DNA was incubated with shrimp alkaline phosphatase (SAP) (ROCHE) before ligation. According to the manufacturer's instructions, the reaction mixture was adjusted with 1/10 volume 10X concentrated dephosphorylation buffer, and incubated with 1µl of SAP at 37ºC for 1 hr. Finally, SAP was inactivated by heating the reaction mixture at 65ºC for 15 min.

DNA Ligation

To carry out the ligation reaction, 50ng of vector DNA were transferred to a microtube with three times the equimolar amount of insert DNA. 2µl of 10X

bacteriophage T4 DNA ligase (PROMEGA) buffer and 1µl of bacteriophage T4 DNA ligase were added to the reaction mix and H_2O was added to a final volume of 20µl. The reaction was carried out for 16 hr at 16ºC. One additional control reaction was set up that contained the plasmid vector alone.

II.2.1.8 Bacteria transformation with plasmid DNA

Preparation of *E. coli* competent cells

A single colony of *E. coli* XL1-Blue was incubated in 10ml of SOB medium at 37ºC overnight. Then, 1ml of this culture was used to inoculate 50ml of SOB and the culture was incubated at 37ºC with shaking 220 rpm for 1-2 hr, until OD_{550nm}=0.3. The culture was cooled on ice for 15 min and centrifuged at 4,000 rpm at 4ºC for 5 min. The supernatant was discarded and then resuspended in 15ml of Solution I. After standing on ice for 15 min, the cells were centrifuged at 4,000 rpm for 5 min at 4ºC and 3ml of Solution II were added to resuspend the cell pellet. The cells were immediately divided in 100µl aliquots and stored at -80ºC.

Bacteria transformation with plasmid DNA

Competent cells (100 µl) were thawed on ice and 0.1-50ng of DNA were added to the cells and gently swirled. The microtube was incubated on ice for 20 min and heat shocked at 42ºC for 90 sec. The microtubes were then incubated on ice for 30 min before adding 0.9ml of SOC medium. The tubes were subsequently incubated at 37ºC for 30 min with shaking at 220 rpm. The culture was centrifuged at 14,000 rpm and the supernatant discarded. The cells were then resuspended in 100µl of the selective medium and spread on the appropriate agar medium. The plates were incubated at 37ºC for 16 hr until colonies appeared. Control transformations were also performed in parallel. These always included a negative control transformation without DNA and a positive control transformation with 0.1ng of a control plasmid, such as pAS2-1.

II.2.1.9 Isolation of plasmids from transformants "Miniprep"

In order to screen for the recombinant plasmid in the transformants, the plasmid DNA was extracted from several isolated bacterial colonies for subsequent restriction fragment analysis and sequencing.

Method 1 – Alkaline lysis "mini-prep"

A single bacterial colony was transferred into 3ml of LB medium containing ampicillin (100µg/ml) and incubated overnight at 37ºC with vigorous shaking (220-250 rpm). 1.5ml of this culture were transferred into a microtube and centrifuged at 14,000 rpm for 1 min at 4ºC and the supernatant was discarded. The cell pellet was resuspended in 100µl of ice-cold solution I by vigorous vortexing. Then, 200µl of freshly prepared solution II were added to the microtube that was mixed by inverting several times. Keeping the microtube on ice, 150µl of ice-cold solution III were added and again the microtube inverted several times. After the microtube was allowed to stand on ice for 5 min, it was centrifuged at 14,000 rpm for 10 min at 4ºC and the supernatant transferred to a clean microtube. The DNA was precipitated by adding 2 volumes of ice-cold ethanol. The mixture was vortexed and placed at -20ºC for 30 min. After centrifugation at 14,000 rpm for 10 min at 4ºC, the supernatant was completely removed and the pellet washed with 70% ethanol. Following centrifugation, the pellet was allowed to air-dry for 10 min. The DNA was dissolved in H_2O containing DNAase-free pancreatic RNAase (20µg/ml) and stored at -20ºC.

Method 2 – QIAGEN "miniprep"

The bacterial pellet was obtained as described above. The pellet was then resuspended in 250µl of buffer P1, 250µl of buffer P2 were added and the microtube was mixed by gently inverting until the solution became viscous and slightly clear. Afterwards, 350µl of buffer N3 were added and the microtube was repeatedly inverted until the solution became cloudy. The microtube was centrifuged for 10 min and the resulting supernatant was applied to a QIAprep

spin column placed in a microtube. After a 1 min centrifugation the flow-through was discarded. The column was washed by adding 0.75ml of buffer PE and centrifuged for 1 min to discard the flow-through, and then a subsequent 1 min centrifugation to remove residual wash buffer. Finally, the column was placed in a clean microtube and 50µl of H_2O were added to elute the DNA by centrifuging for 1 min having let it stand for 1 min. This method gives a cleaner DNA preparation than Method 1 with better yields. This method was used when the DNA was subsequently processed for DNA sequencing. For enzymatic restriction the first method was commonly employed.

II.2.1.10 Restriction fragment analysis of DNA

Plasmid DNA was analysed throughout the digestion with a convenient restriction endonuclease, namelly *Hind III*. For the plasmid DNA digestion the manufacturer's instructions were followed. In a microtube the following components were added:

- 100 µg/ml DNA
- 1X reaction buffer (specific for each restriction enzyme)
- 1U/µg DNA of restriction enzyme

The mixture was incubated at the appropriate temperature (37ºC for *Hind III*) for a few hours.

II.2.1.11 Electrophoretic analysis of DNA

The electrophoresis apparatus was prepared and the electrophoresis tank was filled with enough 1X TAE to cover the agarose gel. The appropriate amount of agarose was transferred to an Erlenmeyer with 50ml 1X TAE. The slurry was heated until the agarose was dissolved and allowed to cool to 60ºC before adding

ethidium bromide to a final concentration of 0.5µg/ml. The agarose solution was poured into the mold and the comb was positioned. After the gel became solid the comb was carefully removed and the gel mounted in the tank. The DNA samples were mixed with the 6X loading buffer (LB) (0.25% bromophenol blue/ 30% glycerol in water) and the mixture was loaded into the slots of the submerged gel using a micropipette. Marker DNA (1kb ladder or ?-Hind III fragments) of known size was also loaded into the gel. The lid of the gel tank was closed and the electrical leads were attached so that the DNA migrated towards the anode. The gel was run until the bromophenol blue had migrated the appropriate distance through the gel. At the end, the gel was examined by UV light and photographed or analysed on a Molecular Imager (Biorad).

II.2.1.12 DNA sequencing

All the DNA samples to be sequenced followed the same protocol. If the DNA was obtained by the "alkaline lysis miniprep" method and had not been purified by QIAGEN miniprep spin column (section II.2.1.9), it was purified in a QIAquick spin column (QIAGEN DNA Purification Kit) as described bellow.

QIAGEN DNA Purification kit

Briefly, 5 volumes of buffer PB were added to 1 volume of the solution to be purified and mixed. The QIAquick spin column was placed in a collection microtube and the sample was applied to the column and centrifuged for 1 min at 14,000 rpm to bind the DNA. The flow-through was discarded and the column was washed with 0.75ml of buffer PE, centrifuged for 1 min at 14,000 rpm and the flow-through discarded. The column was placed back in the same microtube and centrifuged again to remove traces of washing buffer. Then, the column was placed in a clean microtube, 50ml of H2O were added and allowed to stand for 1 min. To elute the DNA the column was centrifuged for 1 min at 14,000 rpm. The DNA was stored at -20ºC.

Sequencing PCR reaction

In a 0.5ml microtube the following components were added:

- 500ng dsDNA
- 4µl of Ready Reaction Mix*
- 3.2pmol primer
- H_2O to a final volume of 20µl

* Ready Reaction Mix is composed of: dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA polymerase, FS, rTth pyrophosphatase, magnesium chloride and buffer (Applied Biosystems).

This reaction mixture was vortexed and spun down for a few seconds. The PCR was then performed using the following conditions:

96ºC 1 min

96ºC 30 sec
42ºC 15 sec
60ºC 4 min
} 25 cycles

Afterwards, the samples were purified by ethanol precipitation (see above). Briefly, 2.0µl of 3 M sodium acetate (pH 4.6) and 50µl of 100% ethanol were added to the reaction microtube. The microtube was vortexed and incubated at RT for 15 min to precipitate the extension products. The microtube was then centrifuged at 14,000 rpm for 20 min at RT. After discarding the supernatant 250µl of 70% ethanol were added, the microtube was briefly vortexed and recentrifuged for 5 min at 14,000 rpm at RT. The supernatant was discarded and the pellet dried. After this procedure the DNA was ready to be applied in an Automated DNA Sequencer (ABIPRISM 310, Applied Biosystems).

II.2.2 Results

The resulting plasmid, named pAS-CYE, was sequenced to check the orientation of the AICD^{Y687E} and validate the reading frame of the fusion protein (Fig. II.1).

```
atgaagctactgtcttctatcgaacaagcatgcgatatttgccgacttaaaaagctcaag
M  K  L  L  S  S  I  E  Q  A  C  D  I  C  R  L  K  K  L  K

tgctccaaagaaaaaccgaagtgcgccaagtgtctgaagaacaactgggagtgtcgctac
C  S  K  E  K  P  K  C  A  K  C  L  K  N  N  W  E  C  R  Y

tctcccaaaaccaaaaggtctccgctgactagggcacatctgacagaagtggaatcaagg
S  P  K  T  K  R  S  P  L  T  R  A  H  L  T  E  V  E  S  R

ctagaaagactggaacagctatttctactgatttttcctcgagaagaccttgacatgatt
L  E  R  L  E  Q  L  F  L  L  I  F  P  R  E  D  L  D  M  I

ttgaaaatggattctttacaggatataaaagcattgttaacaggattatttgtacaagat
L  K  M  D  S  L  Q  D  I  K  A  L  L  T  G  L  F  V  Q  D

aatgtgaataaagatgccgtcacagatagattggcttcagtggagactgatatgcctcta
N  V  N  K  D  A  V  T  D  R  L  A  S  V  E  T  D  M  P  L

acattgagacagcatagaataagtgcgacatcatcatcggaagagagtagtaacaaaggt
T  L  R  Q  H  R  I  S  A  T  S  S  S  E  E  S  S  N  K  G

caaagacagttgactgtatcgccggtattgcaatacccagctttgactcatatggccatg
Q  R  Q  L  T  V  S  P  V  L  Q  Y  P  A  L  T  H  M  A  M

gtgatgctgaagaagaaacagtacacatccattcatcatggtgtggtggaggttgacgcc
V  M  L  K  K  K  Q  Y  T  S  I  H  H  G  V  V  E  V  D  A

gctgtcaccccagaggagcgccacctgtccaagatgcagcagaacggctacgaaaatcca
A  V  T  P  E  E  R  H  L  S  K  M  Q  Q  N  G  Y  E  N  P

accgagaagttctttgagcagatgcagaactgacccggg
T  E  K  F  F  E  Q  M  Q  N  ^^^
```

Figure II.1: *Partial sequence of the pAS-CYE construct. Human AICD^{Y687E} sequence is in blue; pAS2-1 sequence that is fused to AICD is in black; mutation site in Tyr-687 is in red;* Nco *I restriction site is in green and* Sma *I is in grey; Stop codon is in pink.*

II.3 EXPRESSION OF THE BAIT PROTEIN IN YEAST

In order to verify the ability of the recombinant constructs to drive $AICD^{Y687E}$ expression, they were transformed into the yeast strain Y187. The transformed cells were grown on the appropriate media and $AICD^{Y687E}$ expression was confirmed by immunobloting of the corresponding protein extracts. The fusion protein GAL4-BD/$AICD^{Y687E}$ was detected with the 369 antibody (that recognizes AICD), and an anti-rabbit secondary antibody conjugated to peroxidase. Detection of the GAL4-BD/$AICD^{Y687E}$ fusion protein was also carried out with the GAL4 DNA-BD antibody (Clontech) that recognizes the GAL4-BD. A protein extract from Y187 transformed with pAS2-1 plasmid was used as a positive control, and as a negative control the protein extract from untransformed Y187 yeast cells was used.

