Universidade de Aveiro Departamento de Biologia
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Daniela Marques Patrício

Implementação do sistema de dois-híbrido em mamífero para rastreio de drogas em grande escala

Implementation of mammalian two-hybrid system for highthroughput drugs screening

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutora Margarida Sâncio da Cruz Fardilha, Professora Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro e coorientação da Doutora Ana Sofia da Cunha Guimarães, Investigadora de Pós-Doutoramento do Instituto de Biomedicina da Universidade de Aveiro, e da Doutora Virgília Sofia Almeida de Azevedo e Silva, Professora Auxiliar Convidada do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave

Dois-híbrido em mamífero, Interação proteína-proteína, PPP1CC2, AKAP4, mobilidade espermática

resumo

A comunicação celular baseia-se em vias de sinalização, que quando desreguladas podem levar ao desenvolvimento de doenças, desde cancro a doenças autoimunes. As interações proteína-proteína (PPIs) são fundamentais para a regulação de vias de sinalização. Deste modo, a identificação e a modulação de PPIs é crucial para entender mecanismos pato-fisiológicos de diferentes doenças. O sistema reprodutor masculino não é exceção, estando as PPIs no centro da manutenção de funções celulares na produção e funcionamento dos gâmetas. A PPP1CC2 é uma proteína com expressão enriquecida nos testículos e a sua interação com a AKAP4, proteína específica de testículo, aparenta ser um bom alvo para novos fármacos. A monitorização da modulação da interação PPP1CC2/AKAP4 através do sistema de dois-híbrido em mamífero (MTH) é crucial. Os sistemas dois-híbrido são técnicas específicas para validar PPIs *ex vivo.* Desta forma, neste trabalho explorou-se o sistema MTH para uma análise de potenciais drogas para a modulação de PPIs. Isto permitirá identificar um novo alvo terapêutico e/ou desenvolvimento de drogas que afetem especificamente uma PPI. Esta metodologia baseia-se no facto de em eucariotas, a transcrição de genes ocorrer apenas quando o domínio de ligação (BD) e o domínio de ativação (AD) do DNA estão próximos. Ao fundir o DNA complementar de duas proteínas que interagem com o BD e AD, a expressão de um gene repórter é ativada e a interação detetada. O objetivo principal desta tese é implementar o MTH no laboratório de transdução de sinal, para identificar e modular PPIs num sistema que mimetiza o ambiente onde ocorrem PPIs – células de mamíferos.

keywords

Mammalian two-hybrid, Protein-protein interaction, PPP1CC2, AKAP4, sperm motility.

abstract

The base of cell communication is signalling pathways which, when deregulated can lead to several diseases, from cancer to autoimmune diseases. Protein-protein interactions (PPIs) are the backbone of signalling pathways. Consequently, identifying and modulating PPIs can unravel the pathophysiological mechanisms of a disease. When considering the male reproductive system, the same is observed, hence PPIs are responsible and regulate most of the cellular functions. PPP1CC2 is a testis enriched protein, key to sperm motility acquisition. Its interaction with AKAP4, also a testis-specific protein, appears to be a potential new drug target. Therefore, the modulation of PPP1CC2/AKAP4 interaction under mammalian two-hybrid system is crucial.

Two-hybrid system, particularly the mammalian two-hybrid (MTH), is an efficient technique to validate PPIs *ex vivo*. Thus, we will explore this tool for highthroughput drug screening through the modulation of PPIs. Consequently, it will be used to identify either a target to a specific drug or drugs. This methodology relies on the fact that in eukaryotes, gene transcription only occurs when the DNA-binding domain (BD) and DNA activation domain (AD) of a transcription factor come into close proximity. When fusing the cDNA of two interacting proteins with the BD and AD, a reporter gene is expressed and the interaction can be detected.

The main goal of this thesis is to implement the MTH into the Signal Transduction laboratory and to be able to identify and modulate PPIs in a system that can mimic the natural way PPI's occur – mammalian cells.

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List of Abbreviations, symbols and acronyms

AB	Antibody
AD	Activation domain
AKAP	A-kinase anchoring protein
AKAP3	A-kinase anchoring protein 3
AKAP4	A-kinase anchoring protein 4
AKAP11	A-kinase anchoring protein 11
APS	Ammonium persulphate solution
ATP	Adenine triphosphate
BBS	BES buffered solution
BCA	Bicinchoninic acid assay
BD	Binding domain
BES	N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
bp	Base pairs
BSA	Bovine Serum Albumin
cAMP	Cyclic adenosine monophosphate
CAT	Chloramphenicol acetyltransferase
cDNA	Complementary DNA
CT	Cancer/testis antigen
DMEM	Dulecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
FBS	Fetal bovine serum
FS	Fibrous sheath
GFP	Green fluorescent protein
GSK3	Glycogen synthase kinase 3
HTP	Highthroughput
iBiMED	Institute of Biomedical Sciences
JAK-STAT	Janus kinase/Signal transducer and activator of transcription
kb	Kilo base pairs
LB	Luria-Bertani broth medium
MAPPIT	Mammalian protein-protein interaction trap
MDM2	Murine doble minute 2
MTH	Mammalian two-hybrid
NADH	Nicotinamide adenine dinucleotide
ODF	Outer dense fibres

ON	Overnight
ORF	Open reading frame
PBS	Phosphate buffered saline
PFA	Para-formaldehyde
PIP	Phosphoprotein phosphatase 1 interaction protein
PKA	Protein kinase A
PKs	Protein kinases
PPI	Protein-protein Interaction
PPP	Phosphoprotein phosphatase
PPP1	Phosphoprotein phosphatase 1
PPP1C	Phosphoprotein phosphatase 1 catalytic subunit
PPP1CA	Phosphoprotein phosphatase 1 subunit alpha
PPP1CB	Phosphoprotein phosphatase 1 subunit beta
PPP1CC1	Phosphoprotein phosphatase 1 subunit gamma 1
PPP1CC2	Phosphoprotein phosphatase 1 subunit gamma 2
PPP1R	Phosphoprotein phosphatase 1 regulatory subunit
PPP1R1	Phosphoprotein phosphatase 1 inhibitor 11
PPP1R2	Phosphoprotein phosphatase 1 inhibitor 2
PPP1R2P3	Phosphoprotein phosphatase 1 inhibitor 2 pseudogene 3
PPPM	Metallo-phosphatase protein
PP	Protein phosphatase
RT	Room temperature
SDS	Sodium dodecyl sulfate
SEAP	Secrete alkaline phosphatase
SPZ1	Spermatogenic zinc protein 1
STPPs	Serine threonine protein phosphatase
TAE	Tris Acetate-EDTA buffer
TBS	Tris-buffered saline containing
TBST	Tris-buffered saline containing 0,1% Tween 20
TEMED	Tetramethylethylenediamine
WR	Working reagent
YTH	Yeast two-hybrid system

I. Introduction

I.1. Male reproductive system – testis and sperm

In males, gamete (spermatozoa) are produced by spermatogenesis in the testis which are housed inside the scrotum (Figure 1.a). The composition of testicular parenchyma is highly intricate of seminiferous tubules, where the production of spermatozoa occurs, and it is surrounded by the interstitial tissue containing Leydig cells. Leydig cells are responsible for testosterone production, which is essential for spermatogenesis. The seminiferous epithelium is complemented with sustentacular Sertoli cells and a stratified layer of developing male germ cells, as spermatogonia, spermatocytes and spermatids (1). Beside the role in the maintenance of the integrity of seminiferous epithelium, Sertoli cells have an important role in the regulation of male germ cell proliferation and differentiation, as well as in male germ cells nutrition (2).

Spermatogenesis can be divided into three major phases: proliferation and differentiation of spermatogonia; meiosis; and spermiogenesis (Figure 1.b) (3). The spermatogenesis is initiated with several cell divisions and differentiation processes of spermatogonia, resulting in primary spermatocytes. Then, each spermatocyte formed increases markedly in size and undergoes through the first meiotic division (secondary spermatocyte) followed by a second meiotic division (spermatid). The final phase of spermatogenesis is the differentiation of spermatids into spermatozoa known as spermiogenesis. Spermiogenesis involves extensive cell remodelling and results in morphological mature spermatozoa.

The mammalian spermatozoa consist of a head and a flagellum, Figure 1.c. The head comprises a nucleus and an acrosome surrounded by the plasma membrane (4). In the nucleus is the condensed chromatin with genetic information, the acrosome is a result from Golgi complex suppression and covers two thirds of the anterior head surface (5). The flagellum is the longest part of the sperm and is a critical structure for motility, it is composed by the axoneme, fibrous sheath (FS) and outer dense fibres (ODF). While the axoneme function as the motor of the sperm, the FS and ODF modulate the frequency and pattern of flagellar beating. The flagellum is divided into four pieces, the connection of head with flagellum (connecting piece), the region with the mitochondrial sheath that surrounds the axoneme (mid-piece), the main portion of the tail (principal piece) and the end piece (6).

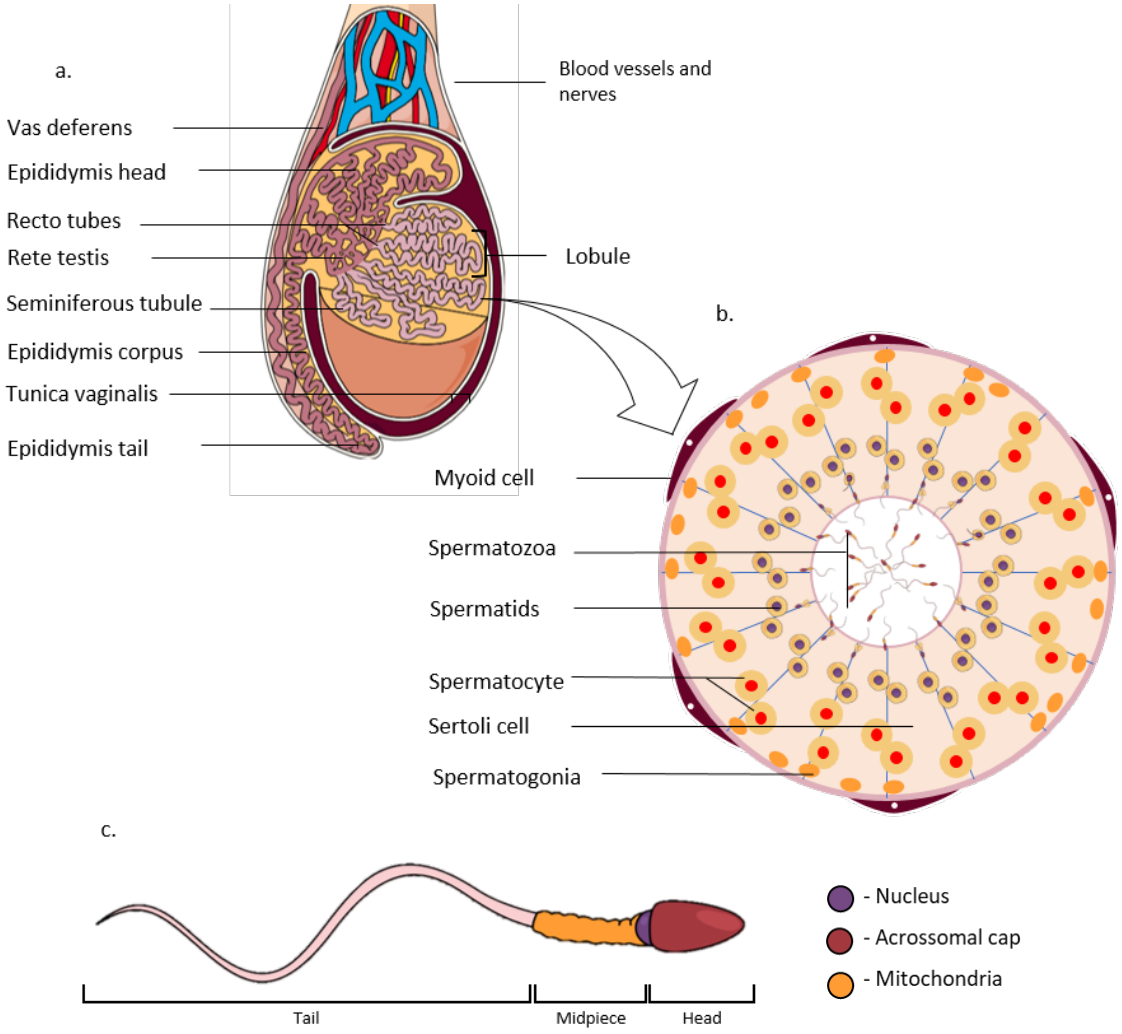

Figure 1 – Diagram of testis and seminiferous tubes. a. Represents a cross section of the testis, revealing the seminiferous tube and its distribution in lobules, epididymis head, corpus and tail. **b.** Shows a section of a seminiferous tube, representing several Sertoli cells and the disposition of spermatogonia before the initiation of the meiotic division I to became a spermatocyte (2n), and further meiotic division 2 to became a spermatid (n) and be able to suffer speciation and became a spermatozoa, present in the seminiferous tube lumen. **c.** Illustrate a spermatozoa cell, with the acrosome cap in the head, the midpiece, where mitochondria are agglomerated and the tail evident Figures were produced using Mind the Graph.

I.2. Protein-protein interactions in male fertility

Protein-protein interactions (PPIs) are one of the most important mechanisms used by mammalian cells to regulate a variety of cellular and molecular activities, such as transcription, translation, signal transduction and enzyme reaction (7). PPIs are dependent on cell type, cell cycle phase, development stage, environmental conditions, protein modifications (e.g. phosphorylation), the presence of cofactors, binding partners and inhibitors. Modifications and regulation of proteins necessarily involve a transient PPIs (8), interactions that quickly bound and unbound, are a key to regulation of protein

functions (9). These interactions are common in signalling pathways as they provide a mechanism for a fast cellular answer to extracellular factors (10) such as drugs. On other hand, stable PPIs are obligatory, with structural or functional roles, this PPIs are related with permanent complexes, such as interactions of cytochrome c (11) or some hetero-oligomeric proteins (12), like subunits of ATPase.

PPIs can interfere with kinetic properties of proteins; act as a general mechanism to tolerate substrate channelling; construct a new binding site for small effector molecules, inactivate or suppress the protein activity; and modify the specificity of a protein for its substrate through interaction with distinct binding partners (13). Characterization of different PPIs might be essential to comprehend diverse cellular functions (8). Over the past decades, many PPIs have been identified, both *ex vivo*, for example by co-immunoprecipitation, and *in vivo*, for example by yeast two-hybrid. PPIs have emerged as promising drug targets, and the interest in identification and modulation of PPIs has grown, since it can unravel the pathophysiological mechanisms of a disease (14–16). On a therapeutic perspective, targeting PPIs should result in a better diagnosis of the disease and lesser side effects of treatments.

In mammalian spermatozoa reversible phosphorylation of structural and regulatory proteins are involved in intracellular control mechanisms responsible for functional modifications, in order to overcome the lack of DNA transcription in spermatozoa (17).

The spermatozoa released from the testis are morphological mature but immotile and unable to fertilize the oocyte (18). To acquire motility, spermatozoa must produce energy to fuel the movement, and several signalling pathways must be activated/inhibited to initiate movement. Spermatozoa needs high and constant amounts of adenosine trisphosphate (ATP) (19). It is believe that oxidative phosphorylation in mitochondria and/or glycolysis in the flagellum and head are the metabolic pathways required to provide energy to spermatozoa (20–22).

Most signalling pathways in spermatozoa are related with motility acquirement, capacitation and acrosome reaction. Smith and colleagues (23) suggested the important role of phosphoprotein phosphatases (PPP) in the regulation of spermatozoa motility through the epididymis. The group showed that inhibition of phosphoprotein phosphatase 1 (PPP1) activity results in the initiation and stimulation of motility. It has been proposed that primary regulation of flagellar beating, that happens in the epididymis, occurs through reversible phosphorylation of axonemal proteins (24,25). Changes in pH; intracellular concentrations of calcium ions; and cyclic adenosine monophosphate (cAMP) biochemical

and morphological changes (26) that leads to alterations of protein phosphorylation (27) and high concentration of cAMP are believed to stimulate the phosphorylation of sperm proteins by protein kinase A (PKA), increasing motility (28).
After maturation, spermatozoa are stored in the distant part of the caudal epididymis where they remain quiescent. Sperm motility is activated during ejaculation when spermatozoa are mixed with fluids from accessory glands (29).

I.2.1. Phosphoprotein phosphatase 1 (PPP1)

Protein phosphorylation is the process that adds a phosphate group (PO_3-) to either hydroxyl group of an amino acid of serine, threonine or tyrosine. It is involved in many cellular processes and is especially important to stablish protein function by its activation or deactivation through modification of enzymes activity, cellular location, or association with other proteins. Protein phosphorylation is the most abundant post-translational modification in eukaryotic organisms. It is believe that 30% of the proteins encoded by the human genome contain covalently bound phosphate, and that abnormal phosphorylation can be a cause or a consequence of many human diseases (30). For phosphorylation and dephosphorylation are necessary protein kinases and protein phosphatases, respectively. This process is not only controlled by the balanced activity of protein kinases and protein phosphatases but also by their restricted localisation (30,31).

Protein kinases (PKs) are involved in transferring of a terminal phosphate group of an ATP molecule to a hydroxyl group of an amino acid (serine, threonine or tyrosine, and histidine in prokaryotes) side chain of a protein. It can be distinguished by the ability of interaction with both aminoacids (Ser/Thr kinase); only with tyrosine (Tyr kinases); or with all three amino acids (dual-specificity kinases) (32). PKs can be activated or deactivated by (1) phosphorylation, (2) binding of activator proteins or inhibitor proteins or small molecules (e.g. regulatory proteins), (3) controlling their location in the cell relative to their substrates. Regulatory proteins and domains allow to restrict specificity proteins into a particular subcellular compartment and can modulate proteins activity (33).

Protein phosphatases (PPs) are the primary effectors of dephosphorylation (removal of the phosphate group). PPs can be grouped into three main classes based on sequence, structure and catalytic function. Those classes are (i) Ser/Thr protein phosphatases (STPPs), (ii) Tyr phosphatases and (iii) the dual specific phosphatases that dephosphorylate all three residues. The largest class of PPs is the phosphoprotein phosphatase (PPP) family comprising PPP1, PPP2A, PPP2B, PPP4, PPP5, PPP6 and

PPP7, and the metallo-phosphatase dependent on Mg2+- or Mn2+- (PPPM) family, composed primarily of PPP2C.

PPP1 family has a role on a variety of cellular processes, such as cell division; transcription; translation; muscle contraction; glycogen and lipid metabolism; neuronal signalling and embryonic development (26). It is estimated to catalyse about a third of proteins dephosphorylation reactions in eukaryotic cells. PPP1 does not exist freely within the cell, being most of the time associated with another protein (34). In human, the catalytic subunit is express from three different PPP1 genes, that give rise to four isoforms α/A, β/B, γ1/C1 and γ2/C2 (35). PPP1C isoforms differ in their C- terminal of the amino acid sequence, being more than 90% identical in its sequence. PPP1CC1 and PPP1CC2 isoforms occurs by alternative splicing from the same gene, *Ppp1cc* (36), as represented in Figure 2, and the expression of both isoforms is distinct, PPP1CC1 is ubiquitously expressed whereas PPP1CC2 is testis-enriched and sperm-specific (37).

In constant interaction with PPP1, there is a vast and heterogenous group of proteins, called PPP1 interacting-proteins (PIPs), PPP1 regulatory subunits. Depending on the PIP, PPP1 isoforms can play different roles in the cell. So far, more than 200 PIPs were identified, resulting in the involvement of PPP1 in various functions of the human physiology (38).

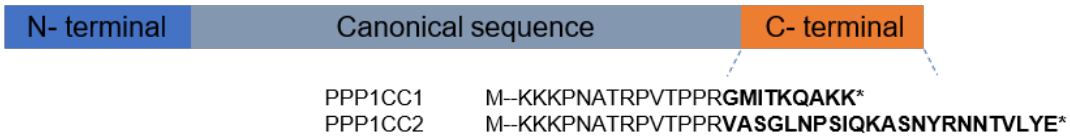

Figure 2 - Schematic representation of human PPP1CC C-terminals isoforms. PPP1CC1 and PPP1CC2 differ in the C-terminus sequence (**in bold**), leading to the specific role of each protein in the cell. These two isoforms arise from alternative splicing of the gene sequence correspondent exon of PPP1CC C-terminus. * denotes the termination codon.

PPP1CA and PPP1CB are expressed in most of mammalian tissues except in skeletal and heart muscles. Levels of PPP1CC1 are higher in brain, small intestine and lung when compared with other tissues, and it is undetectable in heart and spleen. In brain, these three PPP1 isoforms were shown to be present in different regions and revealed also specific subcellular localization. PPP1CC2 expression is enriched in testis and spermatozoa (low expression in brain, lung, spleen and thymus) (39–41).

In human testis, all four PPP1 isoforms are expressed (42). However, their cellular and subcellular distributions are distinct. During mitosis, PPP1CA localizes to the centrosome; PPP1CC1 is associated with microtubules of the mitotic spindle; and PPP1CB is strongly

associated with chromosomes. These differences on subcellular and cellular distribution may be the result of specific interactions of each isoforms and consequently respond to distinct signalling complexes (43,44). PPP1CC2 is predominantly localized in post-meiotic cells, in the cytoplasm of secondary spermatocytes, round spermatids and elongated spermatids (45). In the spermatozoa, this isoform is distributed through the flagellum, midpiece, and posterior region of the head (24), suggesting a role in motility and acrosome reaction.

I.2.1.1. PPP1CC2 Interactions

Most of PIPs have a PP1 docking motif, known as RVxF motif (46). The RVxF binding site is a shallow hydrophobic groove on the surface of the PPP1, away from the catalytic site (47) and it is essential for the binding of many PIPs (46). The presence of this RVxF motif can allow weaker secondary interactions motifs to bind with PPP1, serving as an anchoring point (48). The contribution of the RVxF motif to the binding of PPP1 is interactor-dependent. Some PIPs still interact strongly with PPP1 in the presence of an excess of a synthetic RVxF peptide or despite alterations in their RVxF motif (46). The substrates that interact with PPP1 through this binding motif do not lead to an additional change on the PPP1 conformation, when compared with PIPs without the RVxF motif to bind PPP1 (47).

Interactions of PPP1CC2-PIPs had a notorious role to understand PPP1CC2 function in testis and spermatozoa maturation in the epididymis. The main regulation suggested for PPP1CC2 activity during spermatozoa motility acquisition is the presence of inhibitors, such as PPP1R2 (inhibitor 2, I2), PPP1R7 (sds22) and PPP1R11 (inhibitor 3, I3), but also by protein 14-3-3 and A-kinase anchoring proteins (AKAPs) (38). Moreover, in 2013, Korrodi-Gregório et al. identified a new PPP1R2 (inhibitor 2) isoform in human sperm, PPP1R2 pseudogene 3 (PPP1R2P3). This isoform has the unique feature of Thr_{73} being replaced by Pro avoiding glycogen-synthase kinase 3 (GSK3) phosphorylation. PPP1R2P3 protein binds directly to PPP1CC and the inactive PPP1CC2-inhibitor complex formed cannot be activated by GSK-3 phosphorylation. Korrodi-Gregório et al. hypothesize that PPP1R2P3 is only present in caudal motile sperm, representing a constitutively inhibitor of PPP1, independent of GSK-3 phosphorylation, and therefore responsible for the process of spermatozoa motility acquisition along the epididymis journey (38,49).

Specifically, PPP1CC2 isoform interacts with Spermatogenic zip protein 1 (Spz1), identified as a specific binding partner of this PPP1 isoform. The specificity of this binding partner was confirmed by the loss of interaction when the unique C-terminus of PPP1CC2 was excised, as shown by Hrabchak et al. (2004) (50). Spz1 and PPP1CC2 interaction may be required for proper regulation of spermatogenesis and fertility in mice, since phosphatase assays using recombinant PPP1CC indicate that increasing concentrations of Spz1 are able to inhibit PPP1CC2 (50). Endophilin B1t is a testis-specific splice variant of endophilin B1 and it was shown to interact specifically with PPP1CC2. Despite the presence of a canonical RVxF motif near the N-terminus, Endophilin B1t does not interact with others PPP1C isoforms or with PPP1CC2 mutant lacking the unique C-terminus (51). The A-kinase anchoring proteins (AKAPs) have been also related to testis and sperm function, specifically AKAP3, AKAP4 and AKAP11 (52).

PPP1CC2/PIP complexes are essential regulatory components in the signalling transduction cascades involved in sperm motility acquisition during epididymal transit. Therefore, the study of new PPP1CC2/PIP complexes in testis and sperm are extremely important through a physiological and pathological point of view, as well as a potential contraceptive target.

I.2.2. A-kinase anchor proteins (AKAPs)

AKAPs are a group of structurally diverse proteins, that have the capacity to directly anchor pools of enzymes to a subset location to ensure a particular intracellular signalling event. AKAPs were originally classified based on their ability to bind the cAMP-dependent protein kinase (53). An important role of the AKAPs is the ability to form multi-protein complexes that integrate cAMP signalling with other pathways and signalling events (54,55). AKAPs can integrate diverse signalling pathways that co-ordinately regulate the phosphorylation of specific cellular substrates (56). In this group of proteins 50 AKAPs were already identified (57) from which 20 were cloned (58). Several AKAPs are present in spermatozoa - AKAP1, AKAP3, AKAP4, AKAP8, AKAP11, WAVE-1 and MAP2 (52), and three of them related with PPP1CC2 – AKAP11, AKAP3, AKAP4 (38). Given that many AKAPs have been shown to be present in germ cells and localized to compartments related to motility where PPP1CC2 is also present they might be iinvolved in motility acquisition (59,60).

A-kinase anchor protein 11 (AKAP11) is present in both testis and spermatozoa. In testis, is localized to germ cells and in mature sperm is restricted to the midpiece. Its location in

the midpiece is associated with the cytoskeleton (61). The midpiece associated AKAP11 could serve to anchor PKA and/or PPP1CC2, directly regulating the contractile machinery in the sperm axoneme, as shown by membrane permeable peptides that disruption of RII subunit interaction with AKAPs, causes the arrest of sperm motility (62).

AKAP3 is a testis-specific protein found only in the fibrous sheath and localised in principal piece of the tail (circumferential ribs) of human sperm (63,64). It is reported to participate in protein-protein interactions with the R-subunit of the PKA (63).

In human, A-kinase anchor protein 4 (AKAP4) is the most abundant protein in the fibrous sheath of sperm and its expression is restricted to testis and sperm. Therefore it seems to be a PKA-anchoring testis-specific protein (65). It was shown that AKAP4 gene knockout mice lacks flagellar movement, exhibiting a significant change in the activity and phosphorylation of PPP1CC2. Together, it was inferred that AKAP4 recruits PKA to the fibrous sheath and facilitates local phosphorylation to regulate flagellum functions (66) (Figure 3). Considering AKAP4 localization it was hypothesised its involvement in the regulation of sperm motility. In a AKAP4 knockout male mice, sperm concentration is not affected. However, sperm fails to show progressive motility and consequently the mice is infertile (65). A variation in the activity and phosphorylation of PPP1CC2 is induced, which suggests that AKAP4 is required to regulate the phosphorylation levels of PPP1CC2 in the principal piece of the spermatozoa (69). In a study with human patients whom spermatozoa have low or none motility a screening was performed to assess the status of FS and the axonemal structure. An association between absence or weak AKAP4-labelling in the flagellum and absence or weak motility of spermatozoa was suggested (60).

cAMP analogues and phosphodiesterase inhibitors increase the motility of mature spermatozoa, proposing that elevated cAMP stimulates the phosphorylation of sperm proteins by PKA increasing motility (28). Vieira Silva et. al. (2017) characterized the PPP1CC2/AKAP4 interaction as a potential target for modulation of spermatozoa motility, a decrease of AKAP4 in asthenozoospermic samples when compared with normozoospermic samples is shown. These suggest that the PPP1CC2/AKAP4 interaction has an important role in spermatozoa motility regulation since the loss of PPP1CC2/AKAP4 interaction results in immotile spermatozoa (67).

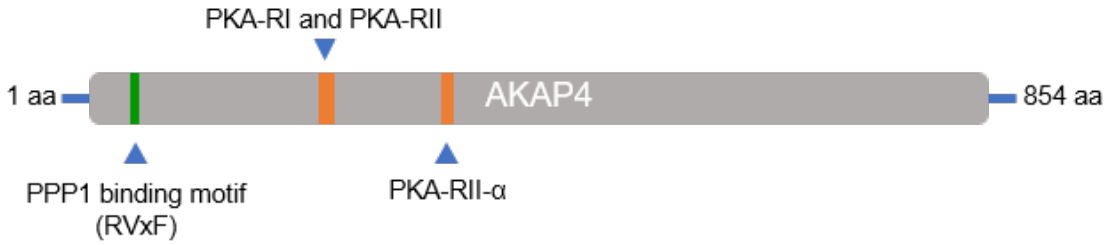

Figure 3 – Schematic representation of human AKAP4. The human protein sequence of AKAP4 has 854 aminoacids (aa) (grey); AKAP4 motifs are represented (▲). In green is highlighted binding motif of PPP1 (^{39}RKVICF^{44}), and in orange is shown the binding motifs of AKAP4 to PKA-RI and PKA-RII subunits.

I.3. Modulation of PPP1 interactions

Modulation of spermatozoa motility has a key role to understand how motility can be increased, in a male infertile situation, or decreased as a contraceptive approach. The development of novel male contraceptives would be pivotal to replace or adjuvate the ones already available, such as condom or vasectomy. PPP1 are involved in the regulation of spermatozoa motility in the epididymis, and it was shown that PPP1 activity inhibition induces motility (23). Targeting PPP1 complexes has already been shown, and natural and chemical compounds can be used in several diseases (68,69).

Okadaic acid and calyculin A are known PPP1 inhibitors. Inhibition of PPP1 activity by these compounds initiates sperm motility in the caput epididymis without requirement for a change in cAMP levels (70). Goto et al (2009) shown that inhibition by calyculin A results in the enrichment of the phosphorylated status at the activation loop of the PKA catalytic subunit in the mouse spermatozoa principal and midpieces (71). Ammosova et al. (2012) demonstrated the viability of inhibiting PPP1 phosphatase activity through regulatory site targeting, by inhibition of the HIV-1 transcription and replication. The authors employed small molecules targeting to the RVxF-binding site of PPP1 and identified a small molecule – 1H4 (patent US 20090264463 A1) – that presumably binds to the RVxF motif of PPP1. This molecule selectively disrupt the Tat-PPP1 interaction without showing cytotoxicity and not affecting other PPP1 holoenzymes (72).