II.3.1 Materials and Methods

II.3.1.1 Yeast transformation with plasmid DNA

Preparation of competent yeast cells

One yeast colony was inoculated into 1ml of YPD medium in a 1.5ml microtube and vortexed vigorously to disperse cell clumps. The culture was transferred into a 250ml flask containing 50ml of YPD and incubated at 30ºC with shaking at 230 rpm overnight, until it reached stationary phase with OD_{600nm}>1. Enough of this culture (20-40ml) was transferred into 300ml YPD in a 2L flask to produce an OD_{600nm}=0.2-0.3. The culture was incubated for 3 hr at 30ºC with shaking at 230 rpm, centrifuged at 2,200 rpm for 5 min at room temperature and the supernatant was discarded and the cells resuspended in 25ml H_2O. The cells were recentrifuged and the pellet was resuspended in 1.5ml of freshly prepared, sterile 1X TE/LiAc.

Yeast transformation- Lithium acetate (LiAc)-mediated method

In a microtube 200ng of plasmid DNA were added to 100µg of herring testes carrier DNA. Then, 100µl of freshly prepared competent cells were added to the microtube, followed by 600µl of sterile PEG/LiAc (40% PEG 4000/ 1X TE/ 1X LiAc). The solution was incubated at 30ºC for 30 min with shaking (200 rpm). After adding 70µl of DMSO the solution was mixed gently and then heat-shocked for 15 min in a 42ºC water bath. The cells were pelleted after being chilled on ice, centrifuged for 5 sec at 14,000 rpm and resuspended in 0.5ml of 1X TE buffer. The cells (100µl) were then plated in the appropriate SD selection medium (e.g. SD/-Trp both for the plasmids pAS2-1 and pAS-CYE), and incubated at 30ºC for 2-4 days, until colonies appear.

II.3.1.2 Expression of proteins in yeast

Preparation of yeast cultures for protein extraction

A colony of each previously transformed yeast was inoculated in 5ml of the appropriate SD selection medium and incubated at 30ºC with shaking at 230 rpm overnight. As a negative control an untransformed yeast colony was inoculated in YPD. The overnight cultures were vortexed and separately added to 50ml aliquots of YPD. These cultures were incubated at 30ºC with shaking (220 rpm) until OD_{600nm}=0.4-0.6. At this point the cultures were quickly chilled by pouring them into a prechilled 50ml centrifuge microtube halfway filled with ice. The tubes were immediately centrifuged at 1,000g for 5 min at 4ºC. The supernatant was discarded and the cell pellet was washed in 50ml of ice-cold water. The pellet was recovered by centrifugation at 1,000g for 5 min at 4ºC and immediately frozen by placing tubes in liquid nitrogen.

Preparation of protein extracts

The cell pellets were quickly thawed by resuspending each one in 100µl of prewarmed cracking buffer (60ºC) per 7.5 OD_{600} units of cells (OD_{600} of a 1ml sample multiplied by the culture volume). The samples were briefly thawed in a 60ºC water bath. After 15 min an additional aliquot (1µl of 100X PMSF per 100µl of cracking buffer) of the 100X PMSF stock solution was added to the samples and every 7 min thereafter during the procedure. Each cell suspension was transferred into a 1.5ml microtube containing 80 µl of glass beads per 7.5 OD_{600} units of cells. The samples were heated at 70ºC for 10 min to release the membrane-associated proteins. Then, the microtubes were vortexed vigorously for 1 min and centrifuged at 14,000 rpm for 5 min at 4ºC. The supernatants were transferred to fresh microtubes and placed on ice. The pellets were boiled for 5 min, vortexed for 1min and centrifuged again, being the resulting supernatants combined with the first ones. The samples were boiled and loaded immediately on a gel.

II.3.1.3 SDS-PAGE

In order to visualize the fusion protein GAL4-BD/AICD^{Y687E}, with molecular weight around 26 kDa, 12% gels were used (Table II.1).

Table II.1: Composition of the running and stacking gels for SDS-PAGE.

Components	**Running gel (12%)**	**Stacking gel (3.5%)**
Water	10.35ml	6.60ml
30%Acryl./8%Bisacryl.	12.00ml	1.20ml
4X LGB	7.50ml	-------
5X UGB	-------	2.00ml
SDS 10%	-------	100.0µl
10% APS	150.0µl	100.0µl
TEMED	15.0µl	10.0µl

The 12% running gel was prepared by sequentially adding the components indicated on Table II.1 (APS and TEMED were added last, as they initiate the polymerising process). The solution was then carefully pipetted down the spacer into the gel sandwich, leaving some space (4 cm) for the stacking gel. Then, water was carefully added to cover the top of the gel and the gel was allowed to polymerise for 1 hr. The stacking gel was prepared according to Table II.1. The water was poured out and the stacking gel was added to the sandwich; a comb was inserted and the gel was allowed to polymerise for 30 min. Then, the samples were prepared by the addition of ¼?volume of loading gel buffer. The microtube was boiled and centrifuged, the combs removed and the wells filled with running buffer. The samples were carefully applied into the wells that were filled with running buffer, and the samples were run at 45mA until the bromophenol blue from the LB reached the bottom of the gel.

II.3.1.4 Immunoblotting

For immunoblotting the tank transfer system was used as follows: 3MM blotter paper was cut to fit the transfer cassette and a nitrocellulose membrane of the gel size was also cut. The gel was removed from the electrophoresis device and the stacking gel removed and discarded. The transfer sandwich was assembled under transfer buffer to avoid trapping air bubbles. The cassette was placed in the transfer device filled with transfer buffer. Transfer was allowed to proceed overnight at 200mA. Afterwards, the transfer cassettes were disassembled; the membrane carefully removed and allowed to air dry prior to further manipulations.

II.3.1.5 Immunodetection by enhanced chemiluminescence (ECL)

ECL™ is a light emitting non-radioactive method for the detection of immobilised antigens, conjugated directly or indirectly with horseradish peroxidase-labelled antibodies. In order to visualize the fusion protein GAL4 DNA-BD/AICD^{Y687E} two distinct blots were probed with different antibodies: GAL4 DNA-BD monoclonal antibody (Clontech) and 369 polyclonal antibody, that recognizes human AICD (a kind gift from Prof. Sam Gandy)

GAL4 DNA-BD monoclonal antibody (Clontech)

The membrane was soaked in 1X TBS for 10 min. Non-specific binding sites were blocked by immersing the membrane in 5% low fat milk in TBST for 2 hr. After washing with 1X TBST, the membrane was incubated with a solution of the primary antibody diluted in 5% low fat milk in TBST for 2 hr with shaking. After three washes of 10 min each in 1X TBST the membrane was incubated with a solution of the anti-mouse secondary antibody diluted (1:5000) in 5% low fat milk in TBST for 1 hr with shaking. The membrane was then washed 3 times for 10 min.

369 polyclonal antibody

The procedure was similar with the described above, but the membrane was blocked by immersing it in 5% low fat milk in TBST for 3 hr, with shaking, plus an overnight incubation at 4ºC. After washing with 1X TBST, the membrane was incubated with a solution of the primary antibody diluted in 5% low fat milk in TBST for 2 hr with shaking. After washing 6 times for 15 min in 1X TBST the membrane was incubated with the anti-rabbit secondary antibody diluted (1:5000) in 5% low fat milk in TBST for 1:30 hr with shaking. The membrane was then washed 6 times for 15 min.

Subsequently the membrane was incubated for 1 min at RT with the ECL detection solution (a mixture of equal volumes of solution 1 and solution 2 from the ECL kit, approximately 0.125ml/cm^2 membrane). Inside the dark room, the membrane was gently wrapped with cling-film, eliminating all air bubbles and placed in a film cassette and an autoradiography film (XAR-5 film, KODAK) was placed on the top. The cassette was closed and the blot exposed for short periods of time. The film was then removed and developed in a developing solution, washed in water and fixed in fixing solution. If needed, a second film was exposed more or less time according to the first result.

II.3.2 Results

The protein extracts produced in section II.3.1.2 were probed with GAL4 DNA-BD monoclonal antibody, which recognised the GAL4 DNA-BD peptide (Fig. II.2, A).

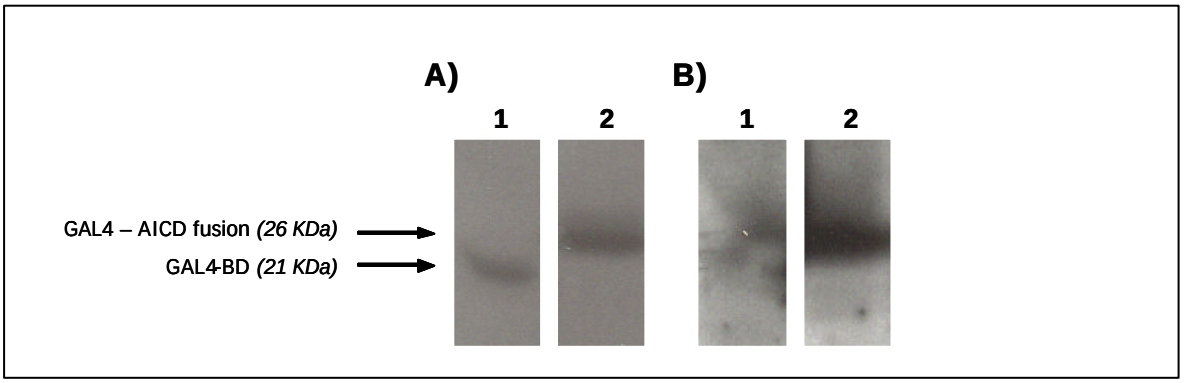

Figure II.2: *Immunoblot analysis of yeast protein extracts, probed with GAL4-BD antibody (**A**), and with 369 antibody **(B)**. Lane 1, control yeast transformed with pAS2-1 vector; Lane 2 , pAS-CYE clones. The calculated molecular mass (in kDa) of the fusion proteins is indicated.*

As expected for the control yeast, the peptide is smaller (21 KDa), because it's encoded by pAS2-1 vector without the fusion construct. When probed with 369

antibody, in the first lane the GAL4 is not detected. A band of the expected molecular mass (26 KDa) was detected in the protein extract from yeast cells containing the pAS-CYE plasmid (Fig. II.2, B, lane 2), demonstrating that the yeast are expressing the fusion protein.

The DNA-BD/Bait protein was tested for transcriptional activation. The bait constructs did not activate transcription from the UAS (the DNA sequence that is recognized by GAL4-BD) as detected by lack of growth of yeast cells containing the bait plasmid pAS-CYE on selective media SD/TDO/X-? -Gal and SD/QDO/ X-? -Gal (data not shown).

Once yeast cell expression of the fusion proteins GAL4-BD/AICD^{Y687E} was confirmed, the yeast two hybrid screen was performed.