The interfaces between PPP1 and PIPs represent an excellent target for pharmacological intervention. McConnell et al. (2009) have shown two drugs - salubrinal and trichostatin A – that modulate PPP1 complexes. Salubrinal is a small molecule that protect the cell from the Endoplasmatic Reticulum stress-induced apoptosis and was shown to diminish the level of PPP1-GADD34 complex in cells treated with it. Trichostatin A can disrupts the interaction between PPP1 and HDAC6 in glioblastoma and prostate cancer cells (73).

Disruption of PPIs by peptides is an appealing strategy due to their affinity and specificity. Chatterjee et al. (2012) developed a cell-penetrating peptide - PDP3 – that competes with PPP1-interacting proteins (PIP) containing RVxF motifs for binding to PPP1 in living cells. The blocking of these interactions by PDP3 promoted PPP1 auto-dephosphorylation resulting in active PPP1 that dephosphorylate their substrates (74).

I.4. Two-hybrid System

Many functions in cells are controlled by PPIs, hence appropriate methodologies have been developed. Yeast two-hybrid (YTH) system was established by Fields and Song in 1989 (75) and it allows the identification of PPIs. When compared to other biochemical methods it has a higher sensitivity, since it allows the identification of weak and unspecific interactions (44). The YTH system uses the reconstitution of the Gal4 transcription factor of the *Saccharomyces cerevisiae* to identify PPIs. As any other eukaryotic transcription factors, Gal4 requires the close proximity between a DNA-binding domain (BD) and an activation domain (AD) (75). Both domains carry out their functions independently, but only when they are brought together the transcription of the reporter gene occurs. The principle of this system lies in the fusion of each domain with a protein of interest, “bait” and “prey” (76). If the interaction between bait and prey proteins occurs, the Gal4 transcription factor reassembles and the transcription of *lacZ* reporter gene is induced, which results in a blue phenotype when in the presence of X-gal substrate (44,75).

The importance of this technique is reflected on the number of high confidence binary interactions identified. Moreover, it is estimated that about 50% of the interactions described in PubMed were identified by YTH screening (44,75,77). Specifically, for PPP1, most of its interacting proteins were identified and characterized by YTH. Endophilin B1t, Spz1, AKAP3 and AKAP11 are examples of PIPs that were identified by this methodology (50,51).This method has contributed to understand male fertility through the characterization of the human testis PPP1 interactome. Fardilha *et al*. (24) performed YTH screens from a human testes cDNA library, using as baits different PPP1C isoforms (PPP1CC1, PPP1CC2 and PPP1CC2-specific C-terminus). They reported the identification of 77 different proteins, some of them unique to a particular PPP1. 35 proteins were found to exclusively bind PPP1CC2-specific C-terminus, which could reflect the PPP1CC2 isoform specificity (44).

Despite the advantages of this method, it also shows limitations such as, (1) high level of false positives; (2) need an identification of more than one reporter gene for identification;

(3) auto-activation of the reporter gene; (4) use of yeast, that do not mimic the environment where the PPIs occurs in mammalian organisms (44,78). In order to overcome some of the YTH drawbacks, mammalian two-hybrid system (MTH) was developed (78).

I.4.1. Mammalian two-hybrid System

In the MTH system, at least three different types of plasmids are necessary to identify PPIs, often called “bait,” “prey,” and “reporter.” Similar with YTH system, the bait encodes a protein of interest that is fused to the BD, while the prey encodes a target protein that is fused to the AD. These plasmids are then co-introduced into appropriate host cells along with the reporter vector, as shown in Figure 4.

The first study of PPIs in mammalian cells was performed by Dang and colleagues in 1991. Dang et.al system was based on Gal4-BD from the YTH system and VP16-AD (from Herpes virus), with chloramphenicol acetyltransferase (CAT) as the reporter gene, to analyse the complex formed between leucine zipper transcription factors (79).

Later, Lou and colleagues (1997) (78) confirmed that interaction of proteins could be studied in mammalian cells. They proved the expression of two chimeric proteins from the simian virus 40 T antigens and its interactions with mouse p53 antitumor protein by expression of CAT gene under the control of five consensus Gal4 binding sites. Since the development of the MTH system, new versions have emerged. Those, were focus on transient transfection of bait, prey, and reporter vectors (79). For example, interaction between the ERa and SRC1 which phosphorylation is PKA-dependent were studied under this method with luciferase as a reporter, to understand the resistance to tamoxifen observed in half of the recurrences in breast cancer (80). Isselbacher and colleagues optimized a new version of the method where a GFP-based reporter and the bait construct were stably integrated into the cells’ genome and the prey expression was propagated from a stable vector by the use of the EBNA-1/ori-P system, this method allows to perform a cDNA library screening using a specific bait (79). Some studies use mammalian two-hybrid as a control technique. In 2010, Naz and Dhandapani (86) use this method to check for false positives after YTH system. In these case, human ZP3 was cloned into YTH vector and used as bait to find reactive proteins in the human testis cDNA library. Six specific clones were identified and the interaction was further confirmed by MTH system. Recently, there are studies that use M2H systems to detect peptide interactions as Leverson et. al. 2015, in which it was identified a small-molecule MCL-1 inhibitors with sufficient potency to induce clear on-target cellular activity in cancer (87).

Molecules as A-1210477, that induces the hallmarks of intrinsic apoptosis and demonstrates single agent killing of multiple myeloma, binds MCL-1 with sub-nanomolar affinities and confirmed an ability to disrupt endogenous MCL-1–BH3-only protein interactions. By using MTH assays, with Gal4-luciferase as a reporter, and a cell imaging platform that tracks BCL-2 family interactions in real time, it was found that A-1210477 could selectively disrupt MCL-1–NOXA and MCL-1–BIM2A complexes in living cells. In 2010, extensive mammalian two-hybrid assay-based PPI maps were established using pairwise analyses of 1,988 human and 1,727 mouse transcription factors, revealing a total of 762 and 877 interactions, respectively (83). The availability of large transcription factors combinatorial networks in both human and mouse will provide many opportunities to study gene regulation, tissue differentiation, and mammalian evolution (80).

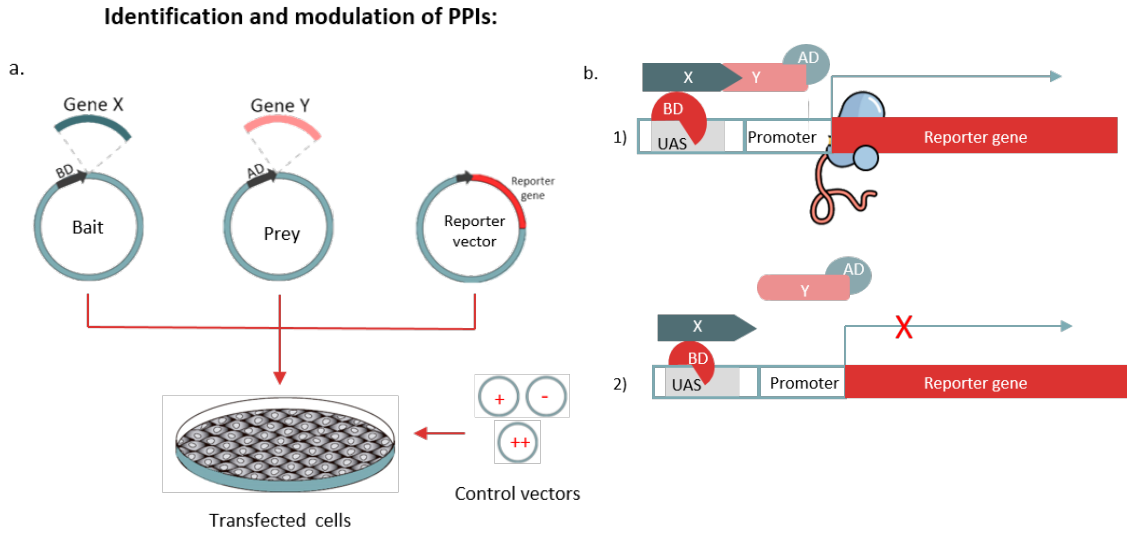

Figure 4 - Mammalian two-hybrid System assay. a. Cloning of the proteins ORF (gene X and Y) of interest and transfection of bait and prey, with the DNA-binding domain (BD) and DNA-activation domain (AD), respectively and the reporter plasmid. The plasmids are transfected together to evaluate the PPI between protein X and Y, control vectors are also transfected to further analyse the interaction. **b**. Interaction between protein X and Y, b.1) reporter gene is expressed when the recognition of upstream activation site (UAS) through the BD in protein X occurs and there is an interaction with protein Y, the interactions ensures that BD and AD are close and recruits RNA polymerase I (I) to start the reporter transcription. b.2) When Protein X and Y do not come in close proximity the transcription of the report gene does not occurs.

The need to identify PPIs that occur in a specific part of the cells, like with transmembrane proteins, lead to adaptation of MTH. Mammalian protein-protein interaction trap (MAPPIT) is a YTH variant that exploits the unique properties of the JAK–STAT (Janus kinase/Signal Transducer and Activator of Transcription) signalling pathway. Since the PPIs occur in the context of an activated receptor, MAPPIT is the method of choice for the analysis of PPIs in signal transduction pathways (81,82).

When compared with yeast, the MTH system presents key advantages, since it can mimic the complex cellular context where the interactions occur in natural circumstances. Furthermore, it allows the study of drugs in an interaction of interest. This system is more flexible, since it can be applied into different cell lines. MTH is not dependent on the expression of an endogenous coactivator since Gal4-coactivator is transfected into the cells to identify if the PPI of interest occurs.

In this assay, the effects of the cell context on transactivation can be determined since ligand-dependent Gal4-coactivator–VP-receptor interactions also rely on endogenous cofactors for transactivation. MTH is more straightforward, the transfection of the reporter vector allows the detections of nuclear and non-nuclear proteins since it is not dependent on transcriptions factors from the nucleus, when compared with YTH where hybrid proteins may not be expressed stably in yeast or may not efficiently move into the nucleus and express the reporter gene, as an example β-galactosidase (83). The use of positive controls with subtracts such as secreted alkaline phosphatase (SEAP) and CAT avoids the need of cell lysis, and allows a fast and convenient analysis of PPIs in mammalian cells. In the perspective of the target proteins, the native physiological context ensures proper folding and provides the necessary cofactors and regulatory proteins involved in post-translational modifications (e.g. phosphorylation, acetylation or ubiquitination) or assisting any conformational alterations that the target proteins need to undergo to allow their interaction (84).

I.4.2. The use of Mammalian two-hybrid in highthroughput drug screening

The highthroughput (HTS) screening is a methodology used for scientific experimentation, with importance for fields like biology or chemistry, especially for drug discovery. HTS allows to perform a high scale test, like a library of different compounds, and discover drugs that inhibit/stabilize PPIs.

Berg et al. (2002) screened four small-molecules antagonists of c-Myc/Max dimerization-domain with 7000 compounds by fusion of fluorophores in the c-Myc/Max complex, when in close proximity the energy transferred between fluorophores increased, and when the complex is inhibited the energy is not transferred, meaning inhibition of the complex (85). Sohn et al (2001) identified 11 potential activators of DPC4 out of a library of compounds. The screening of the compounds on this tumour-suppressor gene mutated, DPC4 signal transduction pathway was tested in a human pancreatic cancer cell line in order to find a therapy that could have a rational role in anticancer therapy (86).

Combining MTH and HTS allows to evaluate drug effect in a specific PPIs, which is an advantage for therapeutic research, since it allows the development of more target specific treatments. As the interface of a particular PPI is determined by the combination of interacting domains of two proteins, it will probably exhibit a high level of specificity when compared to e.g. the catalytic pocket of an enzyme, which is often well conserved throughout a whole enzyme class (84). Several HTP screening assays have been used to discover inhibitors of protein interactions (87). In 2006, Yen and colleagues (88) used HTS to identify small molecules that can serve as inhibitors of ribozyme self-cleavage. The group established cell-based assay in which expression of a luciferase reporter is controlled by ribozyme sequences, and screened 58,076 compounds for their ability to induce the expression of luciferase. Fifteen compounds were able to inhibit ribozyme self-cleavage, with highlights for toyocamycins and 5-fluorouridine. Pattyn et al (2008) performed HTS to modulate the interaction between HIV reverse transcriptase subunits, and analyse the small molecules related to that PIPs (89). Shiu and colleagues found small-compounds, such as ADS-J1, XTT formazan and tannin acid, as inhibitors of trimerization of gp41, an HIV-1 protein involved in cell entrance, by systematic screening for interaction inhibition using 96-well plates and luciferase as a reporter (90). Li et. al. (2011) screened more than 3,000 compounds identified with the mammalian two-hybrid system with luciferase as a reporter gene on the cellular levels of p53. This study was looking for compounds with an inhibitory effect in the interaction of MDM2, an E3 ubiquitin ligase, to the tumour suppressor p53. It is believed that p53 is activated by inhibition of p53-MDM2 interaction, and the effect of nutlin-3, caylin-1 and three hit compounds increased (SL-01, 02, and 03) the levels of p53 protein (91).

I.5. Aims

The mammalian two-hybrid system is without a doubt a powerful tool not only to study protein-protein interactions but also to evaluate the effect of small molecules and drugs in specific protein-protein interactions pairs. Therefore, we believe the MTH is of a great utility in a signalling laboratory. Moreover, since sperm relies on protein-protein interactions to adapt to different environments, the MTH is particularly important to study sperm protein-protein interactions. This work aimed to implement the MTH into the Signal Transduction Laboratory. To accomplish this general goal the following aims were proposed:

Aim 1: To clone PPP1CC2 and AKAP4 ORF into bait and prey MTH system vectors;

Aim 2: To transfect and express the PPP1CC2 and AKAP4 bait and prey vectors into a mammalian cell line.

II. Material and methods

Experimental procedures were performed in the Signal Transduction Laboratory, Institute for Research in Biomedicine (iBiMED), University of Aveiro (Aveiro, Portugal). The details of the solutions used in this work are stated in the Supplementary data.

II.1. Amplification and isolation of plasmids DNA from bacteria

AKAP4 Open Reading Frame (ORF), transcript variant 1, and PPP1CC2 ORF were initially cloned into pCMV6-AC-GFP (RG221765, OriGene, Rockville, MD, USA) and into pAS2-1, respectively. Mammalian two-hybrid (MTH) system vectors, Matchmaker Clontech pM (bait) and pVP16 (prey) plasmids were kindly provided from Dr Folma Buss, Cambridge Institute for Medical Research, Cambridge, United Kingdom. Controls of MTH, pG5SEAP, pM53, pM3-VP16, pVP16-T and pVP16-C were a kind gift from Dr Sharifah Syed Hassan, Monash, Malasya.

Initially, all vectors were transformed into *E. coli* XL1-Blue competent cells. One single and isolated colony was retrieved and incubated in LB (Luria-Bertani) broth medium (ThermoFisher Scientific, Waltham, MA USA), with appropriate antibiotic (Ampicillin), overnight (ON) at 37 ºC with vigorous shaking (220 rpm) for further DNA isolation.

II.1.1. Transformation of *Escherichia coli* - XL1-Blue competent cells

E. coli XL1-Blue competent cells were thawed on ice, and 0.5 µl DNA was added and gently mixed in separate tubes. The cells were incubated on ice for 20 min. Then, the cells were incubated at 42 ºC for 45 s (heat shocked) and after cooled down on ice for 2 min. Next, 500 µl of LB medium was added and incubated for 45 min at 37 ºC with shaking at 180 rpm. The cells were then pelleted for 2 min at 1000 rpm and the supernatant discarded, the cells were then resuspended in 100 µl of LB medium and spread on LB/ampicillin (50 µg/ml) agar plates and incubated at 37 ºC for 16 h, until colonies appeared.

II.1.2. Purification with NucleoBond® Xtra Midi Plus EF

Plasmid DNA purification was performed using NucleoBond® Xtra Midi Plus EF (Machenery-Nagel, Düren, Germany) kit. To prepare a preculture, a single bacterial colony was transferred into 3 ml of LB medium with ampicillin (50 µg/µl) and incubate ON at 37 ºC and 180 rpm. After the saturated culture was transferred into 200 ml of LB

medium containing ampicillin (50 µg/ml) and incubated in the same conditions. The culture was transferred into a falcon and centrifuged at 4500 xg for 15 min at 4°C. The supernatant was discarded. The bacterial pellet was resuspended in 8 ml of Resuspension Buffer RES-EF with RNase A (0.06 µg/µl) and vortexed. Then, 8 ml of Lysis Buffer LYS-EF was added to the suspension and mixed gently by inverting 5 times. The suspension was incubated at room temperature (RT) for 5 min. After, 8 ml of neutralization buffer NEU-EF was added and the mixture left on ice for 5 min. The NucleoBond® Xtra Midi column was prepared by adding 15 ml of Equilibration Buffer EQU-EF onto the filter and left to empty by gravity. The suspension was inverted 3 times to homogenize and loaded onto the column. To the assemble 5 ml of Filter Wash Buffer FIL-EF was added and allowed to empty by gravity. The filter was then discarded and the column washed with 35 ml of Wash Buffer ENDO-EF, followed by adding 15 ml of Wash Buffer WASH-EF. After emptied, the plasmid DNA was eluted to a new 50 ml centrifuge tube by adding 5 ml of Elution Buffer ELU-EF. To precipitate the eluted plasmid DNA, 3.5 ml of RT isopropanol was added to the samples and vortex thoroughly. The precipitate was centrifuged at 15000 ×g for 30 min at 4 °C and the supernatant carefully discarded. 2 ml of endotoxin-free RT 70% ethanol was added to the pellet and gently mixed. The suspension was centrifuged at 12000 ×g for 5 min at RT. Ethanol was discarded and the pellet left to dry at RT. After, the pellet was resuspended in an appropriate volume of endotoxin-free H_2O. Plasmids yield was determined by UV spectrophotometry (DS-11 FX+ Spectrophotometer - DeNovix, Wilmington, DE 19810 SA).

II.2. Confirmation of Control vectors

To confirm the size of the vectors, these were submitted to endonuclease restriction. Control vectors are based on pM and pVP16 vector; thus *Nde*I (New England Biolabs, Herts, UK) enzyme was chosen, since both vectors sequence present the *Nde*I restriction site. 300ng of each DNA were cut according to manufacturer's instructions.

II.3. PPP1CC2 and AKAP4 cloning into MTH vectors

II.3.1. PPP1CC2 Cloning

To clone PPP1CC2 ORF into the pM and pVP16 vectors the DNA of interest was extracted from the original vectors, pAS2-1-PPP1CC2 (92). 5 µg of pAS2-1-PPP1CC2 was first cut with *Sal*I (New England Biolabs, Herts, UK) and after gel extraction and purification all the eluted DNA was enzyme restricted with *Sam*I (ThermoFisher Scientific, Waltham, MA USA). Enzymatic reactions were performed as recommended by the

manufacture's in a volume of 50 µl and incubated ON at 37 ºC (*Table 1*). MTH vectors, pM and pV16, were endonuclease restricted with *Sal*I (New England Biolabs, Herts, UK), and were electrophoretic purified from the agarose gel to perform a second endonuclease restriction with all the DNA recovered from the gel with *Sma*I (ThermoFisher Scientific, Waltham, MA USA), these reactions were performed under manufactures instructions for a volume of 50 µl, at 37 ºC ON. Linearized vectors (pM and pVP16) and PPP1CC2 fragment were then electrophoretic purified and extracted from the gel.

Table 1 – Reaction to linearize the bait and prey MTH vectors.

	pM (µl)	**pVP16 (µl)**
DNA	1	1.3
10x Enzyme buffer	5	5
Enzyme	2	2
Deionised H_2O	42	41.7
Final volume	50 µl	

II.3.1.1. Electrophoretic purification

To visualize the DNA fragments an agarose gel electrophoresis was prepared and the DNA fragments were separated by molecular size. For a 1% agarose gel, the appropriate amount of agarose powder (Invitrogen, LifeTechnologies S.A., Madrid, Spain) was dissolved in TAE 1x (Supplementary data 2), and GreenSafe Premium (NZYTech, Lda., Lisboa, Portugal) was added as manufacturer's instructions. The solution was poured into the appropriate tray and the comb was placed. The gel was allowed to polymerize for 30 min. The samples and GeneRuler 1 kb DNA Ladder (ThermoFisher Scientifics, Waltham, MA USA) were mixed with 6x loading buffer (ThermoFisher Scientifics, Waltham, MA USA). Once the gel was solidified it was placed into the tank field with TAE 1x and the comb removed carefully. The samples and the marker were loaded into the wells and run at 70 V. When the dye had migrated the appropriate distance, the gel was examined under a UV light, using Gel Doc™ XR+ System (Bio-Rad Laboratories, Lda, Almada, Portugal) and the bands were cut from the gel.

II.3.1.2. Plasmid DNA Gel Clean-up

Extraction of DNA from agarose gel was performed by following the instructions of NucleoSpin Gel and PCR Clean-up kit (Machenery-Nagel, Düren, Germany). For each 100 mg of gel 200 µl of NTI binding buffer was added on a 2 ml tubes, heated at 50°C and shacked for 7 min. The result mixture (maximum 700 µl) was loaded to the column and centrifuged for 30 s at 11000 xg and the liquid was discarded. This step was repeated until all the volume passed through the column. 700 µl of NT3 wash buffer was added and

centrifuged for 30 s at 11000 ×g. A second wash of the column was performed in the same manner. In order to remove any traces of ethanol, the column was centrifuge for 1 min at 11000 ×g and the ethanol collected on the bottom discarded. To collect the DNA a new tube was used. 20 µl of NE elution buffer was added to the column and incubated at RT for 1 min. Finally, DNA was eluted by centrifuging for 1 min at 11000 ×g and stored. Plasmids yield was determined by UV spectrophotometry (DS-11 FX+ Spectrophotometer - DeNovix, Wilmington, DE 19810 SA).

II.3.1.3. PPP1CC2 ligation into pM and pVP16

Linearized PPP1CC2 ORF was inserted into linearized pM and pVP16 by performing a 1:3 (vector:insert) reaction in the presence of T4 DNA ligase (New England Biolabs, Herts, UK) at 16ºC ON, originating pM-PPP1CC2 and pVP16-PPP1CC2 vectors. Controls of ligation were performed as described on Table 2.

The two ligations were transformed into *E. coli* XL1-Blue competent cells as described in section II.1.1. Ten positive colonies were selected, each colony was transferred into 3 ml of LB medium with ampicillin (50 µg/µl) and incubate overnight at 37 ºC and 180 rpm.

Table 2 – Controls performed for pM-PPP1CC2 and pVP16-PPP1CC2 ligation

	Control 1	**Control 2**	**Control 3**	**1:3 reaction**
Vector	✓	✓	✓	✓
Insert	✓	-	-	✓
T4 DNA ligase	-	-	✓	✓

II.3.1.4. Alkaline lysis “mini prep”

1.5 ml of bacterial culture from positive clones pM-PPP1CC2 and pVP16-PPP1CC2 were transferred into a microtube and centrifuged at 14000 xg for 1 min at 4°C. The supernatant was discarded. The bacterial pellet was resuspended in 100 µl of ice-cold Resuspension solution through vigorous vortexing. Then, 200 µl of freshly prepared lysis solution was added to the microtube and mixed by inverting several times. 150 µl of ice-cold Neutralization solution were added and the microtube gently inverted 20 times. The microtube was then left on ice for 5 min, centrifuged at 14000 xg for 10 min at 4°C and the supernatant transferred to a clean microtube. DNA was precipitated by adding 2× the volume of absolute ethanol at -20 °C and vortexed. The mix was incubated for 10 min at -20 °C. Then, it was centrifuged at 14000 xg for 5 min at 4°C, the supernatant was completely removed and the pellet washed with 70% ethanol. After centrifugation, at 14000 xg for 5 min at 4 °C, the pellet was allowed to air-dry for 10 min. The DNA was

dissolved in H_2O containing DNAse-free pancreatic RNAse (20 mg/ml). Transformants of PPP1CC2 were endonuclease restricted with *Xho*I and AKAP4 with *Xho*I. When separated by agarose gel electrophoresis, the fragment pattern allowed the identification of positive colonies resulting from cloned vectors. The plasmids that generated the expected DNA fragments were transformed in XL1-Blue competent cells (described on section II.1.1) for a purified extraction by NucleoSpin plasmid and further analysed by sequencing.

II.3.2. AKAP4 Cloning

In order to clone AKAP4 ORF into MTH vectors In-Fusion Cloning (Clontech, Saint-Germain-en-Laye, France) kit was used, this kit allows a rapid and efficient cloning of one or more fragments of DNA into any vector. The DNA of interest was extracted from the original vectors, pCMV6-AKAP4-GFP through PCR. The PCR products were then cleaned by the Cloning Enhancer reagent, and the DNA of interest was ligated to the plasmids, pM and pVP16 by In-Fusion Cloning Reaction with the same primers used to extract the fragment of DNA.

Both vectors were linearized with *Sal*I (New England Biolabs, Herts, UK) restriction endonuclease and purified as descried on section II.3.1. The primers, were designed as suggested in the Kit User Manual and the online platform of Clontech to perform the In-Fusion HD Cloning, sequence can be find on Supplementary table 3.

II.3.2.1. Extraction and Amplification of ORFs

AKAP4 ORF was amplified according to manufacturer's instruction using the CloneAmp™ HiFi PCR Premix. This polymerase ensures a high sensitivity, specificity, priming efficiency and extension efficiency. To perform the PCR a Master Mix was done with concentrations described on *Table 3*.

Table 3 – Reaction of AKAP4 ORF amplification

Reaction component	Volume	Final concentration
CloneAmp HiFi PCR Premix	12.5 µl	1X
Primer 1	5–7.5 pmol	0.2 – 0.3 µM
Primer 2	5–7.5 pmol	0.2-0.3 µM
DNA template	< 1 ng	
Deionized water	Up to 25 µl	
Final volume	25 µl	

The reaction to amplify AKAP4 ORFs from the original plasmids was performed on a thermal cycler with the following cycling conditions:

AKAP4 PCR

95 ºC	10 s	
55 ºC	15 s	x35 cycles
75 ºC	13 s	

II.3.2.2. PCR product purification

To purified PCR product, 2 µl of Cloning Enhancer (CE) were added to 5 µl of the PCR product in a new PCR tube. After, was incubated on a ThermoCycler at 37 °C for 15 min, then at 80 °C for 15 min. The In-Fusion Cloning Procedure for Cloning Enhancer-Treated PCR Fragments was then performed for each plasmid, pM and pVP16, as described on *Table 4*. The reaction occurs at 50 °C for 15 min, it was stored at -20 ºC until transformation of competent cells.

Table 4 – In-Fusion Cloning Reaction

Reagent	Volume for pM vector	Volume for pVP16 vector
5X In-Fusion HD Enzyme Premix	2 µl	2 µl
Linearized vector	5 µl (75 ng)	5 µl (50 ng)
Purified PCR fragment	2 µl	2 µl
Deionized water	1 µl	1 µl
Final volume	10 µl	10 µl

II.3.2.3. Transformation of Stellar Competent cells

Stellar Competent Cells were thaw on ice just before use. After thawing the cells, were mix gently to ensure even distribution, and then 50 µl of competent cells were placed into a 15 ml round-bottom tube. To each 50 µl of competent cells 2.5 µl of the In-Fusion reaction was added. The tubes were incubated on ice for 30 min. The competent cells were heat shocked for 45 s at 42 ºC and placed on ice for 2 min. 450 µl of SOC medium was added to bring the volume up to 500 µl and incubated for 1h at 37 ºC with shaking (180 rpm). After incubation, 20 µl of the cells were pippeted and the volume was brought up to 100 µl. Each diluted transformation reaction was spread on a separate LB plate containing the appropriate antibiotic for the cloning vector (50 µg/ml of ampicillin). The remaining of each transformation reaction was centrifuge at 6000 rpm for 5 min. The supernatant was discarded and each pellet was resuspended in 100 µl of fresh SOC medium. Every transformation reaction was spread on a separate LB plate containing appropriate antibiotic for the cloning vector (50 µg/ml of ampicillin). All plates were incubated overnight (~16 h) at 37 ºC.

To perform the control, ~200 µg of pM vector, pVP16 vector, pM linearized with *Sal*I and pVP16 linearized with *Sal*I were transformed in 16.6 µl of Stellar competent cells, as described previously.

II.3.2.4. Analysis of In-Fusion cloning products

To identify positive clones, isolated colonies of pM-AKAP4, pVP16-AKAP4, were picked. To screen the recombinant plasmid in the transformants, the plasmid DNA was extracted from 3 isolated colonies using an alkaline lysis "mini prep" approach as described on section II.3.1.4. and digested with the restriction endonuclease. Transformants of AKAP4 were digested with *Eco*RI and *Pvu*II. When separated by agarose gel electrophoresis, the fragment pattern allowed to identify the positive clones resulting from transformation of the bait and prey vectors. The plasmids that generated the expected DNA fragments were transformed in *E. coli* XL1-Blue competent cells for a purified extraction by NucleoSpin plasmid and further analysed by sequencing.

II.4. Amplification and purification of PPP1CC2 and AKAP4 vectors by NucleoSpin® Plasmid

To ensure a proper cloning of PPP1CC2 and AKAP4 with the vectors, sequencing of all plasmids was performed. To ensure DNA purity a positive clone was transformed into *E. coli* XL1-Blue competent cells (as described in II.1.1) and DNA was extracted and purified using the NucleoSpin Plasmid kit. Briefly, a single bacterial colony was transferred into 3 ml of LB medium containing ampicillin (50 µg/ml) and incubated overnight at 37 °C with vigorous shaking. 1.5 ml of saturated culture was collected into a microtube and centrifuged at 11000 xg for 30s. The supernatant was discarded and removed as much as possible. The pellet was resuspended in 250 µl of Buffer A1 with RNase A (0.4 µg/µl) by vortexing vigorously. 250 µl of Buffer A2 was added to the mix and the tube inverted 8 times, turning the samples blue. The mix was incubated at RT for 5 min, allowing the lysis to occur. After incubation of the lysate 300 µl of Buffer A3 (neutralization) was added and mixed thoroughly by inverting until the blue samples became colourless. Lysate was centrifuged for 5 min at 11000 ×g to clarify and to remove bacterial debris. The lysate was loaded in a NucleoSpin® Plasmid Column in a Collection Tube (2 ml) and centrifuged at 11000 ×g for 1 min to bind DNA of interest in the silica membrane. The flow-through was discarded from the tube. This step was repeated until all the remaining lysate was load. The silica membrane was washed with 600 µl of Buffer A4, supplemented with ethanol, and centrifuged at 11000 ×g for 1 min. The flow-through was discarded and the column centrifuged at 11000 ×g for 2 min to remove ethanol residues from the silica membrane.

The DNA was collected into clean microtube, by adding 30 µl of Elution buffer (AE) to the membrane and left in incubation for 1 min at RT and centrifuged at 11000 xg for 1 min. This step was repeated with 20 µl of Elution buffer (AE) in order to collect as much DNA as possible from the membrane. Each extraction of the DNA obtained by In-Fusion reaction was then analysed by restriction digestion. Bait and prey vectors fused with AKAP4 ORF were digest with restriction enzymes *Eco*RI and with *Pvu*II, and the vectors fused with PPP1CC2 ORF were digest with restriction enzymes *Xho*I. The mix for each sample were done as described below:

Table 5 – Reactions of constructed plasmids with endonuclease restriction.