II.4 TWO-HYBRID LIBRARY SCREENING USING YEAST MATING

II.4.1 Methods

II.4.1.1 cDNA library screening by yeast mating

A concentrated overnight culture of the bait strain (Y187+pAS-CYE) was prepared by inoculating a colony of the bait strain into 50ml of SD/-Trp and incubating it at 30ºC overnight with shaking at 250 rpm. The next day, when OD_{600}>0.8, the culture was centrifuged at 1,000g for 5 min, the supernatant was decanted and the pellet was resuspended in the residual liquid (5ml) by vortexing. The cells concentration (> $1X10^9$ cells/ml) was verified in a haemocytometer. Just prior to use, a frozen aliquot (1ml) of the library culture (Pretransformed Human Brain MATCHMAKER cDNA Library) was thawed in a room temperature water bath. The library was gently mixed and 10µl were set aside for later tittering (see below). The entire bait strain culture was combined with the 1ml library aliquot in a 2L sterile flask, 45ml of 2X YPDA were added and gently swirled. This culture was incubated at 30ºC for 20-24 hr, with shaking at 40 rpm. After 20 hr of mating a drop of the mating culture was checked under a phase-contrast microscope to check for the presence of zygotes, thereafter allowing the mating to proceed for more 4 hr. The mating mixture was transferred to a sterile 50ml tube and the cells spun down at 1,000g for 10 min. The mating flask was rinsed twice with 2X YPDA (50ml) and the rinses were combined and used to resuspend the first pellet. The cells were centrifuged again at 1,000g for 10 min, the pellet resuspended in 10ml of 0.5X YPDA and the total volume (cells + medium) was measured. Half of the library mating mixture was plated on SD/QDO (SD without Leu, Trp, Ade and His), and the other half on SD/TDO (SD without Leu, Trp and His), at 200µl per 150mm plate. For mating efficiency controls, 100µl of 1:10,000, 1:1,000; 1:100 and 1:10 dilutions of the mating mixture were plated in 100mm SD/-Leu, SD/-Trp and SD/-Leu/-Trp plates. All plates were incubated at 30ºC until colonies appeared,

generally 3-8 days on TDO and 8-21 days on QDO medium. Then, growth of the control plates was scored and the mating efficiency and number of clones screened were calculated. All positive clones were replated twice in SD/QDO medium containing X-a-Gal and incubated at 30ºC for 3-8 days. True positives formed blue colonies. The master plates were sealed with parafilm and stored at 4ºC. Glycerol stocks were prepared for all the positive clones.

II.4.1.2 Library titering

A library aliquot (10µl) was transferred to 1ml of YPDA in a 1.5ml microtube – dilution A (dilution factor 10^{-2}). 10µl from dilution A were added to 1ml of YPDA in another microtube and mixed gently – dilution B (dilution factor 10^{-4}). From dilution B, 100µl were spread onto three SD/-Leu plates. All the plates were incubated at 30ºC for 3 days after which the number of colonies was counted. The titer of the library was calculated using the following expression: ?#colonies?/?plating volume (ml)x dil factor? = cfu/ml.

II.4.2 Results

II.4.2.1 Mating efficiency and number of clones screened

After transforming the bait plasmids into the appropriate yeast strain, Y187 (mat ?), the next step was to obtain the desired library pretransformed in a yeast strain of the opposite mating type. In our case we used a pretransformed pACT-2 library in the yeast strain AH109 (mat a) containing human brain cDNA sequences fused to the GAL4 activation domain (AD).

The YTH screen was performed by yeast mating, whereby more unique positive clones are obtained, due primarily to the “jump-start” that the new

diploids receive before being plated on selective medium. Additionally, diploid yeast cells are more vigorous than haploid cells and can better tolerate the expression of toxic proteins. Also, in diploids, the reporters are less sensitive to transcription activation than they are in haploids, reducing the incidence of false positives from transactivating baits.

The human brain cDNA library was screened to identify new interacting partners for APP C-Terminal, in a phosphorylation-state dependent manner. The mating culture was observed under a phase-contrast microscope to check for the occurrence of zygotes (Fig. II.3), indicative that mating was occurring as expected.

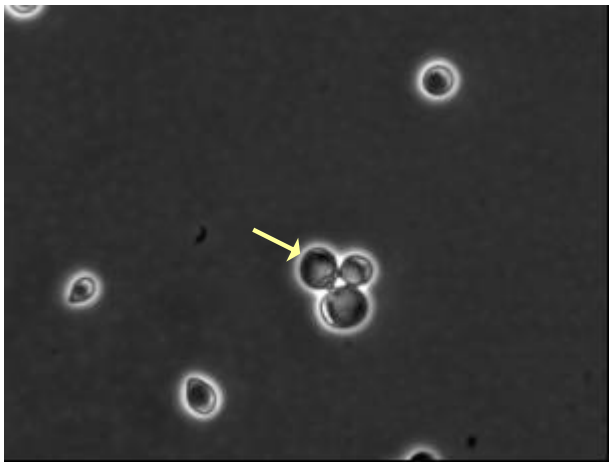

Figure II.3: *Zygote formation in the mating mixture with its typical three-lobed shape. The arrow is pointing the budding diploid cell. The other two lobes are the two haploid (parental) cells. This picture was taken using an inverted microscope in phase contrast mode, during the mating event (40X magnification).*

After plating the mating mixture in the appropriate selective media and waiting several days for colonies to appear, the growth on the control plates was scored and the mating efficiency and number of clones screened were calculated (Table II.2).

Table II.2: *Results from the yeast two hybrid screen.*

Positive clones	Mating efficiency (% diploids)	Clones screened
53	10%	1.1 X 10^8

To calculate de number of clones screened the following equation was used [# cfu/ml of diploids X resuspension volume]. To calculate the mating efficiency the following equation was used [# cfu (in SD –Leu/-Trp) X 1000µl/ml/ volume plated (µl) x dilution factor] / [# cfu (in SD -Trp) X 1000µl/ml/ volume plated (µl) X dilution factor] X 100.

II.4.2.2 Identification of AICD^{Y687E} interacting clones

In the YTH screen 239 clones were isolated, 206 from SD/TDO and 33 from SD/QDO plates (Fig. II.4). All the colonies were re-streaked in SD/QDO plates in order to test for the expression of the nutritional reporter genes HIS3 and ADE2. These clones were further tested for MEL1 expression, another reporter gene, by growing these putative primary positive clones in SD/QDO media with X-?-Gal. True positive clones turn a blue colour (Fig. II.5), resulting in 55 true positive clones, thus identified in this screen. The positive clones were named *CYE#*.

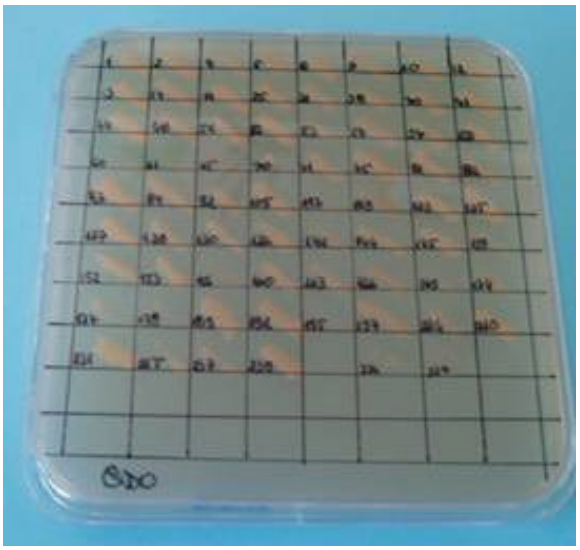

Figure II.4: *Positive clones identified in the yeast two hybrid screen. The clones were recovered from the original SD/TDO or SD/QDO plates.*

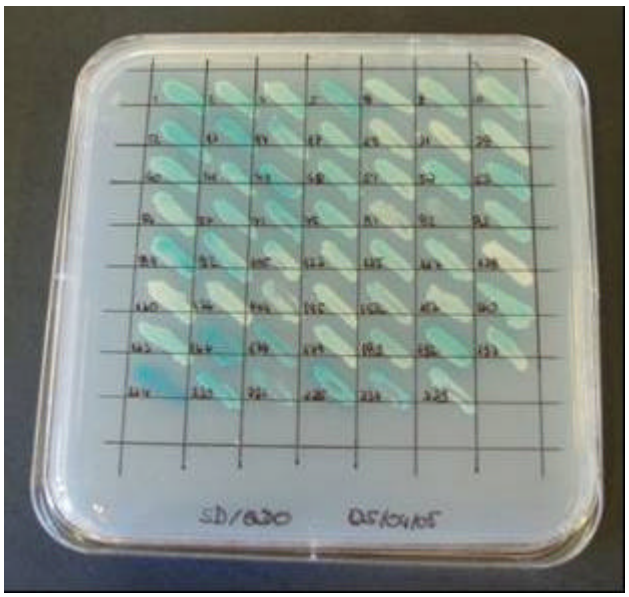

Figure II.5: *MEL-1 expression test of the positive clones obtained in the YTH screen. Light blue colonies also represent positive interactions, but took longer to turn blue in the presence of X-? -GAL.*

II.5 DISCUSSION

As with all detection methods, the YTH system is known to result in the detection of some false positives. This was a relatively serious problem in the early days of the YTH method but the elimination of such false positive results has been greatly improved recently. False positive signals result from cells in which the reporter genes are active even though the bait and prey do not interact. There are several classes of false positives. For example, false positives may arise from prey that interact with DNA upstream of the reporter genes or with proteins that interact with promoter sequences. These two classes of false positives can be eliminated by the use of more than one reporter gene under the control of different promoters, as was the case with the present work. Another inherent problem with the system is that not all proteins will be efficiently folded and/or post-translationaly modified in the yeast nucleus, which may result in the protein not interacting with the true partner. In the same way, the protein may adopt a different tertiary structure when expressed as fusions with the transcription factor domains. Also, some proteins may be toxic when expressed as fusions in yeast, inhibiting growth when expressed at high levels. This can be avoided to some extent by the use of inducible expression plasmids. Other false positive results include interactions that occur in the YTH screen but not in a physiological context, because the partners are not expressed in the same cellular or subcellular environment at the same time.

By screening 1.1 X 10^8 clones from a human brain cDNA library with $AICD^{Y687E}$ as bait 55 positive clones were obtained as accessed by their ability to grow on SD/QDO selective media and to turn blue in the presence of X-a-Gal. Given the conditions of our screen, these should include the AICD interactome when the former is phosphorylated. At this stage however interactions should be validated and the protein interactions may be confirmed. As such, supplementary data from other sources should be used to evaluate the credibility of interactions in an YTH screen. Thereby, the verification of a putative interaction can be achieved in a variety of ways. One approach is to mix the recombinant proteins

and verify binding *in vitro* through a variety of biochemical assays. Another approach is to express both proteins in cells by transfection and analyse interactions by immunoprecipitation studies. However, even if co-immunoprecipitation is successful, there is still the possibility that the proteins only interact under the conditions used. So, a crucial validation of the two-hybrid results is to prove that the two proteins exist in the same subcellular environment, by doing immunoprecipitation in the tissue of interest.

III CHARACTERIZATION OF THE POSITIVE CLONES

III.1 INTRODUCTION

The majority of the APP binding proteins that have been identified to date were discovered using the yeast Two-Hybrid (YTH) method. Often, when performing an YTH screen, only a few clones are selected and further characterized. Using such an approach many of the rarer positive clones are never analyzed, and some important potential interacting proteins may be missed.

In this screen 55 positive clones were obtained and we decided to analyse all the positive clones in order to identify not only the most abundant clones but also the more interesting ones, even though they may have been detected only once or twice in the screen. This does not mean they are not important but may simply reflect the low abundance of the mRNA in the library used or its low abundance in the tissue from which the library was made.

All positive clones were partially sequenced and some interactions were further confirmed.

III.2 MATERIALS AND METHODS

For the complete composition of all reagents, media and solutions used, see Appendix I. All reagents were cell culture grade or ultrapure.

III.2.1 Plasmid isolation from yeast and transformation into bacteria

"Breaking Buffer" Method

Yeast plasmid DNA was extracted by resuspending cells in 0.2ml of breaking buffer, adding 0.3g of 0.5mm acid-washed glass beads plus 0.2ml 25:24:1(v/v/v) phenol/chloroform/isoamyl alcohol and vortexing for 4 min before centrifuging for 5 min. The upper layer was transferred to a new microtube and once more, 0.2ml 25:24:1(v/v/v) phenol/chloroform/isoamyl alcohol were added, followed by vortexing for 2 min and centrifugation for 5 min. The upper layer was transferred to a new microtube and 0.2ml chloroform were added, vortexed for 1 min and centrifuged for 5 min. The DNA in the upper layer was ethanol precipitated.

"High Efficiency Yeast Plasmid Rescue" Method

An alternative method was used for isolating yeast plasmid DNA: 3ml of yeast cells were pelleted and the DNA extracted using the QUIAGEN kit. The pellet was resuspended in 250µl of buffer P1, added about 250µl of 0.5mm acid-washed glass beads and vortexed on high for 5 min. Afterwards, 250µl of buffer P2 were added and the microtube was mixed by gently inverting until the solution became viscous and slightly clear. Then 350µl of buffer N3 were added and the microtube was repeatedly inverted until the solution became cloudy. The microtube was centrifuged for 10 min and the resulting supernatant was applied to a QIAprep spin column placed in a microtube. After a 1 min centrifugation the flow-through

was discarded. The column was washed by adding 0.75ml of buffer PE and centrifuging 1 min to discard the flow-through. The column was centrifuged for an additional 1 min to remove residual wash buffer. Finally, the column was placed in a clean microtube and 50µl of H_2O were added to elute the DNA by centrifuging for 1 min having let it stand for 1 min.

Both methods were performed to carry out yeast plasmid extraction, the Breaking buffer method was extensively used, except for clones CYE163, CYE166 and CYE174, in an attempt to improve the transformation efficiency.

Plasmid DNA was transformed in *E. coli* XL1-Blue competent cells and plasmid DNA was isolated from single colonies as described in sections II.2.1.8-9.

III.2.2 Analysis of the positive plasmids by restriction digestion, sequencing and database searching

Plasmid DNA was digested with the restriction endonuclease *Hind III*, and fragments produced were separated by agarose gel electrophoresis as described in Chapter II.

The vector used (pACT-2) produced a characteristic pattern of fragments that allowed its differentiation from colonies resulting from transformation by the bait vector. Plasmids generating DNA fragments characteristic of the pACT-2+library insert digested with *Hind* III were further sequenced. Sequencing was performed as described in section II.2.1.12, using GAL4-AD primer (Clontech).

A search for similar sequences in the Genbank database was performed using the BLAST algorithm (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990).

III.2.3 Verifying protein interactions in yeast by yeast mating

To verify protein interactions, yeast mating was performed combining the bait plasmid pAS-CYE in Y187 with the AD/library positive clones in AH109. Additional Two-Hybrid tests were carried out with the wild-type AICD and the $AICD^{Y687F}$, to compare the relative strength of the interaction. The $AICD^{Y687F}$ mutant mimics the dephosphorylated state of Y-687. To achieve this assays, wild-type AICD cDNA and $AICD^{Y687F}$ cDNA were inserted in PAS2-1 vector, in frame with GAL4-BD (named pAS-CWT and pAS-CYF, respectively) and transformed in Y187 yeast strain (data not shown).

The AD/library plasmids to be tested were transformed into AH109 and selected on SD/-Leu. For each one, pair wise yeast matings were set up with Y187 containing the plasmids pAS-CWT, pAS-CYF and the bait pAS-CYE. One colony of each type were combined in 1.5ml YPD medium, vortexed and incubated at 30ºC for 24 hrs with shaking (180 rpm). A 100µl aliquot of each mating culture was spread on SD/-Trp/-Leu and incubated at 30ºC for 3 days to allow diploid cells to form visible colonies.

To confirm protein-protein interactions, the fresh diploid colonies were assayed for growth on SD/QDO plates and for X-a-Gal activity.

III.3 RESULTS

III.3.1 Preliminary analysis of the positive clones

In order to identify the library insert present in a given positive clone, the plasmid DNA was first isolated from yeast. Thus, a mixture of different plasmid DNAs can be isolated from a single yeast clone, namely the bait plasmid (Figure III.1, lanes 3 or 4) and one or more library plasmids (Figure III.1, lane 6), being that each yeast cell can incorporate more than one library plasmid. Hence, in order to obtain single plasmids and pure DNA for sequence analysis, the plasmid DNA isolated from yeast cells was used to transform *E. coli* XL1-Blue. The plasmid DNA obtained from the resulting transformants was further analysed by restriction digestion with endonuclease *Hind III*. The restriction fragments were then separated by agarose gel electrophoresis. Figure III.1 exemplifies a typical result obtained after this procedure:

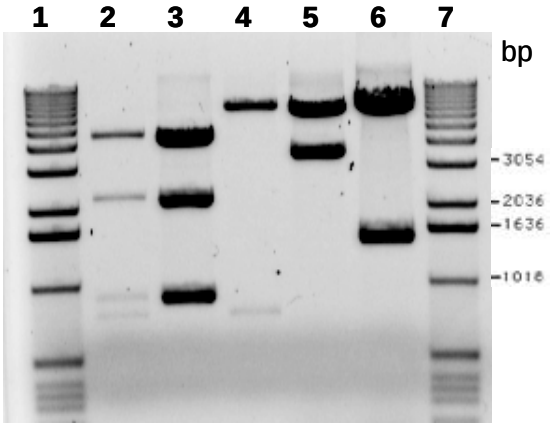

Figure III.1: *Hind III restriction analysis of yeast two hybrid plasmids. Lanes:*
1, 7: *1Kb ladder marker;*
2: *pAS2-1 vector (4.6+2.2+0.9+0,7 Kb);*
3: *pAS-CYE bait plasmid (4.6+2.2+0,9 Kb);*
4: *pACT-2 vector (7.4+0.7Kb);*
5: *pACT-2+library insert CYE12 (7.4+(0.7+ ~2.8 insert)Kb);*
6: *pACT-2+library insert CYE189 (7.4+(0.7+ ~0.7 insert)Kb).*

The same strategy was adopted for each positive clone. After identifying transformants carrying the cDNA library plasmids, their respective inserts were sequenced with the GAL4-AD primer (Appendix II). Figure III.2 is a representative example obtained with one of the positive clones, CYE12:

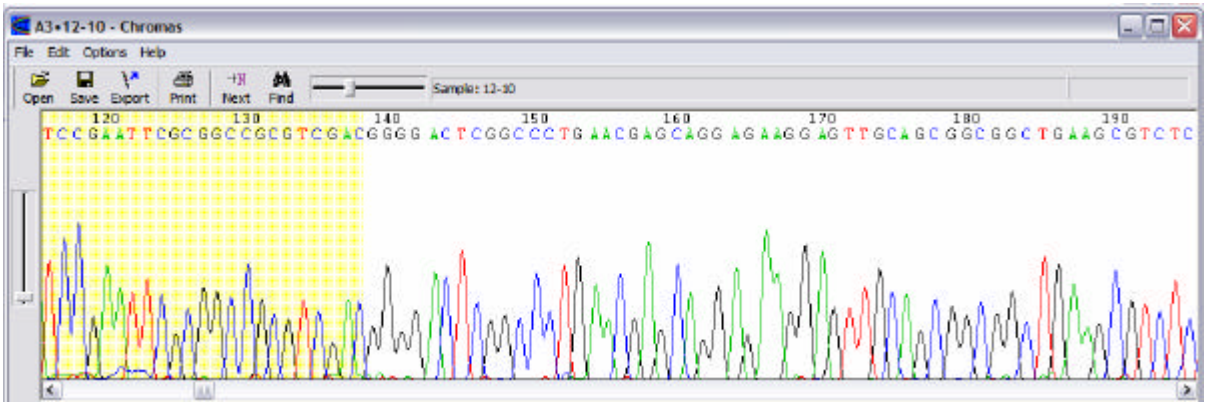

Figure III.2: *Nucleotide sequence of the positive clone CYE12. The yellow shaded area limits the vector pACT-2 and the library linker "GCGGCCGCGTCGAC".*

The nucleotide sequence of each clone was then converted to FASTA format (Fig. III.3). In this format, in the first line the signal ">" precedes the name or additional information on the sequence and the sequence itself starts on the second line.

```
>CYE12sequence
GGGGACTCGGCCCTGAACGAGCAGGAGAAGGAGTTGCAGCGGCGGCTGAAGCGTCTCTAC
CCGGCCGTGGACGAACAAGAGACGCCGCTGCCTCGGTCCTGGAGCCCGAAGGACAAGTTC
AGCTACATCGGCCTCTCTCAGAACAACCTGCGGGTGCACTACAAAGGTCATGGCAAAACC
CCAAAAGATGCCGCGTCAGTTCGAGCCACGCATCCAATACCAGCAGCCTGTGGGATTTAT
TATTTTGAAGTAAAAATTGTCAGTAAGGGAAGAGATGGTTACATGGGAATTGGTCTTTCT
GCTCAAGGTGTGAACATGAATAGACTACCAGGTTGGGATAAGCATTCATATGGTTACCAT
GGGGATGATGGACATTCGTTTTGTTCTTCTGGAACTGGACAACCTTATGGACCAACTTTC
ACTACTGGTGATGTCATTGGCTGNTGNGNTAATCTTATCAACAATACCTGCTTTTACNCC
AAGAATGGACATAGTTAAGGTATTGCTTTCCCTGACCTACCCCCAANTTGTATCCTACTG
NGGGGCTTTNAACCCCAGGANAAGGGTCGATGCCAANTTTGGGCAAATCCTTTCNGTTTG
ANAAAAAAAATNTTGCNGGGGNGGGNAACCNAATCCNGCCAAANANATCGATTTCNTTCG
AAATCGAAGGAAAANGGCNACCTTTNCCAAAAAGGTTNCNTTTTTANCCCCCGGGTCTGG
NCCAAAAAGCCTTTCAATTNAAANCAACGTTTAAAAATTNTNCTTNAAAANAAAATNAAA
TTGTTTCCGGAAAGGAG
```

Figure III.3: *Sequence of clone CYE12 in the FASTA format.*

The sequence in FASTA format was then copied to the BLAST window (www.ncbi.nlm.nih.gov/BLAST/) (Fig. III.4A) to be compared with the GenBank Database of nucleotide sequences. The identity of the positive clones isolated was

thus obtained. Figure III.4B shows a representative example of the results obtained for clone CYE12.

A) **B)**

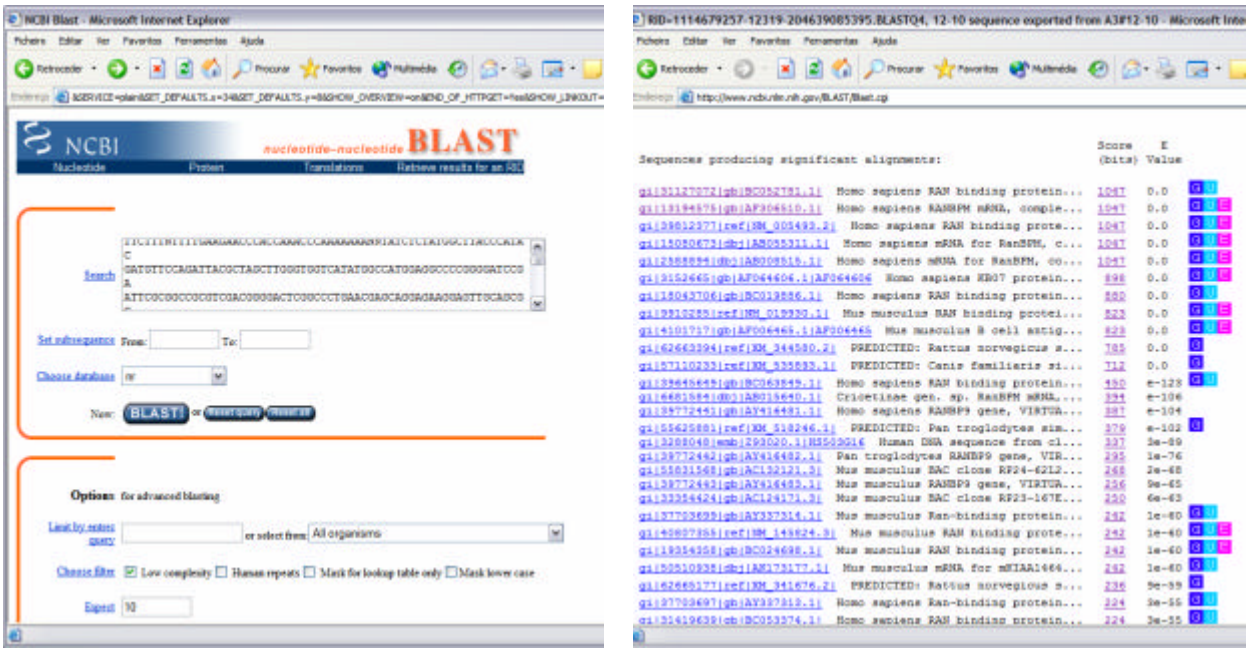

Figure III.4: *A, Blast window to introduce the query sequence; B, Blast results.*

III.3.2 Identification of the positive clones

Of the original 55 positive clones identified in the YTH screen, 53, were recovered and definitively identified by partial or full DNA sequence analysis. The results obtained are summarized in Table III.1.