Reagent	**pM-AKAP4 (µl)**	**pVP16-AKAP4 (µl)**	**pM-PPP1CC2 (µl)**	**pVP16-PPP1CC2 (µl)**
DNA	1	1	1	1
10x Enzyme buffer	1	1	1	1
Enzyme	0.3	0.3	0.3	0.3
MQ-H2O	7.7	7.7l	7.7	7.7
Final volume	10	10	10	10

II.5. Plasmid sequencing

After confirmation of pM-PPP1CC2, pVP16-PPP1CC2, pM-AKAP4 and pVP16-AKAP4 by restriction endonucleases, the samples were analysed by sequencing. pM vector with AKAP4 and PPP1CC2 was sequenced with Gal4-BD primer (forward) and with T7 primer (reverse). pVP16 vector with AKAP4 and PPP1CC2 were sequenced with Sp6 primer (forward) and with T7 primer (reverse). To obtain the full sequence of AKAP4, pM-AKAP4 and pVP16-AKAP4 vectors were further sequenced using primers that recognized the middle of the AKAP4s sequence, primer 5 and 6 on Supplementary table 3.

II.6. DNA sequence analysis

Clones with PPP1CC2 and AKAP4 ORF were sent to Eurofins genomics (Ebersberg, Germany). The nucleotide sequence obtained from each clone was visualized and analysed using Finch TVTM software (http://www.geospiza.com/Products/finchtv.shtml) that enables the visualization of chromatograms and a direct BLAST search.

II.7. Cell culture

HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) medium supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics (1% penicillin/streptomycin). Cells were grown in 10cm plates in a 5% CO_2 humidified incubator at 37 ºC and sub cultured every 2-3 days.

II.7.1. Endogenous AKAP4 expression in HeLa cells

To identify if AKAP4 was expressed endogenously in HeLa cells, 1×10^6 cells were plated in a 100mm plate and incubated ON in 10 ml of DMEM supplemented with 10% FBS and 5% penicillin/streptomycin with coverslips on plate's bottom. The coverslips were then collected from the plate and an immunofluorescence protocol was performed.

II.7.1.1. Immunofluorescence protocol

Coverslips were collected to 12 wells plate and washed 2 times with 1x PBS. The cells were fixed with 4% Para-formaldehyde (PFA), for 20 min at RT. PFA was removed and the coverslips wash 3 times with 1x PBS. In order to permeabilize the cells, 2 ml of 0.2% of Triton X-100 was added, for 10 min at RT. After, coverslips were washed 3 times with 1x PBS and unspecific binding places in cells were blocked using 5% bovine serum albumin (BSA), 30 min at RT. The cells were treated with 50 µl of primary antibody (AB), for 1h at RT, anti-AKAP4 (ab56551) (1:1000), respectively. The AB was washed 3 times with 1xPBS and the respective secondary AB (anti-mouse (ab32723), Invitrogen (1:300)) was distributed per each coverslip, and incubated for 1h at RT, wrapped in wet paper. Negative controls were performed, HeLa cells with no secondary AB, HeLa cells with Anti-mouse secondary AB. The antibody was washed 3 times with 1xPBS and each coverslip was dyed with Hoesch (1:8000) to coloured the cell's nucleus. To assemble the slides the coverslips were dip in dH_2O and mounted with Mowiol solution. Slides were analysed and pictures obtain on a Motorized inverted system microscope IX81 (Olympus Portugal - Opto-Digital Tecnologias, S.A., Lisboa, Portugal) on a 100x objective (oil emerged).

II.7.2. PPP1CC2 and AKAP4 HeLa transfection

To identify if the proteins were expressed in HeLa cells, firstly, 1×10^6 cells were plated in a 100mm plate and incubated in 10 ml of DMEM supplemented with 10% FBS without antibiotics one day before transfections process. Around 70-90% confluent at the time of transfection was established. On samples with GFP coverslips were added for microscopy analysis. For each transfection, the DNA/lipofectamine complexes were prepared under

manufacturer's instructions. 50 µl of lipofectamine® 2000 were diluted in 1450 µl of Opti-MEM™ I Reduced Serum Medium (ThermoFisher Scientific, Waltham, MA USA) and added to 20 µg of plasmid DNA previously diluted in 1500 µl of the same medium. The mixture was allowed to complex for 25 min at RT. Then, 3 ml of the complexes were added to the plate drop-by-drop and the plate was mixed gently by rocking it back and forth. 24 h after incubation at 37 ºC in a CO_2 incubator, the cells were washed twice in 1× PBS, coverslips were collected for a 6 well plates and cell extracts were prepared. To prepare the extracts, 100 µl of boiled SDS 1% was added to each plate and cells were collected with a scrapper. Cells were on constant shack at 4 ºC for 30 min to lysate, lysed cells were then centrifuged at 16000 xg for 20 min and the supernatant was collected to a new tube. All the controls of transfections were done under the same protocol, such as HeLa cells without DNA addition, empty pM vector, empty pVP16 vector, pEGFP-N1 vector and pCMV6-AKAP4-GFP vector.

Coverslips with pCMV6-AKAP4-GFP and pEGF-N1 transfections, the cell were fixed with 4% PFA, as described on section II.7.1.1.

II.7.3. Bicinchoninic acid assay

Extracts were mass normalized using the bicinchoninic acid (BCA) assay (Fisher Scientific, Loures, Portugal). Samples were prepared by adding 5 µl of each sample plus 20 µl of 1% SDS in a 96-well plate. Standard protein concentrations were prepared as described in Supplementary table 1. Samples and standards were prepared in duplicate. The Working Reagent (WR) was prepared by mixing BCA reagent A with BCA reagent B in the proportion of 50:1. Then, 200 µl of WR was added to each well (standards and samples) and the plate was incubated at 37ºC for 30 min. Once the 96-well cooled to RT the absorbance was measured at 562 nm using an Infinite® 200 PRO (Tecan, Switzerland). A standard curve was obtained by plotting BSA standard absorbance *vs* BSA concentration, and used to determine the total protein concentration of each sample.

II.7.4. Western Blotting

The protein extracts of HeLa cells were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and proteins were electrotransferred onto nitrocellulose membranes. The gel was run at 200 V and transferred at 200 mA for 2 h. Non-specific protein-binding sites on the membrane were blocked for 1h with 5% BSA in Tris-buffered saline containing 0,1% Tween 20 (TBS-T) or 5% Milk in TBS-T, for AKAP4 and PPP1CC2 blots, respectively.

The membranes for endogenous and transfected PPP1CC2 and AKAP4 were then washed with TBS-T and incubated with primary antibodies rabbit anti-PPP1CC2 (G502) and rabbit anti-AKAP4(ab123415) overnight at 4 ºC. After the incubation, the membranes were washed three times with TBS-T for 10 min each and incubated with the appropriate secondary antibody for 1h at RT, infrared IRDye®680RD anti-rabbit (926-68071) secondary antibodies (1:5000) from LI-COR Biosciences (Lincon, NE, USA). Blots were washed three times for 10 min with TBS-T and once with Tris-buffered saline (TBS: 25 mM Tris-HCl, pH 7.4, 150 mM NaCl) and immunodetected using the Odyssey Infrared Imaging System (LI-COR® Biosciences, US). As a control for protein charge in the wells, the membranes were incubated with mouse anti-β-tubulin (32-2600) (1:2000), 40 min at RT. After the incubation, the membranes were washed three times with TBS-T for 10 min each and incubated with the appropriate secondary antibody for 1 h at RT, IRDye®800CW anti-mouse (926-32210) secondary antibody (1:5000) from LI-CORBiosciences (Lincon, NE, USA) and immunodetected using the Odyssey Infrared Imaging System (LI-COR® Biosciences, US).

III. Results

The work performed aimed to implement the MTH into the Signal Transduction Laboratory. To accomplish such goal, MTH control vectors were prepared for transfection and PPP1CC2 and AKAP4 were cloned into MTH vectors. Two methodologies were used. PPP1CC2 plasmids construction was performed under a "traditional" cloning protocol. For AKAP4 plasmids construction In-Fusion HD Cloning Plus CE was used. Finally, assessment of endogenous and transfected PPP1CC2 and AKAP4 expression on mammalian cell lines was performed. The next section presents a detailed description of the results.

III.1. MTH control vectors are correct

Since MTH control vectors were a gift, sent on paper, we ensured the purity of plasmids after isolation and extraction from E. coli XL1-Blue competent cells. DNAs were endonuclease restricted with *Nde*I, since the restriction site for this enzyme is expected to be present in all MTH plasmids (no vector map available).

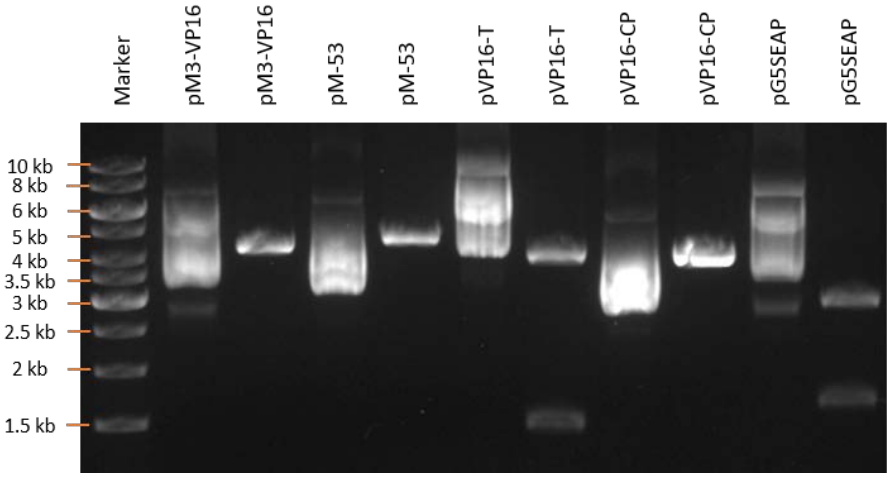

Figure 5 – Endonuclease restriction of MTH control vectors. It shows the patterns of all the control vectors for the MTH system undigested (first lane of each plasmid) and digested with *Nde*I endonuclease (second lane of each plasmid). Marker – GeneRuler 1kb ThermoFisher.

After restricted with *Nde*I, pM3-VP16 presents one band at 4.4 kb, pM-53 at 4.6 kb, pVP16-T at 4.0 kb and at 1.3 kb (total 5.5 kb), pVP16-CP at 4.5 kb and pG5SEAP presents two bands, at 3.0 kb and 1.5 kb (total 4.5 kb). Therefore, patterns observed in Figure 5 revealed that the vectors are correct and well amplified since the bands were as expected. Therefore, the MTH control vectors can be further used in MTH assay.

III.2. PPP1CC2 plasmids construction

To validate and characterize the PPP1CC2/AKAP4 interaction by MTH, PPP1CC2 ORF was extracted from pAS2-1-PPP1CC2 (previously generated in our lab) by digestion with *Sma*I/*Sal*I and directionally subcloned into *Sma*I/*Sal*I-digested pM and pVP16 vectors. The resulting plasmid was sequenced to check for mutations and confirm proper cloning (fusion protein in the correct reading frame).

III.2.1. PPP1CC2 was successfully cloned into pM and pVP16 vectors

pM and pVP16 vectors, as well as, pAS2-1-PPP1CC2 vector suffered a sequential endonuclease restriction, firstly with *Sal*I. It was then electrophoretic purified and extracted. After, DNAs had a second endonuclease restriction with *Sma*I, resulting in the linearized vectors and PPP1CC2 fragment, as shown in Figure 6. After that, DNA was extracted from the gel.

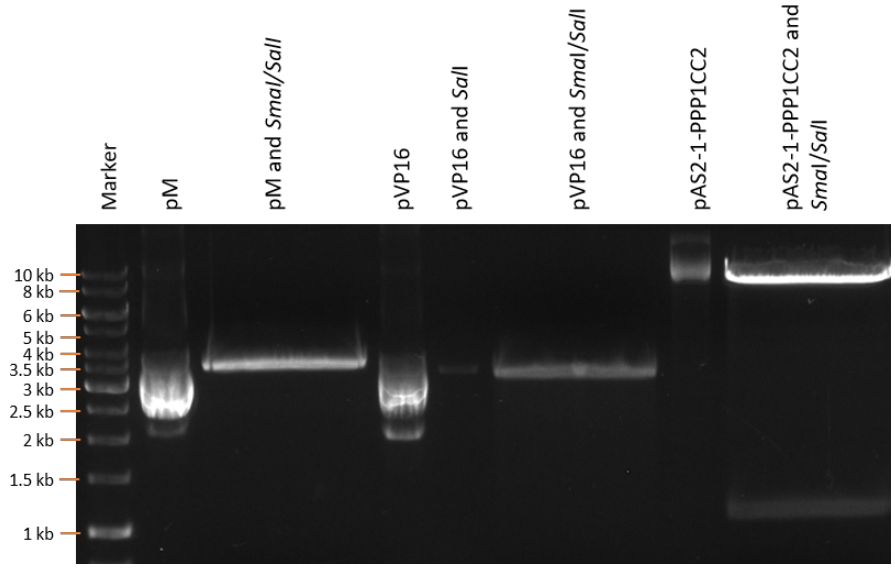

Figure 6 - Purified pM and pVP16 vectors and pAS2-1 restricted with *Sma*I/*Sal*I. The figure shows the purified vectors (pM and pVP16) and the PPP1CC2 fragment (lower fragment in pAS2-1-PPP1CC2 (~1.1 kb)) for extraction. Marker – GeneRuler 1kb ThermoFisher.

After extraction, PPP1CC2 fragment was cloned into the vectors, pM and pVP16, by T4 DNA ligase reaction. The resulting vectors were transformed into XL1-Blue competent cells and isolated colonies were selected to incubate and extract DNA to confirm positive clones by endonuclease restriction with *Xho*I. It was expected for pM-PPP1CC2 to digest into two fragments of 3.2 kb and 1.3 kb; and for pVP16-PPP1CC2 was expected that the vector became linearized with 4.2kb of size.

Figure **7**.a. shows positive clones for pM-PPP1CC2 and figure 6.b. shows a positive clone for pVP16-PPP1CC2. pVP16-PPP1CC2 clone 2 appears to be a negative clone, it

presents the same pattern as the empty pVP16 vector, which indicate that PPP1CC2 was not cloned into the plasmid. Positive clones were then sent for sequencing to confirm the correct reading frame of the inserts.