Analysis of the 53 positives resulted in the identification of nucleotide sequences that could be divided into the following categories:

26 Clones matching a protein coding sequence (cds);

26 Clones aligning with non-coding and other genomic sequences;

1 Mitochondrial clone.

Table III.1: *Complete list of positive clones.*

Clone ID (CYE#)	N. clones	Selection Stringency	Insert (Kb)	Chromosome
known proteins				
1,9,10,12,13,14,17,25,40, 48,54,57,71,75,160,192	16	Medium	2,8	6
189,197	2	Medium	0,7	7
8,31,38,127	4	Medium	0,5	1
224,230,235	3	High	2,2	21
5	1	Medium	3,2	5
after CDS				
145	1	Medium	0,7	17
84,92	2	Medium	1,3	17
231	1	High	1,7	12
2	1	Medium	0,5	2
123	1	Medium	2,2	1
153	1	Medium	0,9	1
genomic				
51,53	2	Medium	1,5	X
177	1	Medium	1,1	19
239	1	High	1,8	17
136	1	Medium	2,8	15
82	1	High	3,1	12
144,152,174	3	Medium	3,1	12
237	1	High	1,8	12
130	1	Medium	1,5	9
4	1	Medium	1,3	9
44,52	2	Medium	0.5	7
105	1	Medium	1,8	5
41,81,125	3	Medium	1,5	3
83	1	Medium	2,8	2
mitochondrial				
128	1	Medium	0,9	
not analysed				
166	1	Medium		
163	1	High		

The identified clones were divided in five groups: proteins already known although not associated with APP (orange panel), clones that align with known proteins but outside of the coding sequence (CDS) (green panel), clones present in the database only at the genomic level (blue panel), a mitochondrial DNA sequence (yellow panel) and clones that, despite all efforts, analysis was not possible (grey panel). All clones were subject to the same analysis. They were

partially or completely sequenced, special features of the amino acid sequence were searched for using Motif and Domain search databases. The existence of ESTs (expressed sequence tags) was also ascertained by database searching.

Some of the positive clones identified were analyzed more thoroughly and these are discussed below.

III.3.3 Clones aligning with non-coding and 3'-UTR sequences

In various cases the searches performed did not reveal any homologies within the coding sequences (CDS) of know protein, or predicted gene product, for the sequenced insert portion of 26 clones (Table III.1, after CDS and genomic). Of these, 19 showed significant similarity only to human genomic clones. The remaining 7 clones (Table III.1, *in green*) matched mRNA sequences, although aligning with the 3'-UTR (untranslated regions).

The translation of these clones in all three forward reading frames revealed premature stop codons, therefore it may be possible that these small DNA fragments encode peptides with strong affinity to the C-terminal domain of APP. However, it cannot be ruled out that such a small peptide may instead interact directly with the GAL4-DNA Binding Domain. Further testing would be required to clarify this issue, such as co-expressing the plasmid with different bait proteins.

III.3.4 Clones matching a protein CDS

Of the 53 clones identified, 26 were assigned to known proteins (Table III.1, orange and yellow panels). The number of clones for each identified binding protein varied considerably, from 1 clone (CYE5) to 16 clones (e.g. CYE12) as discussed below. This group of clones include positives identified from high and

low stringency selection. The following sections address the characterization of these AICD interacting proteins.

Despite being considered a false positive, the mitochondrial clone (Table III.1, yellow panel), which matches a protein CDS, will also be discussed in this section.

Mitochondrial clone

The clone CYE128, obtained from the medium stringency selection, was found to match a mitochondrial DNA sequence, encoding an insert assigned to the Citochrome Oxidase 1 gene (COX1). Translation using the standard genetic code revealed premature stop codons. Mitochondria have a specific genetic code, different from the standard one used by the nuclear translation machinery, and as a result some mitochondrial amino acid codons are read as stop codons in cytoplasmatic translation (Anderson et al., 1981). Hence, in order to be detected in a two-hybrid assay, the plasmid encoded inserts must be expressed in the cytoplasm as GAL4-AD fusion proteins and exported to the nucleus, where they interact with a GAL4-BD-bait fusion to activate reporter gene expression, mitochondrial translation is unlikely to be occurring. Mitochondrial clones (including citochrome oxidase and 16S rRNA) have previously been described as common false positives in two-hybrid screens (Serebriiskii *et al.*, 2001). The presence of premature stops in all reading frames, corroborates the hypothesis that this clone is likely to be a false positive.

SDHB

Four positive clones (CYE8, CYE31, CYE38 and CYE127) were identified using the GenBank database as SDHB (succinate dehydrogenase complex subunit B), a component of Complex II of respiratory electron-tranport chain. SDHB is

localized in the mitochondrial inner membrane. Although SDHB a nuclear-encoded mitochondrial protein, the interaction with AICD is unlikely to occur in a physiologically relevant context, because the partners are not expressed in the same subcellular environment.

RanBPM/RanBP9

Sixteen out of the 53 identified clones encoded a fragment of the RanBPM protein (clones CYE1, CYE9, CYE10, CYE12, CYE13, CYE14, CYE17, CYE25, CYE40, CYE48, CYE54, CYE57, CYE71, CYE75, CYE160 and CYE192). The large cDNA fragment (approximately 2,8Kb) alignment was within the coding region, with the fusion protein being expressed in the correct reading frame. The first nucleotides after the linker sequence, GGG, encoded amino acid G-136, on the sequence with the NCBI accession number AB055311 (in green in Fig. III.5). The RanBPM gene maps to chromosome 6p23, and the RanBPM transcript is widely expressed, evaluating by EST (Expressed Sequence Tag) data.

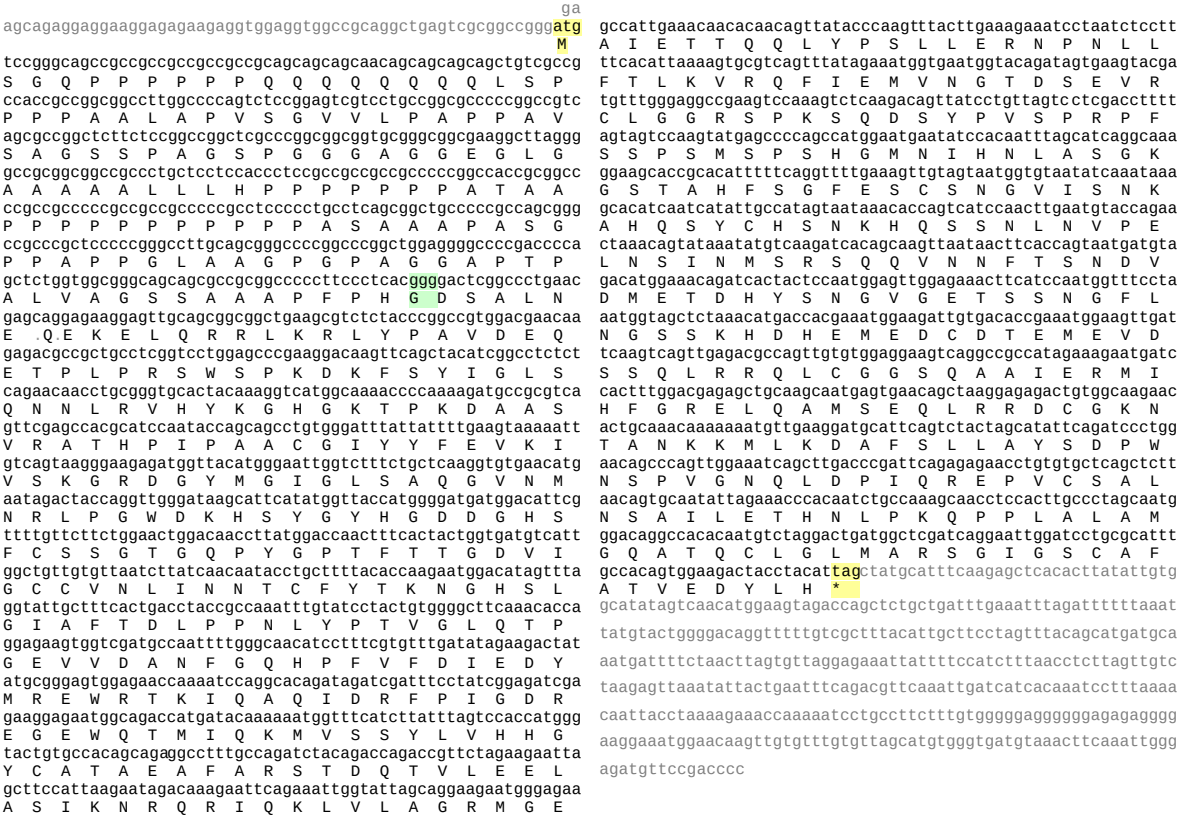

Figure III.5: *Nucleotide and amino acid sequence of RanBPM (Accession #* AB055311*). Highlighted in green is the first codon of the identified positive clone (CYE12).*

The RanBPM/RanBP9 protein (Ran binding protein 9) was initially identified in a two-hybrid screen using Ran as bait, where Ran is a small GTP binding protein involved in nuclear export and import, and spindle formation (Nakamura *et al.*, 1998). Overexpression of this protein induced microtubule nucleation, suggesting a role in microtubule assembly. The full-length RanBPM is a 90kDa protein of 729 aa residues, possessing a long stretch of proline and glutamine residues in the N-terminal region (Nishitani *et al.*, 2001), and three signalling domains (SPRY, LisH and CTLH domains) (Fig. III.6).

Figure III.6: *Diagram of human RanBPM protein.*

SPRY domains were originally identified on Ryanodine receptors and on the SP1A kinase. The SPRY domain is around 140 aa and its function is unknown, but it is thought to be involved in protein-protein interactions or RNA-binding. The suggested functions for some SPRY-containing proteins are RNA-binding, cell growth and differentiation (Ponting *et al.*, 1997).

Another important RanBPM domain is LisH (Lissencephaly type-1-like homology motif). The LisH motif is 33 aa long and the predicted secondary structure consists of two alpha-helices. LisH is likely to possess a conserved protein-binding function and it is present in several proteins involved in microtubule dynamics. These motifs are thought to contribute to the regulation of microtubule dynamics, by mediating dimerization or by binding microtubules or dynein heavy chain (Ponting *et al.*, 1997; Emes and Ponting, 2001).

C-terminally to LisH motif there is a predicted alpha-helical sequence of unknown function that is adjacent to the LisH motif in several proteins, termed CTLH (Ponting *et al.*, 1997; Emes and Ponting, 2001).