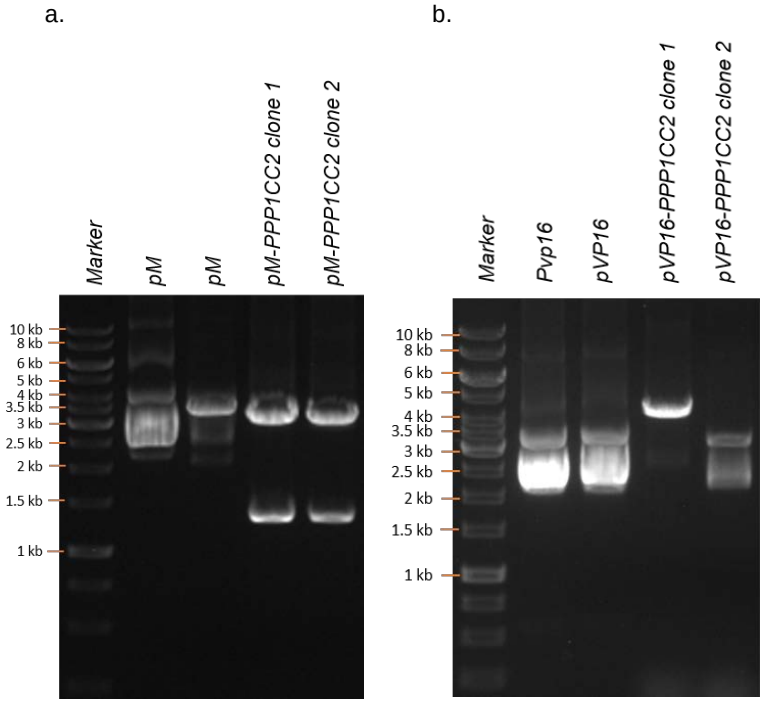

Figure 7 – Positive clones of pM-PPP1CC2 and pVP16-PPP1CC2. **a. pM-PPP1CC2 enzymatic restriciton with *Xho*I.** pM-PPP1CC2 clones digested with *Xho*I. **b. pVP16-PPP1CC2 enzymatic restriciton with *Xho*I.** pVP16-PPP1CC2 clones digested with *Xho*I. Marker - GeneRuler 1kb ThermoFisher.

III.2.2. pM-PPP1CC2 and pVP16-PPP1CC2 are in frame with Gal4-BD and VP16-AD

To check the sequence of the plasmids pM-PPP1CC2 and pVP16-PPP1CC2 cloned with *Sma*I/*Sal*I DNAs were sequenced. This allowed to confirm that PPP1CC2 ORF was correctly inserted into the plasmids and in the correct reading frame, Figure 8.

Note that, this PPP1CC2 sequence results from fusion of human PPP1CC1 with the C-terminus of rat PPP1CC2 (92). There is a non-translated part of the sequence in PPP1CC2 sequence (green). When compared with the human C-terminus the sequence has one alteration, an alanine (A) is replaced by a glycine (G, orange arrow in Figure 8).

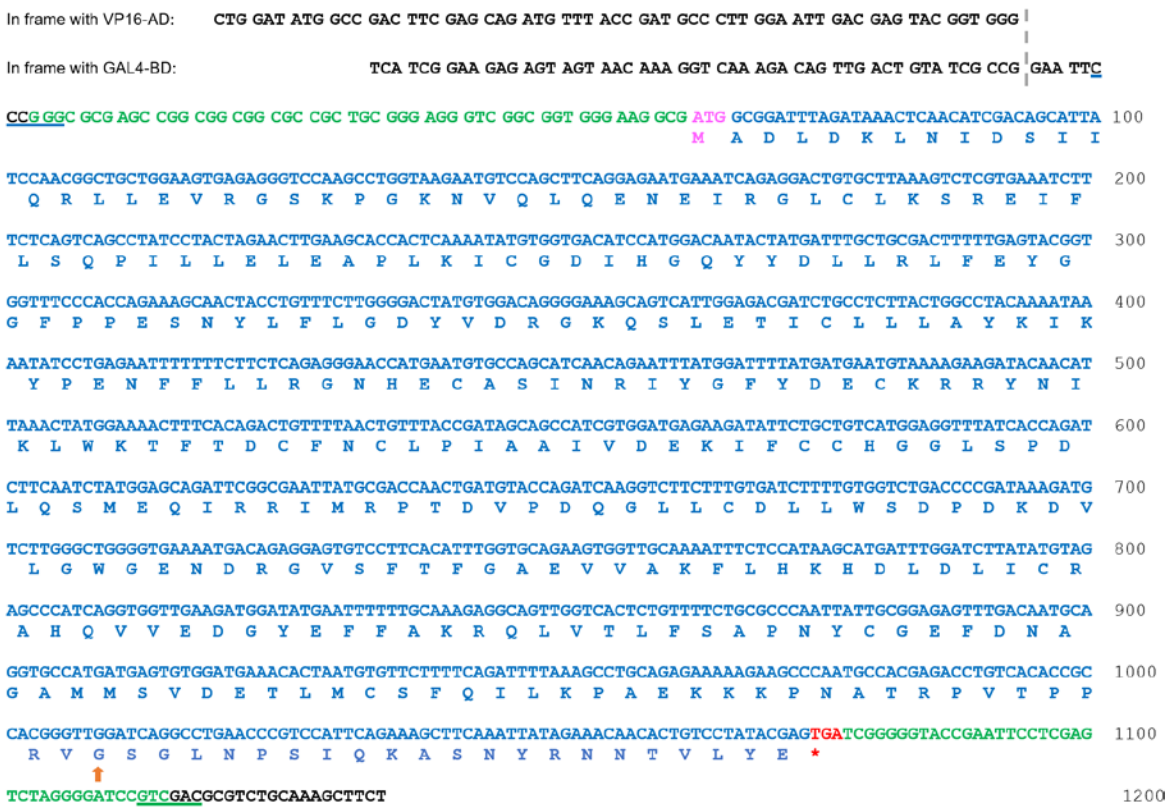

Figure 8 - Partial sequence of PPP1CC2 in pM and pVP16 vectors. The sequence of PPP1CC2 extracted from pAS2-1 vector (in blue) after *Sma*I/*Sal*I restriction (underlined in blue and in green, respectively) and its introduction into pM and pVP16 vectors. The non-translated PPP1CC sequence is in green. Methionine codon of PPP1CC2 reading frame is in pink and stop codon in red (*).

III.3. AKAP4 plasmid construction

Similarly to PPP1CC2, AKAP4 ORF was cloned into the appropriate vectors however, a different cloning method was used. Consequently, the DNA of human AKAP4 was extracted from pCMV6-AKAP4-GFP with a PCR reaction and cloned into pM and pVP16 linearized vectors through In-Fusion Cloning reaction. The resulting plasmids were sequenced to check for mutations and confirm proper cloning (fusion protein in the proper reading frame).

III.3.1. AKAP4 was successfully cloned into pM and pVP16 vectors

pM and pVP16 vectors were endonuclease restricted with *Sal*I, becoming linearized. They were then electrophoretic purified and extracted, Figure 9. AKAP4 ORF was amplified from the original vector with primer 1 and 2 (Supplementary table 3), Figure 10 confirmed that AKAP4 ORF amplification went as expected, resulting in a DNA fragment with 2.6 kb.

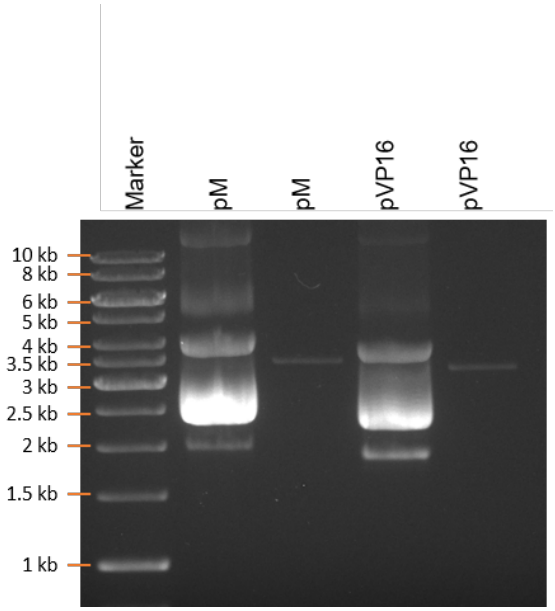

Figure 9 – MTH linearized vectors with *Sal*I. Marker - GeneRuler 1kb ThermoFisher., First lane of each plasmid in uncatted and the second lane shows the linearized plasmid, pM linearized (~3.5 kb) and pVP16 linearized (~3.3 kb).

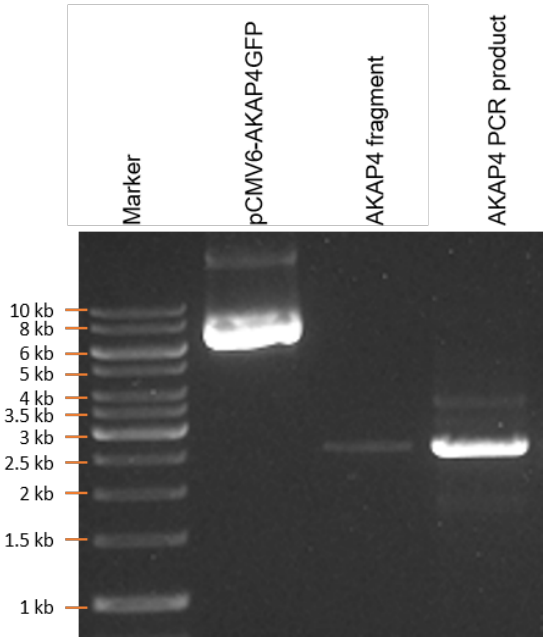

Figure 10 – AKAP4 PCR amplification. AKAP4 was extracted and amplified by a PCR reaction, AKAP4 PCR product resulted from CloneAmp Hi-Fi reaction. Marker – GeneRuler 1kb ThermoFisher,

III.3.2. AKAP4 cloned into pM and pVP16

To obtain single plasmids, the DNA was first transformed into bacteria Stellar Competent Cells, provided with In-Fusion kit and analysed by restriction digestion. Plasmids with the AKAP4 insert were restricted with *EcoR*I and *Pvu*II endonucleases, it was expected for pM-AKAP4 to result into two fragments of 5.7 kb and 369 bp for *Pvu*II digestion and 4.6 kb and 1.4 kb with *Eco*RI digestion. For pVP16-AKAP4 was expected two fragments of 5.5 kb and 369 bp for *Pvu*II digestion and 4.4 kb and 1.4 kb for *Eco*RI digestion.

In Figure 11.a and 11.b, show positive clones for pM-AKAP4 plasmid when cut with *Pvu*II and positive clones for restriction of *Eco*RI. These patterns prove the cloning of AKAP4 into pM. Figure 11.c and 11.d show positive clones for pVP16-AKAP4 endonuclease restricted with the same enzymes, while pVP16-AKAP4 clone 2 DNA may result from a false positive.

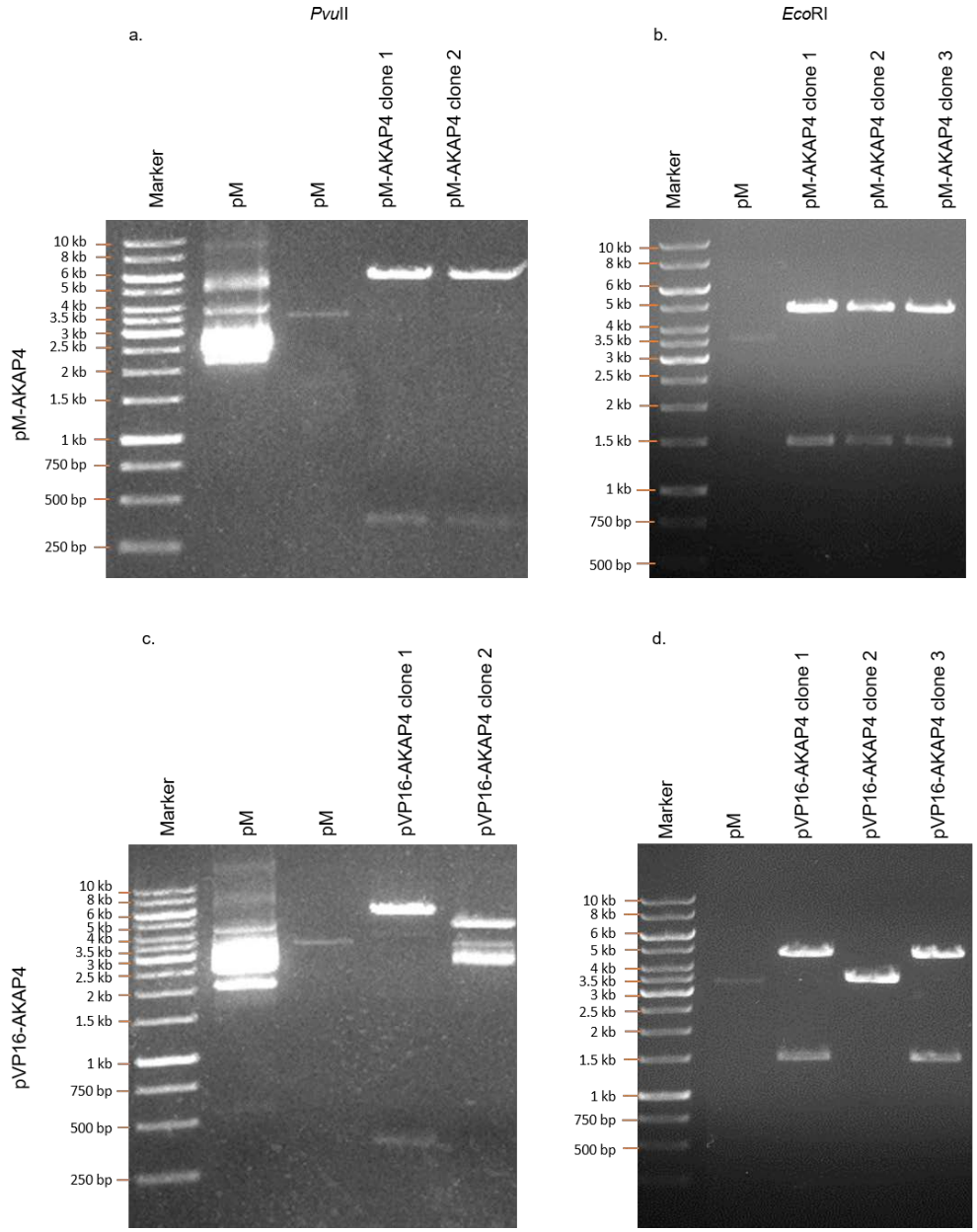

Figure 11 - Fragment pattern produced by endonuclease digestion of In-Fusion products. Marker – GeneRuler 1kb ThermoFisher. **a.** pM-AKAP4 enzymatic digestion with *Pvu*II. **b.** pM-AKAP4 enzymatic digestion with *Eco*RI. **c.** pVP16-AKAP4 enzymatic digestion with *Pvu*II. **d.** pVP16-AKAP4 enzymatic digestion with *Eco*RI.

III.3.3. pM-AKAP4 and pVP16-AKAP4 are in frame with Gal4-BD and VP16-AD

To check the successfully cloning of AKAP4 into pM and pVP16, pM-AKAP4 and pV16-AKAP4 were sequenced. Figure 12 shows that AKAP4 ORF was cloned into the MTH vectors. It allowed to confirm that AKAP4 ORF was successfully cloned into both vectors in the correct reading frame, Figure 12.

Figure 12 - Partial sequence of AKAP4 in pM and pVP16 vectors. The sequence of AKAP4 extracted by PCR reaction with primer 1 and 2 from pMCV6-AKAP4-GFP vector is in blue and its correct introduction into pM and pVP16 vectors. Methionine codon of PPP1CC2 reading frame is in pink and stop codon in red (*).

III.4. AKAP4 is expressed in HeLa cells and presents cytoplasmic distribution

To evaluate the presence and determine the subcellular location of endogenous AKAP4, immunofluorescence studies were performed in HeLa cells, with mouse anti-AKAP4 and respective secondary AB. HeLa cells are expected to express AKAP4 endogenously since it is a carcinoma cell line and AKAP4 has been identified as a cancer/testis (CT) antigen. Figure 13 shows the images captured after immunofluorescence for AKAP4. It is possible to see that AKAP4 is endogenously expressed in this cell line, and appears to have a cytoplasmic and plasma membrane localization.