Additionally, protein interactions were verified by yeast mating of Y187 harbouring the bait plasmid pAS-CYE with the AD/library positive clones in AH109. Further, YTH tests were carried out with the wild-type AICD and the AICD^{Y687F}, to compare the interaction (Fig. III.7):

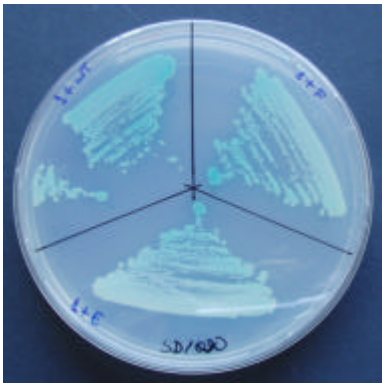

Figure III.7: *X-?-Gal activity assay in SD/QDO plates of diploids with the bait plasmid pAS-CYE or pAS-CWT or pAS-CYF and the prey plasmid pACT2-CYE12.*

The results indicate that RanBPM also interacts with both AICD^{WT} and AICD^{Y687F}. The interaction strengths look similar, since the diploids grew and

develop the blue colour simultaneously. Quantitative analysis will be the logical next step.

The full-length RanBPM is localized within the nucleus, perinuclear region and cytoplasm (Nishitani *et al.*, 2001). Since its initial identification, other two-hybrid assays have shown RanBPM interaction with several other proteins, and these interactions have been confirmed by independent methods. RanBPM interactors include, besides Ran-GTP, the androgen receptor, a steroid hormone receptor whose transactivational function is enhanced by RanBPM (Rao *et al.*, 2002), the nuclear protein HIPK2 (homeodomain-interacting protein kinase-2), a transcriptional repressor (Wang *et al.*, 2002b); hMuskelin, HSMpp8 and a novel nuclear protein Twa1 (Umeda *et al.*, 2003). hMuskelin, and Twa1 also possess the LisH and CTLH motifs involved in microtubule dynamics. RanBPM also interacts with USP11, being deubiquitinated specifically by this enzyme, that prevents its degradation (Ideguchi *et al.*, 2002). RanBPM also binds to MET, the receptor protein-tyrosine kinase, activating the Ras-Erk-SRE (serum response element) signalling pathway by inducing Ras-GTP association and Erk 1/2 phosphorylation (Wang *et al.*, 2002a). Denti et al. reported that RanBPM is a phophoprotein (phosphorylated on serine residues) that interacts with *ß2* integrin LFA-1 *in vivo* and *in vitro* and co-localize at the cell membrane, suggesting a contribution of RanBPM to coupling LFA-1 and other integrins with intracellular signalling pathways (Denti *et al.*, 2004). Immunoprecipitation of this protein from HeLa cells revealed a 670kDa protein complex that included very little Ran, possibly suggesting new roles for RanBPM/RanBP9 namely in nuclear transport.

Transportin-SR2

In the YTH screen, two out of 53 clones (CYE189 and CYE197 encoded a fragment of Transportin-SR2, a nuclear import receptor for SR proteins. The cDNA fragment (approximately 0,7Kb) alignment was observed to be within the coding region of isoform 2, with the fusion protein being expressed in the correct reading

frame. The first nucleotides in the library insert after the linker sequence, ATT, encoded amino acid I-803, on the sequence with the NCBI accession number NM_012470 (Fig. III.8). Transportin is also termed: TRN-SR, TRN-SR2, Transportin 3, TNPO3, Importin 12, IPO12. In this work the designation Tranportin-SR2 will be used. Transportin-SR2 is an alternative splicing product of *TNPO3* gene. The Transportin-SR2 maps to chromosome 7q32.

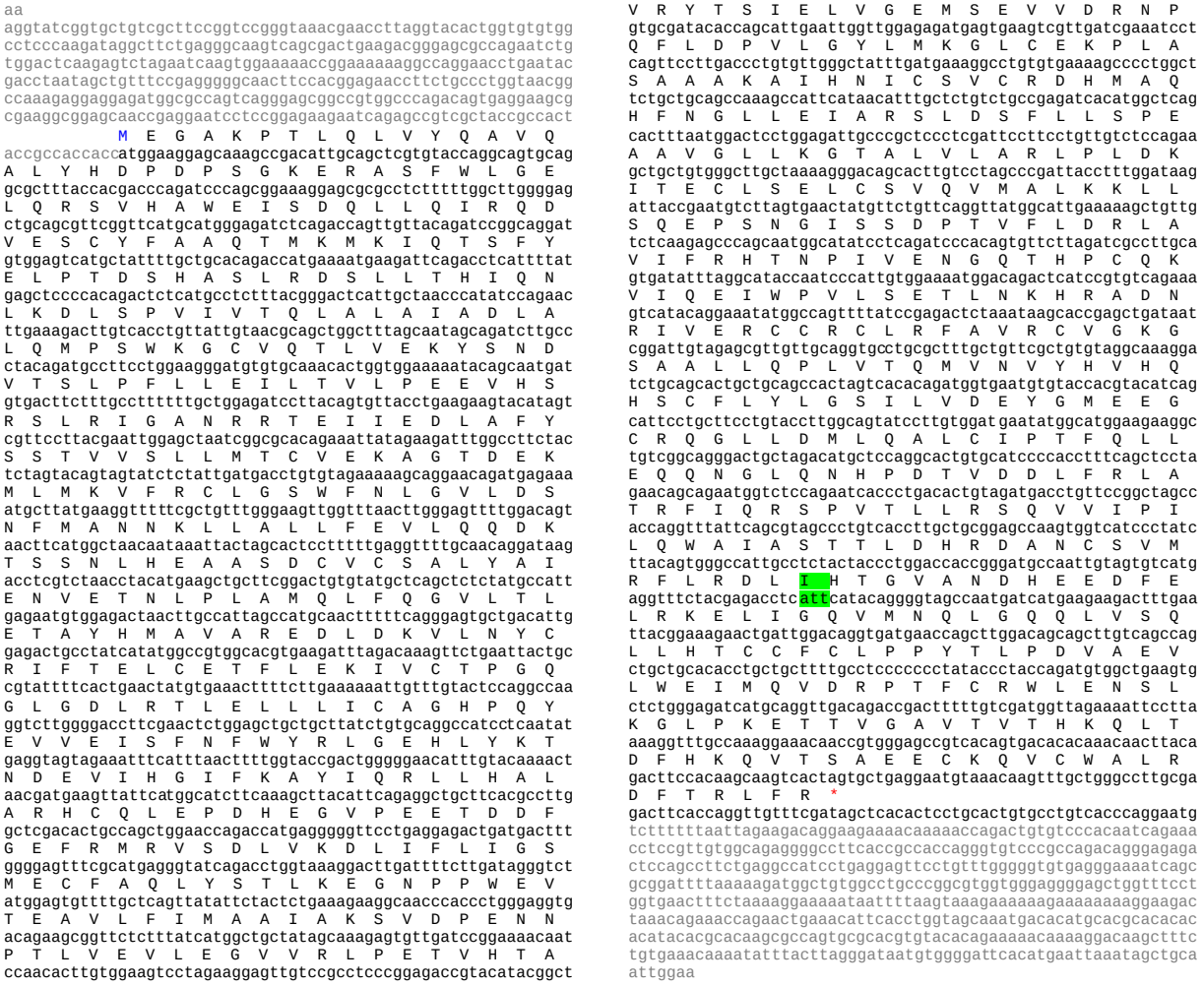

Figure III.8: *Nucleotide and amino acid sequence of Transportin-SR2, isoform 2 (Accession #* NM_012470*). Highlighted in green is the first codon of the identified positive clone (CYE189).*

The full-length Transportin-SR2 is a 104kDa protein of 923 amino acids, possessing a long RS domain interaction in its C-terminal region. RS domains are domains with Arginine/Serine dipeptide repeats and proteins containing RS domains are known as SR proteins (Allemand *et al.*, 2002). Transportin-SR2 has a region that shares similarities with human exportin-t and another region similar to importin-?1 and RanBP5 (Fig. III.9) (Lai *et al.*, 2000).

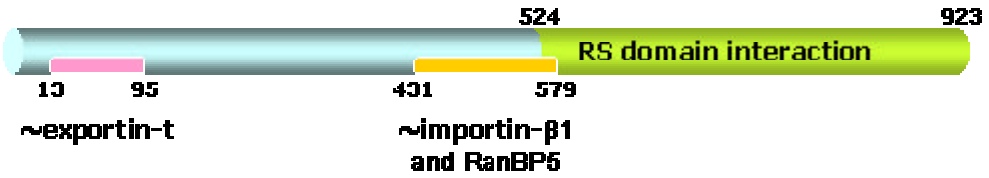

Figure III.9: *Diagram of human Transportin-SR2, isoform 2.*

The novel identified protein interaction between our bait and Transportin-SR2 was verified by yeast mating. Additional YTH tests were carried out with the wild-type AICD and the $AICD^{Y687F}$, to compare the interaction. The results indicate that Transportin-SR2 also interacts with both $AICD^{WT}$ and $AICD^{Y687F}$. The interaction strength looks similar, since the diploids grew and developed the blue colour simultaneously (Fig. III.10).

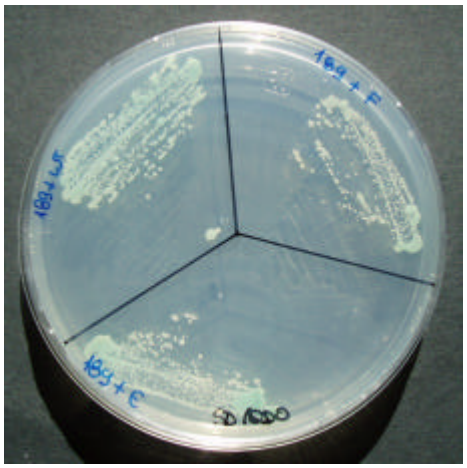

Figure III.10: *X-?-Gal activity assay in SD/QDO plates of diploids with the bait plasmid pAS-CYE or pAS-CWT or pAS-CYF and the prey plasmid pACT2-CYE189, identified as Transportin-SR2.*

Transportin-SR2 is localized in the cytoplasm and in the nucleus, and belongs to the Importin-?/transportin protein family. It was demonstrated to interact *in vitro* with phosphorylated, but not with unphosphorylated, RS domains (domains with Arginine/Serine dipeptide repeats) (Lai *et al.*, 2000; Allemand *et al.*, 2002). SR proteins, which contain RS domain(s), are mainly involved in the splicing of precursor mRNA and in other aspects of gene expression (Kataoka *et al.*, 1999).

Translocation of macromolecules across the nuclear envelope occurs through the nuclear pore complex (NPC) and requires additional factors, such as Ran (small GTPase) and NTF2. Cargoes possessing a prototypical nuclear localization signal (NLS) (cluster(s) of basic residues) are recognized by importin-?, which in turn interacts with importin-? for NPC docking. Non-typical NLS (e.g. Gly-rich, Arg-rich) interact directly with the corresponding import receptor. Receptor-cargo complexes subsequently dock on the cytoplasmic part of NPC and then translocate through the pore into the nucleus. The RS domain acts as the NLS for SR proteins (Poon and Jans, 2005).

To date, Transportin-SR2 interacting proteins comprise the splicing factors SFRS1 and SFRS2 (only with the phosphorylated-state) and NUP62, a nuclear pore glycoprotein. Transportin-SR2 was recently reported to also interact with several non-SR proteins (Lai *et al.*, 2003), directing them to an import pathway to the

nucleus identical to SR proteins. For instance RBM4, a pre-mRNA splicing regulator, was shown to interact with Transportin-SR2, in a Ran-sensitive manner (Lai *et al.*, 2003).

III.4 DISCUSSION

In the YTH screen several positive clones corresponded to independent hits on the same protein, therefore the $AICD^{Y687E}$ interactome comprised 5 different proteins. Interestingly, the most abundant interaction detected for $AICD^{Y687E}$ was towards a single protein. Thus, 16 positives out of the 53 identified clones encoded RanBP9/RanBPM protein.

Two positive clones encoded a fragment of the nuclear import receptor Transportin-SR2. The number of hits on this protein, compared with RanBPM, may denote that either Transportin-SR2 mRNA was less expressed in the tissues used to produce the cDNA library or the interaction of AICD with Transportin-SR2 is not so strong as with RanBPM.