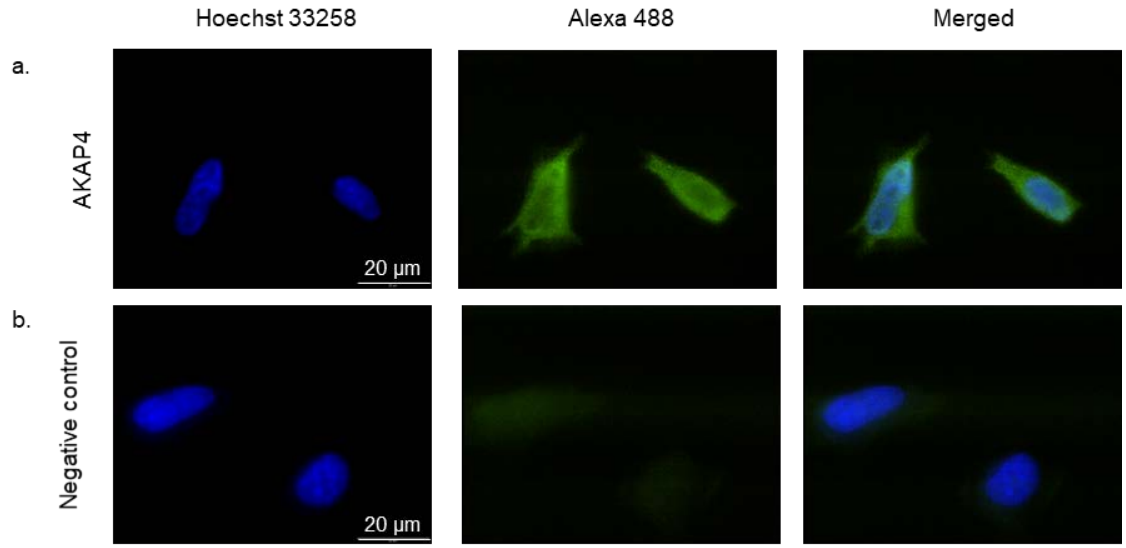

Figure 13 – Endogenous AKAP4 expression in HeLa cells. a. Shows the cell nucleus stained with Hoechst 33258 (blue), the AKAP4 was probed with mouse anti-AKAP4 (primary AB) and anti-mouse (green) (secondary AB), the third column shows the merged pictures. **b.** The control for the secondary AB, the nucleus cells are dyed in blue and the secondary antibody does not present unspecific ligations.

To identify AKAP4 subcellular location when overexpressed, AKAP4 fused with a GFP (pCMV6-AKAP4-GFP) was transfected into HeLa cells. In Figure 14 is possible to observe that AKAP4-GFP is localized throughout the entire cell, suggesting a cytoplasmic subcellular localization.

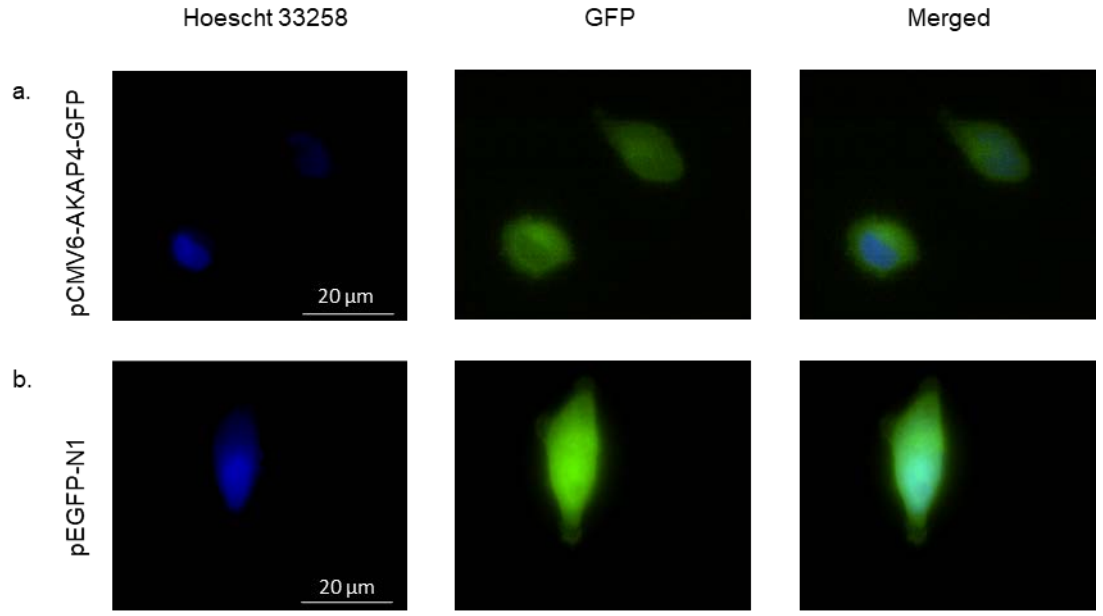

Figure 14 – Transfection of AKAP4 fused with GFP into HeLa cells. a. Shows the control for GFP, with pEGFP-N1 transfected, and the nucleus cell dyed with Hoechst 33258. **b.** Shows the transfection of pCMV6-AKAP4-GFP in HeLa cells.

III.5. Gal4BD-PPP1CC2 pVP16AD-PPP1CC2, Gal4BD-AKAP4 and pVP16AD-AKAP4 are expressed in HeLa cells

To check if PPP1CC2 and AKAP4 cloned into pM and pVP16 vectors are expressed in HeLa cells, pM-AKAP4, pVP16-AKAP4, pM-PPP1CC2 and pVP16-PPP1CC2 were transfected into HeLa cells. To identify expression of PPP1CC2 and AKAP4 by HeLa cells the extracts were run in a 10% SDS-PAGE gel. Blots were incubated ON with primary antibody, Anti-PPP1CC2 (1:5000) and Anti-AKAP4 (1:5000) and the immunoblot with Mouse anti-β-tubulin (1:2000) to confirm that all the protein loaded in the wells of each blot were in identical quantities, Figure 15.

Endogenous PPP1CC2 if expressed in HeLa cells should have a molecular weight of approximately 39 kDa, while when fused with Gal4-BD or VP16-AD, should present a molecular weight of 61 kDa and 53 kDa, respectively. Endogenous AKAP4 if expressed in HeLa cells should have a molecular weight of approximately 94 kDa, while when fused with Gal4-BD and VP16-AD, should present a higher molecular weight. On pM-AKAP4 the expressed protein has attached the BD from Gal4-BD, appearing around 117 kDa and on pVP16-AKAP4 the protein has attached the AD from VP16-AD, revealing the protein expression on 109 kDa. In Figure 15.a. is possible to observe that HeLa cells do not express endogenous PPP1CC2. However, when transfected, PPP1CC2 is expressed as a hybrid protein with Gal4-BD or VP16-AD. In Figure 15.c. is possible to observe that endogenous AKAP4 is expressed in this cell line, as well as when transfected AKAP4 is expressed as a hybrid protein with Gal4-BD or VP16-AD. Therefore pM-PPP1CC2 and pVP16-PPP1CC2 can be used in MTH.

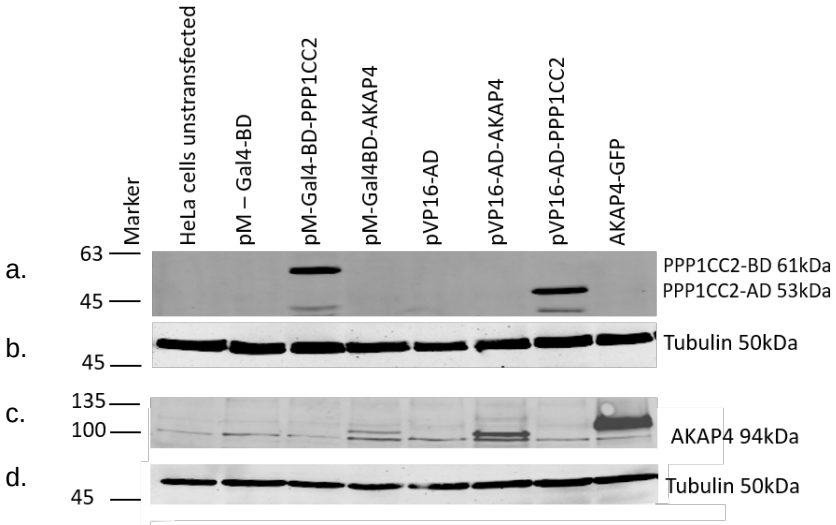

Figure 15 – PPP1CC2 and AKAP expression in HeLa Cells. Marker – NZYColour Protein Marker II (Nzytec, Lisboa, Portugal), **a.** PPP1CC2 immunodetected using an anti-PPP1CC2 (G502, homemade (1:5000)). **c.** AKAP4 immunodetected using an anti-AKAP4. **b. and d.** Tubulin immunodetected using anti-β-tubulin ((1:2000) (32-2600) Invitrogen).

IV. Discussion

The goal of these work was to construct the molecular tools to study the interaction between PPP1CC2 and AKAP4 using the mammalian two-hybrid system and implement this system in the Signal Transduction Laboratory.

Identifying PPIs has a key role to understand signalling pathways responsible for all cellular process and the molecular mechanism behind many diseases. Currently, many techniques are available to identify PPIs, from co-immunoprecipitation to yeast two-hybrid system. The mammalian two-hybrid is able to mimic the complex cellular context where the interactions occur in natural circumstances. It is more flexible, since it can be applied into different cell lines, allowing studies on a specific tissue. Moreover, the identification of PPIs is not dependent on the expression of an endogenous coactivator, as in YTH systems, since it is transfected into the cells. This allows to detect PPIs that have a nuclear as well as a non-nuclear cellular localization. The fact that MTH system requires co-transfection of at least 3 vectors can be a limitation. Different transfection efficiency between vectors can lead to false negatives. On a pharmacologic and therapeutic view, this system can be more advantageous for results extrapolations on human proteins roles and interactions, as well as, protein modulation.

To successfully implement the MTH into the Signal Transduction Laboratory, first it was necessary to construct the MTH bait and prey plasmids with PPP1CC2 and AKAP4 ORFs. These tools must be generated in order to study others PPIs. PPP1CC2/AKAP4 interaction was chosen to implement the MTH in the laboratory, since this interaction appears to be related with motility acquisition and regulation of flagellum functions (66). Some studies have suggested that AKAP4 is necessary to regulate the phosphorylation levels of PPP1CC2 in the principal piece of spermatozoa (70). Modulating the interaction of these proteins might be important to understand and clarify sperm motility acquirement and function. Two approaches were undertaken to construct the plasmids of MTH system, traditional cloning method and commercial available fast cloning kit (In-Fusion HD Cloning Plus CE, 638919, Clontech). PPP1CC2 plasmids were constructed under a “traditional” cloning method, where restriction enzymes are used to linearized the vectors and extract PPP1CC2 ORF from a pre-existing plasmid. This is a time-consuming method, that requires electrophilic purification and extraction taking several days until the DNA is suitable for ligation. Besides, restriction ligations are constricted by sequence limitations when choosing the endonuclease cutting site and all the variabilities that can interfere with

the endonuclease performance. In other hand, In-Fusion cloning kit, the method used to construct AKAP4 plasmids, can amplify the sequence of interest without adding additional information into the sequence of interest. This kit is a fast and directional way to clone any DNA fragment into a plasmid, recommended for long fragments or the fusion of more than one fragment, that sometimes under traditional cloning is challenging. The use of primers is required to amplify and ligate the DNA into the vectors, purification of fragments is done directly into the PCR product. One of the drawbacks of the In-Fusion Cloning method is correlated with the primers. Primers for this protocol can get up to 40 nucleotides of length, which can induce a low amplification rate of the insert because the melting temperatures (Tm) have to be precisely calculated, the percentage of GC ratio has to be consider and the possibility of dimers formations in a long primer is higher. On the other hand, the major advantage of the In-Fusion Cloning when compared to traditional is the duration of the protocol. When compared with T4 DNA ligation, that can take up to 16h to ligate the DNA into the backbone, In-Fusion Cloning reaction only takes 15 min and is highly efficient. Therefore, the procedure can be done within a day and then the DNA is ready to transform into competent cells. The kit is more expensive, which can be a disadvantage to use to generate a reduce number of plasmids.

PPP1CC2 is testis enriched proteins, so their expression in HeLa cells would not be expected. Indeed, in Hela cells PPP1CC2 is not endogenously expressed (Figure 14), being that it is only expressed when transfected. Some of the other 3 isoforms of PPP1C are known to be expressed in HeLa cells. On the other hand, AKAP4, also a testis enriched protein, is shown to be express endogenously on HeLa cells (Figure 16). Cancer/testis (CT) antigens are a category of tumour antigens with normal expression restricted to male germ cells in the testis but not in adult somatic tissues (93). AKAP4 was identified as a cancer/testis antigens, and this protein is expressed in cancer cells, such as cervical cancer(94), multiple myeloma(95), breast cancer (96), prostate cancer (97) or on ovaries carcinoma (98). Siani et al (2013) determined the expression and subcellular localization of AKAP4 in four cervical cancer cells (HeLa, C-33A, CaSki and SiHa). It was detected in the cytoplasm, plasma membrane, endoplasmic reticulum, Golgi complex and mitochondria, but not in the nuclear envelop. Therefore, HeLa cells, as a cervical carcinoma cell line, expresses AKAP4 endogenously as shown by Western blot (Figure 15) and immunofluorescence (Figure 13). We also observed that AKAP4-GFP tag, when transfected into HeLa cells appears to have an effect on nucleus conformation, since it presents a lobulated structure. Further studies to assess the effect of AKAP4 overexpression on cell physiology are necessary

V. Conclusions and future perspectives

The goal of these work was accomplished, molecular tools to study the interaction between PPP1CC2 and AKAP4 to identify with mammalian two-hybrid system and implement this system in the signal transduction laboratory is now possible. All the vectors are cloned and ready to use, cell lines are stablished fortransfection of MTH vectors and to perform the MTH assay. The bait and prey DNAs were inserted into the MTH vectors on the right direction and reading frame. Two different methods were used for construction of PPP1CC2 and AKAP4 plasmids.

The focus of this work was to implement the mammalian two-hybrid system to confirm the PPI between PPP1CC2 and AKAP4 in a more physiological system, closer to spermatozoa. Moreover, the system in place will be pivotal to identify possible compounds that can interfere and modulate that interaction through a highthroughput drug screening. Future work should focus upon the optimization of triple transfection of vectors into HeLa cells, to perform the MTH system. Several transfection protocols can be tested, to enable a faster and more efficient transfection than the recommended on MTH manual (Calcium phosphatase protocol). Determination of subcellular location of PPP1CC2 and AKAP4 when cloned into MTH vectors is important, since the hybrid plasmid would be targeted to the nucleus by the SV40 nuclear localization signal (AD – pVP16) and/or by the GAL4 nuclear localization sequence (BD – pM) for the transcription to occur. The activation and binding domain need to be in close proximity, for the transcription to occur. SEAP reporter gene, which is downstream and under control of GAL4-responsive element, will be activated when the interaction of the two proteins (PPP1CC2 and AKAP4) happens. To measure the interaction between these two proteins the us of Great Escape SEAP Chemiluminescence protocol will be necessary. On a therapeutic approach, it would be interesting to establish this protocol to perform a highthroughput drug screening and modulate this protein interaction, since it is proved by our lab to have a role on sperm motility when the interaction is inhibited.