Four positives were identified as SDHB. Although being a nuclear-encoded protein, SDHB is localized in the mitochondrial inner membrane, as a result this interaction is unlikely to occur in a physiological context, because the partners are not expressed in the same subcellular environment. However, the number of hits for this protein and given that it is associated with oxidative stress in AD (Bubber *et al.*, 2005), suggest that SDHB may potentially be an interesting clone.

None of the previously identified APP binding proteins were isolated in this YTH screen, probably due to the fact in the AICD bait Tyrosine-687 is mutated to Glutamate. Nevertheless RanBPM and Transportin-SR2 were shown to interact also with wild-type AICD and phosphomutant $AICD^{Y687F}$.

IV DISCUSSION AND CONCLUSIONS

Adequacy of the YTH strategy

Of the $1.1x10^8$ clones screened 55 positives clones were isolated. Of these, 2 could not be sequenced. Of the remaining 53 library plasmids analysed, 16 were single hits, but only 4 of these arose from the high stringency selection. Only 5 different proteins were found inside the CDS in the correct reading frame. Among the analysed positive clones, two proteins were found that have central roles in nuclear targeting, RanBPM and Transportin-SR2. Despite the intrinsic limitations of YTH, these results confirm that it is an important tool for identifying new proteins that interact with a protein of interest. However, this method requires some caution in the analysis of the results, and one should be aware of its limitations when discussing the cellular significance of the detected interactions.

The present screen identified many DNA sequences, several of which did not match predicted protein sequences or even protein motifs. These interacting peptide sequences may in the future be analysed to reveal a specific AICD binding motif. The occurrence of mitochondrial clones might simply reflect the presence of similar peptide sequences in the multiple short mitochondrial fusion proteins.

The recovery of two distinct library plasmids from the same clone is likely to happen, because in the library transformation, yeast can occasionally acquire more than one plasmid after several rounds of cell division even without selective pressure. This situation was overcome by restreaking the clones 2 or 3 times, thus selecting for the plasmids that allow the cells to grow on the selective culture media used. Nevertheless, in order to verify that an isolated plasmid is responsible for the interaction, it should be tested by mating in suitable yeast strains of opposite mating type, thus confirming reporter expression of the resulting diploids. This procedure was carried out for the selected positive clones CYE12 (RanBPM) and CYE189 (Transportin-SR2). Subsequently to characterization of positive

clones, all interactions need to be confirmed by a different method, usually coimmunoprecipitation of cellular extracts using specific antibodies to the interacting partners.

To confirm interactions with the complete APP protein, the full-length sequence can also be used in similar assays to confirm interaction with the cellular protein. These will be forthcoming. The YTH may also be used to test the effect of phosphorylation in the novel interactions reported here with AICD, by verifying the interaction with $AICD^{WT}$ and $AICD^{Y687F}$ phosphomutant. Both RanBPM and Transportin-SR2 interact with $AICD^{WT}$, $AICD^{Y687E}$ and $AICD^{Y687F}$ as showed by the positive X-Gal assays. To detect slightly differences in these interactions, a quantitative X-Gal assay should be subsequently carried out.

Another important validation of the putative interactions discovered is the confirmation of coexpression of the two binding partners either in the same cell-type or in the same subcellular compartment. As already mentioned, a condition for in vivo interaction of two proteins is their simultaneous presence in the same subcellular compartment. It is therefore important to determine the subcellular location where these proteins are present, the particular structures they associate with and, mainly in case of proteins involved in AD, their expression levels in AD samples. For example, SDHB, which is a nuclear-encoded protein localized in the mitochondrial inner membrane, is improbable to interact with AICD in a physiological context, because they are not expressed in the same subcellular compartment. Although the activity of SDHB was reported to increase in AD (Bubber *et al.*, 2005) the interaction of AICD with SDHB is more likely to be a false positive. The same happens with the mitochondrial clone CYE128, which codes for Cytochrome c oxidase, since the activity of this enzyme was reported to decrease in AD platelets (Cardoso *et al.*, 2004).

Two novel interactions relevant for AICD nuclear targeting

Here we report two novel interactions for AICD, which relative to previously described interactions were found to have central roles in nuclear targeting, RanBPM and Transportin-SR2.

Among the proteins reported to bind RanBPM, several are important signalling molecules, thus by its interaction with AICD it links the latter to receptor-mediated signalling pathways. RanBPM, for instance, through binding to the cytoplasmic domain of Integrin LFA-1, may constitute a molecular scaffold to coupling integrins with intracellular signalling pathways. Additionally, RanBPM was also reported to bind MET, activating the Ras/Erk signalling pathway. Upon activation, the Erks phosphorylate cytoplasmic targets and translocate to the nucleus were they activate transcription factors. By interacting with RanBPM, AICD may be involved in this signalling cascade. Furthermore, sAPP has been shown to increase MAP kinase activity and tyrosine phosphorylation in a Ras-dependent manner (Greenberg *et al.*, 1994). Concomitantly, RanBPM binds Ran that belongs to the Ras superfamily and plays a role in the translocation of proteins trough the nuclear pore complex. Of relevance to AD in the fact that the N-terminus of RanBPM, which is required for recruiting Sos and Ras activation, has a Poly-PQ stretch. This stretch of proline and glutamine residues have the tendency to aggregate, forming aggresomes.

The novel interaction reported in this thesis between AICD and Transportin-SR2 provides supportive evidence for mediated nuclear import of AICD through the nuclear pore complex. Therefore AICD is likely to be a novel Transportin-SR2 cargo, even though it harbours no RS dipeptide repeats, thus adding further insights to the nuclear import of AICD. Transportin-SR2-mediated nuclear import can also be regulated by Ran, since GTP-bound Ran is known to dissociate cargo from importin-?. For instance RBM4, a pre-mRNA splicing regulator, interacts with Transportin-SR2 in a Ran-sensitive manner, in which Ran-GTP abolishes the binding of RBM4 to Transportin-SR2.

In the present YTH screen we report two novel AICD interacting proteins, RanBPM and Transportin-SR2. In our model (Fig. IV.1) AICD is a cargo for Transportin-SR2 import receptor. The latter docks on nucleoporins and translocates through the nuclear pore. Presumably AICD does not go through facilitated diffusion because it is complexed with RanBPM. At the nucleoplasmic side of the nuclear pore complex, Ran-GTP, which binds RanBPM, dissociates Transportin-SR2 and nucleoporins. These protein-protein interactions may target AICD to the nucleus and link it to signal transduction pathways, culminating in gene transcription events.

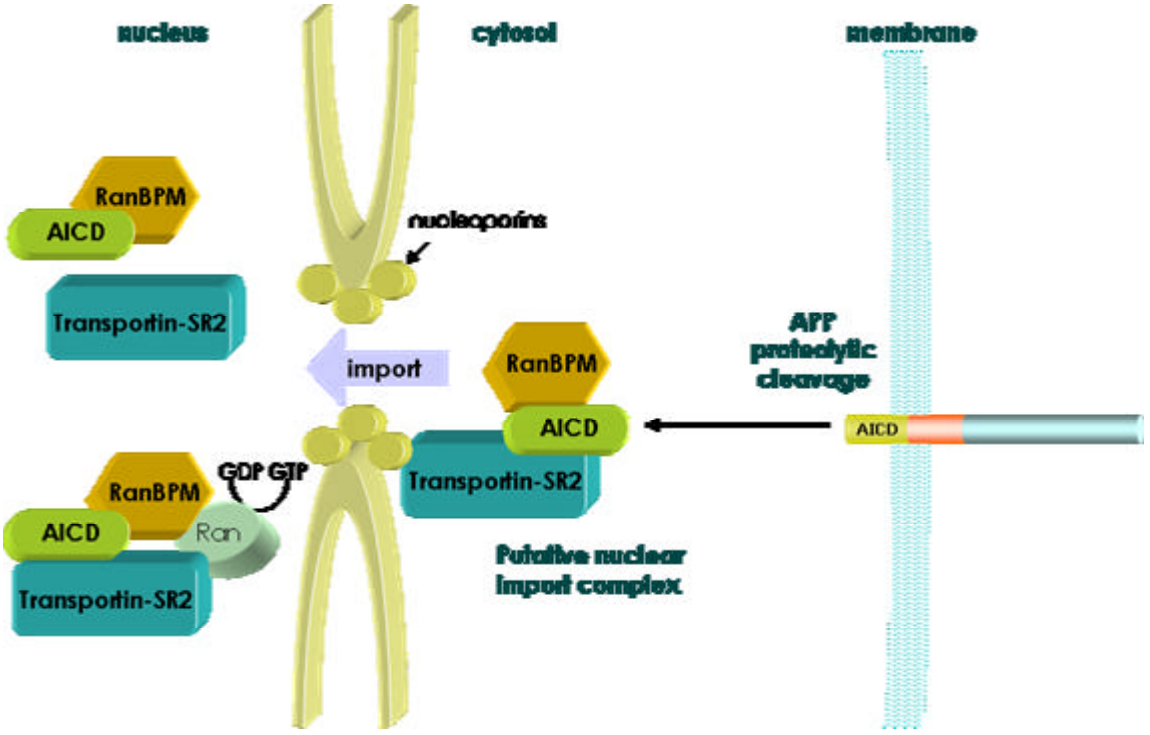

Figure IV.1: *Putative model of a nuclear import complex.*

This data supports the functional similarity between AICD and the intracellular domain of transmembrane proteins, such as Notch, that are released by ?-secretase cleavage and migrate to the nucleus to regulate gene transcription. AICD was already reported to migrate to the nucleus complexed with FE65 and

Tip60 to activate specific gene targets, such as KAI1/CD82 (Cao and Sudhof, 2001; Baek *et al.*, 2002; Telese *et al.*, 2005). Our data provide further evidence as to how AICD nuclear import is mechanistically carried out and suggest that AICD migration to the nucleus can potentially be achieved in absence of FE65.

Together RanBPM and Transportin-SR2 suggest that the AICD fragment released after ?-secretase cleavage is involved in multiple signalling pathways by means of protein-protein interactions, which target AICD to the nucleus in a Notch-like manner. As such the such the work here described brings us nearer to unravelling the physiological functions of APP. This in turn is of potential significant relevance in the pathology of AD, for the design of effective therapeutic strategies.

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Appendix I - Culture media and solutions

Bacterial Media

LB (Luria-Bertani) Medium

To 950ml of deionised H_2O add:

LB 25g

Agar 12g (for plates only)

Shake until the solutes have dissolved. Adjust the volume of the solution to 1 liter with deionised H_2O. Sterilize by autoclaving.

SOB Medium

To 950ml of deionised H_2O add:

Bacto-tryptone 20g

Bacto-yeast extract 5g

NaCl 0.5g

Shake until the solutes have dissolved. Add 10ml of a 250mM KCl (prepared by dissolving 1.86g of KCl in 100ml of deionised H_2O). Adjust the pH to 7.0 with 5N NaOH. Adjust the volume of the solution to 1 liter with deionised H_2O. Sterilize by autoclaving. Just prior to use add 5ml of a sterile solution of 2M $MgCl_2$ (prepared by dissolving 19g of $MgCl_2$ in 90ml of deionised H_2O; adjust the volume of the solution to 100ml with deionised H_2O and sterilize by autoclaving).

SOC Medium

SOC is identical to SOB except that it contains 20mM glucose. After the SOB medium has been autoclaved, allow it to cool to 60ºC and add 20ml of a sterile 1M glucose (this solution is made by dissolving 18g of glucose in 90ml of

deionised H_2O; after the sugar has dissolved, adjust the volume of the solution to 100ml with deionised H_2O and sterilize by filtration through a 0.22-micron filter).

Yeast Media

10X dropout solution (DO10X)

This solution contains all but one or more of the following components:

	10X concentration (mg/L)	SIGMA #
L-Isoleucine	300	I-7383
L-Valine	1500	V-0500
L-Adenine hemisulfate salt	200	A-9126
L-Arginine HCl	200	A-5131
L-Histidine HCl monohydrate	200	H-9511
L-Leucine	1000	L-1512
L-Lysine HCl	300	L-1262
L-Methionine	200	M-9625
L-Phenylalanine	500	P-5030
L-Threonine	2000	T-8625
L-Tryptophan	200	T-0254
L-Tyrosine	300	T-3754
L-Uracil	200	U-0750

10X dropout supplements may be autoclaved and stored for up to 1 year.

YPD medium

To 950ml of deionised H_2O add:

20g Difco peptone

10g Yeast extract

20g Agar (for plates only)

Shake until the solutes have dissolved. Adjust the volume to 1L with deionised H_2O and sterilize by autoclaving. Allow medium to cool to 60ºC and add glucose to 2% (50ml of a sterile 40% stock solution).

⅂SD synthetic medium

To 800ml of deionised H_2O add:

6.7g Yeast nitrogen base without amino acids (DIFCO)

20g Agar (for plates only)

Shake until the solutes have dissolved. Adjust the volume to 850ml with deionised H_2O and sterilize by autoclaving. Allow medium to cool to 60ºC and add glucose to 2% (50ml of a sterile 40% stock solution) and 100ml of the appropriate 10X dropout solution.

⅂2X YPDA

Prepare YPD as above. After the autoclaved medium has cooled to 55ºC add 15ml of a 0.2% adenine hemisulfate solution per liter of medium (final concentration is 0.003%).

Solutions

⅂50X TAE Buffer

242g Tris base

57.1ml glacial acetic acid

100ml 0.5M EDTA (pH 8.0)

⅂TE Buffer (pH 7.5)

10mM Tris-HCl (pH 7.5)

1mM EDTA, pH 8.0

↴Competent Cell Solutions:

Solution I (1L):

9.9g $MnCl_2.4H_2O$

1.5g $CaCl_2.2H_2O$

150g glycerol

30ml KHAc 1M;

adjust pH to 5.8 with HAc, filter through a 0.2µm filter and store at 4ºC

Solution II (1L):

20ml 0.5M MOPS (pH 6.8)

1.2g RbCl, 11g $CaCl_2.2H_2O$

150g glycerol;

filter through a 0.2µm filter and store at 4ºC

↴Miniprep Solutions:

Solution I:

50mM glucose

25mM Tris.HCl (pH 8.0)

10mM EDTA

Solution II:

0.2N NaOH

1%SDS

Solution III:

3M potassium acetate

2M glacial acetic acid

↴Megaprep Solutions:

Cell Resuspension Solution:

50 mM Tris-HCl (pH 7.5)

10 mM EDTA

100 µg/ml RNAase A

Cell Lysis Solution:

0.2 M NaOH

1% SDS

Neutralization solution:

1.32 M potassium acetate (pH 4.8)

Column Wash Solution:

80 mM potassium acetate

8.3 mM Tris-HCl (pH 7.5)

40µM EDTA

55% ethanol

SDS-PAGE Solutions

↴LGB (Low Gel Buffer)

To 900ml of deionised H_2O add:

181.65g Tris

4g SDS

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

↴UGB (Upper Gel Buffer)

To 900ml of deionised H_2O add:

75.69g Tris

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

- 30%Acrylamide/0.8% Bisacrylamide

  To 70ml of deionised H_2O add:

  29.2g Acrylamide

  0.8g Bisacrylamide

  Shake until the solutes have dissolved. Adjust the volume to 100ml with deionised H_2O. Store at 4ºC.

- Loading Gel Buffer

  250mM Tris-HCl (pH 6.8)

  8% SDS

  40% Glycerol

  2% 2-mercaptoethanol

  0.01% Bromophenol blue

- 1X Running Buffer

  25mM Tris-HCl (pH8.3)

  250mM Glycine

  0.1% SDS

- 1X Transfer buffer

  25mM Tris-HCl (pH8.3)

  192mM Glycine

  20% Methanol

- 10X TBS

  10mM Tris-HCl (pH 8.0)

  150mM NaCl

- 10X TBST

  10mM Tris-HCl (pH 8.0)

150mM NaCl

0.05% Tween

Yeast Two-Hybrid Solutions

↴Yeast plasmid rescue – Breaking buffer

2% Triton X-100

1% SDS

100mM NaCl

10mM Tris-HCl (pH 8.0)

↴Solutions for preparation of yeast protein extracts

a) Protease inhibitor solution

Always prepare solution fresh just before using. Place on ice to prechill. To prepare 688µl add in a microfuge tube:

66µl Pepstatin A (1mg/ml stock solution in DMSO)

2µl Leupeptin (10.5mM stock solution)

500µl Benzamidine (200mM stock solution)

120?l Aprotinin (2.1mg/ml stock solution)

b) PMSF (phenylmethyl-sulfonyl fluoride) stock solution (100X)

Dissolve 0.1742g of PMSF (SIGMA) in 10ml isopropanol. Wrap tube in foil and store at RT.

c) Cracking buffer stock solution

To 80ml of deionised H_2O add:

48g Urea

5g SDS

4ml 1M Tris-HCl (pH6.8)

20?l 0.5M EDTA

40mg Bromophenol blue

Shake until the solutes have dissolved. Adjust the volume to 100ml with deionised H_2O.

d) Cracking buffer

To prepare 1.13ml add in a microfuge tube:

1ml Cracking buffer stock solution (recipe above)

10?l ß-mercaptoethanol

70?l Protease inhibitor solution (recipe above)

50?l 100X PMSF stock solution

Appendix II - Primers

PRIMER	SEQUENCE (5':::: 3')	Nt No.	MT (°C)
NAAPC	ATCACCATGGTGATGCTGAAGAAG	24	64
APPCTERMIII	GTGGCCCCGGGCTAGTTCTGCATCTGCTCAAAG	33	86
GAL4 AD	TACCACTACAATGGATG	17	48
GAL4 BD	TCATCGGAAGAGAGTAG	17	50
Amplimer 3' (reverse)	ATCGTAGATACTGAAAAACCCCGCAAGTTCAC	32	84
Amplimer 5'	CTATTCGATGATGAAGATACCCCACCAAACCC	32	94

Appendix III - Bacteria and yeast strains

- E. coli XL1- blue: *recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F' proAB lacZ?M15 Tn10(Tet^r)]*

- S. cerevisiae AH109: MATa, trp1-901, leu2-3, 112?ura3-52, his3-200, gal4?, gal 80?, LYS2:: $GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, URA3::$MEL1_{UAS}$-$MEL1_{TATA}$-lacZ, MEL1

- S. cerevisiae Y187: MATa?ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4?, met-, gal 80?, URA3:: $GAL1_{UAS}$-$GAL1_{TATA}$-lacZ, MEL1

Appendix IV - Plasmids

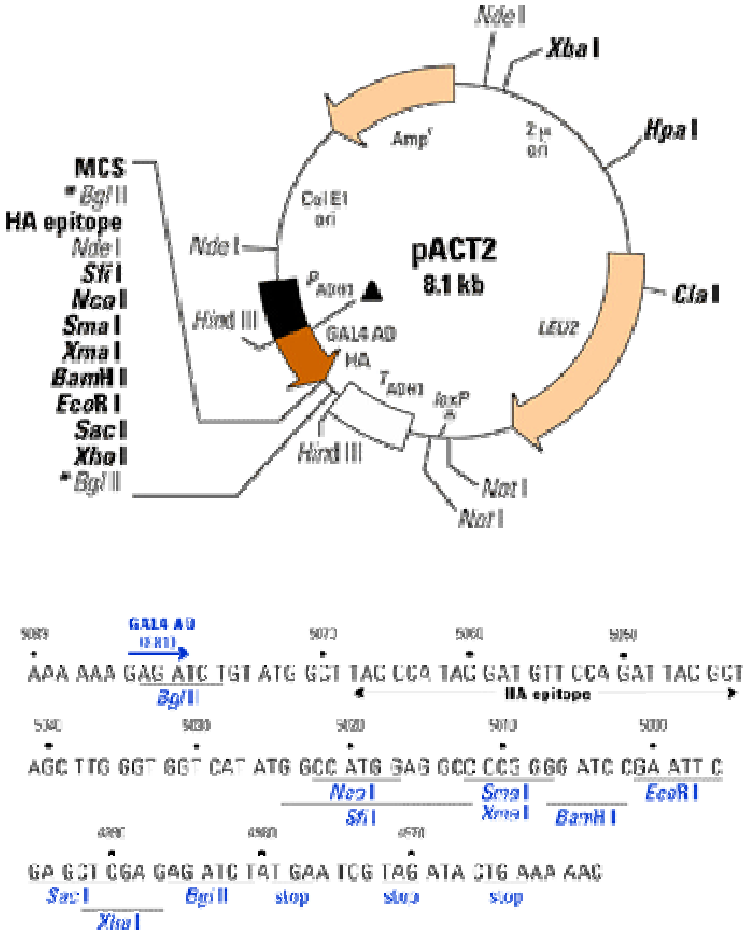

Figure 1: *pACT2 (CLONTECH) map and MCS. Unique sites are coloured blue. pACT2 is used to generate a hybrid containing the GAL4 AD, an epitope tag and a protein encoded by a cDNA in a fusion library. The hybrid protein is expressed at medium levels in yeast host cells from an enhanced, truncated ADH1 promoter and is target to the nucleus by the SV40 T-antigen nuclear localization sequence. pACT2 contains the LEU2 gene for selection in Leu⁻ auxotrophic yeast strains.*

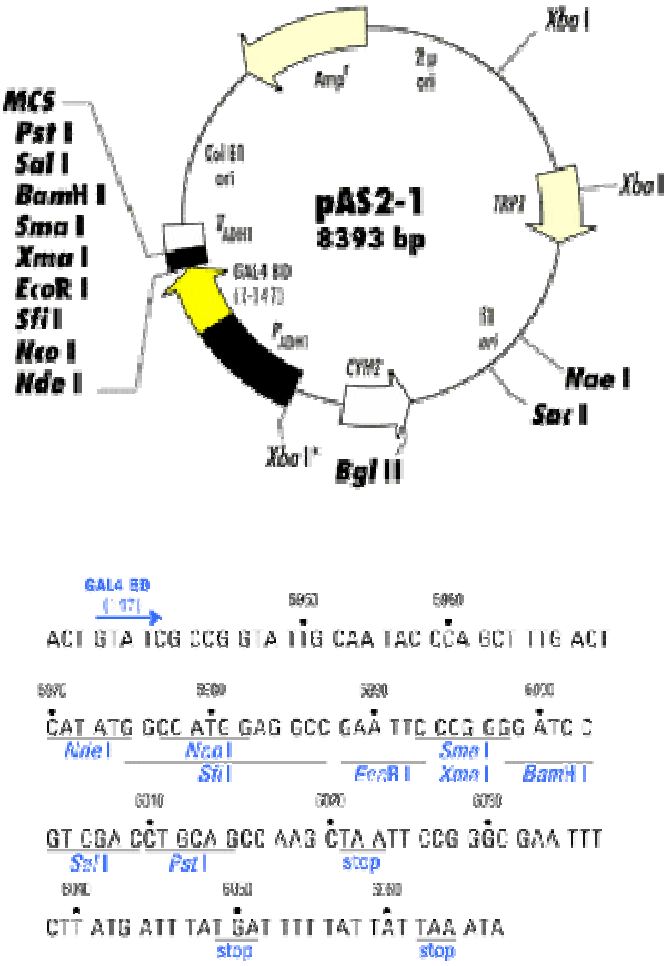

Figure 2: *pAS2-1* (CLONTECH) *map and MCS. Unique sites are coloured blue. pAS2-1 is a cloning vector used to generate fusions of a bait protein with the GAL4 DNA-BD. The hybrid protein is expressed at high levels in yeast host cells from the full-length ADH1 promoter. The hybrid protein is target to the yeast nucleus by nuclear localization sequences. pAS2-1 contains the TRP1 gene for selection in Trp⁻ auxotrophic yeast strains.*