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Supplementary data

Culture Media and Solutions

1. Bacteria Media

LB (Luria-Bertani) Broth medium (ThermoFisher Scientific, Waltham, MA USA) with Ampicillin (50 µg/µl)

In 950 ml of deionised water add:
LB broth 25 g
Agar 15 g (for plates only)

Shake until the solutes have dissolved. Adjust the volume of the solution to 1 liter with deionized H_2O. Sterilize by autoclaving. Add ampicillin (Sigma-Aldrich Química, S.A., Sintra) to a final concentration of 50 µg/ml after LB cool down to ≈ 60 ºC.

2. Agarose Gel

50x TAE buffer

To 950 ml of denoised H_2O add:
242 g Tris Base
7.1 ml Glacial acetic acid
100 ml 0.5 M EDTA (Ph 8.0)

1x TAE buffer

20 ml of 50x TAE buffer
Deionized water up to 980 ml.

3. Alkaline lysis solutions

Resuspension Solution
50 mM Glucose
25 mM Tris-HCl (pH 8.0) (NZYTech Portugal, Lisboa, Portugal)
10 mM EDTA (Sigma---Aldrich Química, S.A., Sintra)

Lysis Solution
0.2N NaOH
1% SDS (Amresco, Ohio, USA)

Neutralization Solution
3 M Potassium acetate (Sigma---Aldrich Química, S.A., Sintra)
2 M Glacial acetic acid (Sigma---Aldrich Química, S.A., Sintra)

Supplementary table 1 - Standards for BCA assay

Standards	BSA (µl)	1% SDS (µl)	Protein (µg)
P_0	-	25	0
P_1	0.5	24.5	1
P_2	1	24	2
P_3	2.5	22.5	5
P_4	5	20	10
P_5	10	15	20

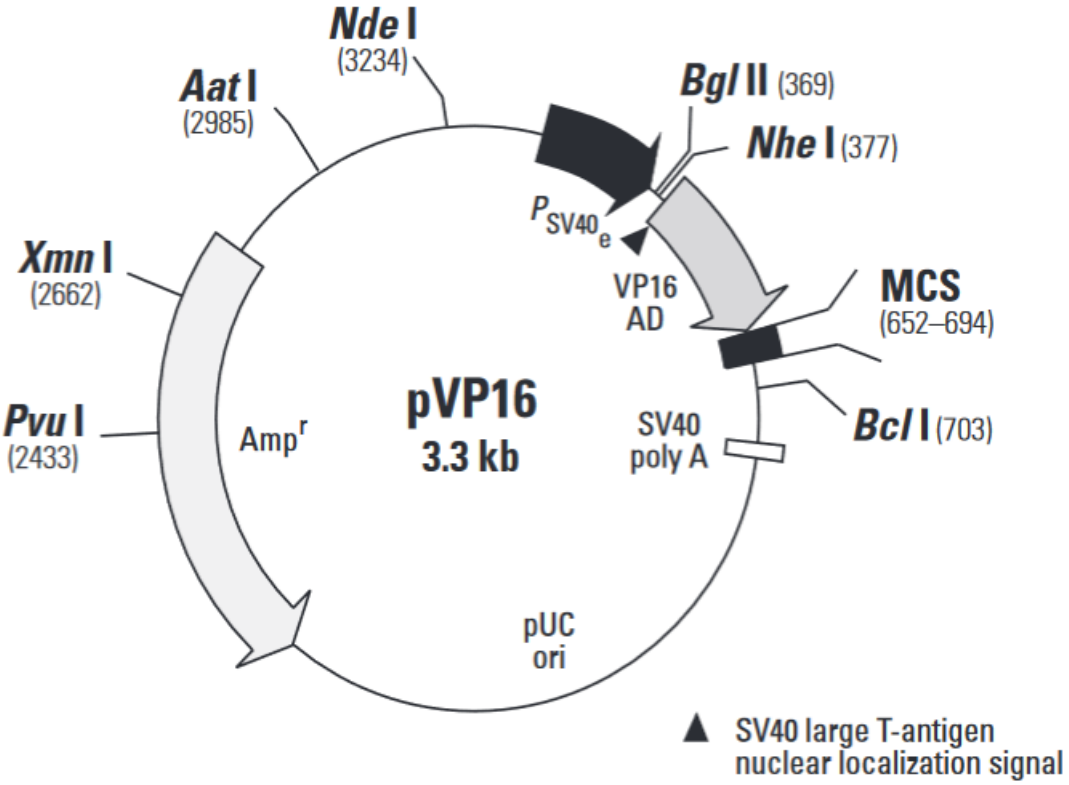
Supplementary table 2 - Solutions for Western blot

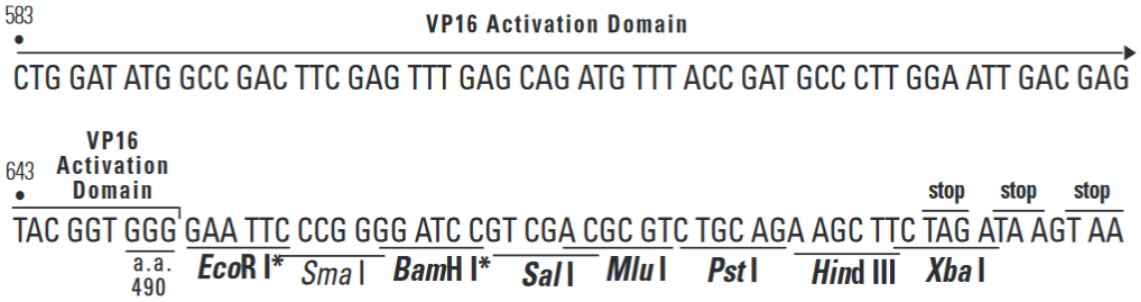
Running gel 10% (2 gels, 1.5 mm thickness)	ddH2O	7.720 ml
	Tris 1.5 M pH8.8	5.000 ml
	Acrylamide 40%	4.920 ml
	Bisacrylamide 2%	1.960 ml
	SDS 10%	0.200 ml
	APS 10%	0.100 ml
	TEMED	0.020 ml
Stacking gel 4% (2 gels, 1.5 mm thickness)	ddH2O	4.736 ml
	Tris 0.5 M pH6.8	2.000 ml
	Acrylamide 40%	0.784 ml
	Bisacrylamide 2%	0.320 ml
	SDS 10%	0.080 ml
	APS 10%	0.040 ml
	TEMED	0.008 ml
Tris-HCl 1.5 M pH 8.8 buffer	For 1 L dissolve 181.5 g Tris in 800 ml deionized water. Adjust pH at 8.8 with HCl and make up to 1 L with deionized water.	
Tris-HCl 0.5 M pH 6.8 buffer	For 1 L dissolve 60 g Tris in 800 ml deionized water. Adjust pH at 6.8 with HCl and make up to 1 L with deionized water.	
10% APS (ammonium persulfate)	For 10 ml of deionized water add 1 g of APS.	
10% SDS (sodium dodecilsulfate)	For 500 ml of deionized water dissolve 50 g of SDS.	
4X Loading gel buffer	For 10 ml add 44 ml glycerol, 250 µl Tris-HCl 0.5 M pH 6.8 buffer, 0.8 g SDS, 0.2 ml β-mercaptoethanol and 3.3 ml deionized water. Add bromophenol blue (a small amount). Keep it at RT for short periods or at 4ºC for longer periods.	
Tris-Gly 10X Stock	For 1 L dissolve 30.30 g Tris (250 mM) and 144.10 g Gly (1.92 M) in 1 L of deionized water.	
Running buffer 1X	For 1 L add 800 ml deionized water, 100 ml Tris-Gly 10X and 10 ml 10%SDS. Make up to 1 L with deionized water.	
Transfer buffer 1X	For 1 L add 100 ml Tris-Gly 10X to 700 ml of deionized water and 200 ml methanol.	
10X TBS Stock (Tris buffered saline)	For 0.5 L dissolve 6.055g Tris in deionized water and adjust pH at 8.0. Add 43.8325 g NaCl and make up to 500 ml with deionized water.	
1X TBST (TBS + Tween 20)	For 1 L add 100 ml TBS 10X and 500 µl Tween-20 to 900 ml of deionized water.	
5% BSA in TBST 1X	For 100 ml of solution dissolve 5 g of BSA in TBST 1X.	

Supplementary table 3- Primers used to extract interest cDNA's from original vectors and insertion of cDNA's in the M2H plasmids (pM and pVP16) and sequencing of positive clones.

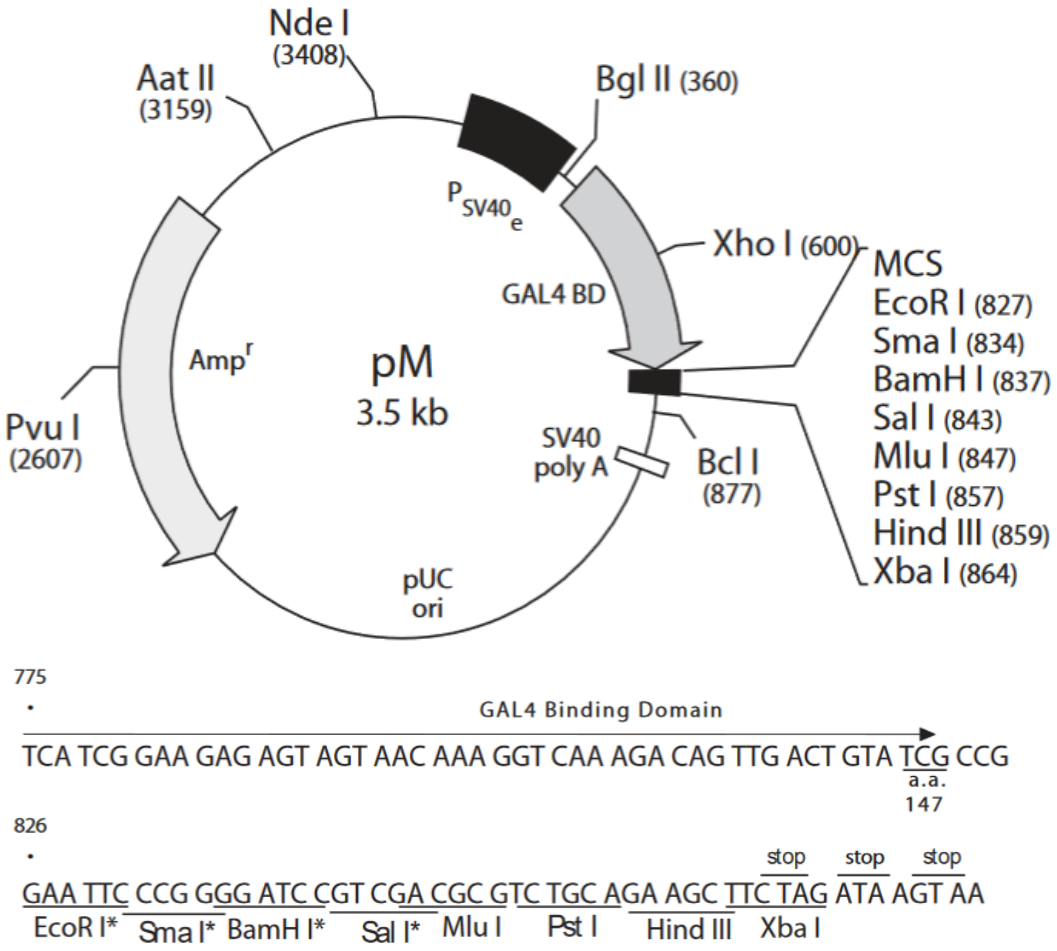
Name	Sequence	Sense	
Primer 1	CCGGGGATCCGTCGAATGATGGCGTACTCTGATACTACAATGAT	Fw	AKAP4
Primer 2	CTGCAGACGCGTCGACCTACAGGT**TAG**CGAGCAGC	Rv	AKAP4
Primer 5	GTGGAGACAAACAGATGTCCC	Fw	pM-AKAP4
Primer 6	CCCTCAACCTCTACCTG	Fw	pVP16-AKAP4
Gal4-BD	TCATCGGAAGAGAGTA	Fw	-
SP6	CATTTAGGTGACACTATA	FW	pVP16
T7	TAATACGACTCACTATAGG	Fw	-

*ATG – start codon; **Bolt** – stop codon; Fw- forward; Rv- Reverse

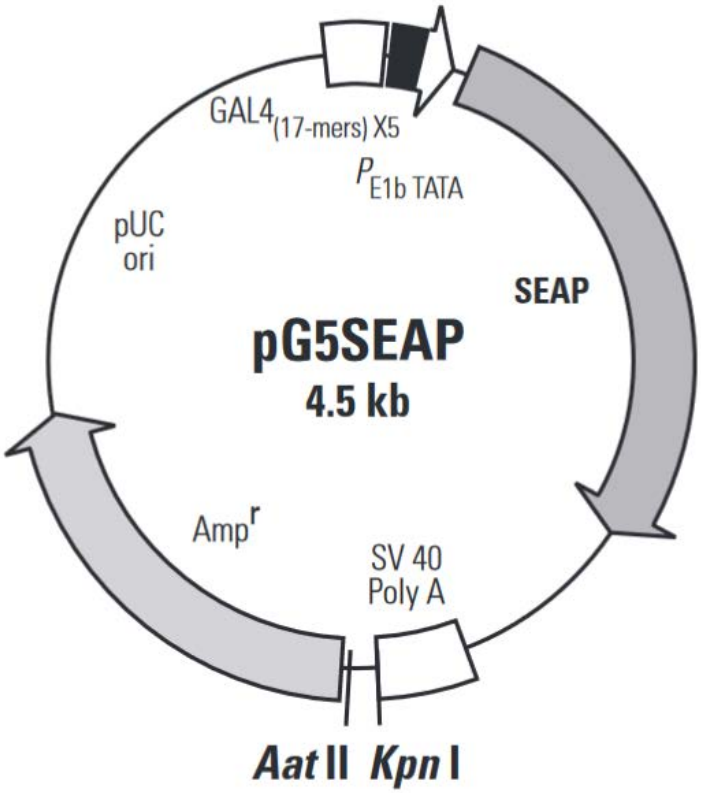




Supplementary figure 1 - pVP16 map (Clontech, Saint Germain en Laye, France). pVP16 was used to generate a fusion protein of VP16-AD and AKAP4 or PPP1CC2. The hybrid protein is targeted to the cell's nucleus by the SV40 nuclear localization sequence. Transcription is initiated from the constitutive SV40 early promoter (PSV40e); transcription is terminated at the SV40 poly A transcription termination signal. Adapted from Matchmaker Mammalian Assay Kit 2 User Manual from Clontech.



Supplementary figure 2 - pM map (Clontech, Saint Germain en Laye, France). pM was used to generate a fusion protein of Gal4-BD and AKAP4 or PPP1CC2. The hybrid protein is targeted to the cell's nucleus by the Gal4 nuclear localization sequence. Transcription is initiated from the constitutive SV40 early promoter (P SV40e); transcription is terminated at the SV40 poly A transcription termination signal. Adapted from Matchmaker Mammalian Assay Kit 2 User Manual from Clontech.



Supplementary figure 3 - pG5SEAP map Reporter Vector. pG5SEAP contains five consensus Gal4 binding sites (UASG 17-mer (x 5)) and an adenovirus E1b minimal promoter upstream of the secreted alkaline phosphatase (SEAP) gene. The DNA-BD portion of a hybrid protein expressed from a pM-derived plasmid localizes to the Gal4 binding sites in pG5SEAP. If the hybrid test protein X interacts with test protein Y (expressed as a hybrid protein from a pVP16-derived plasmid), the SEAP gene will be transcribed. Adapted from Matchmaker Mammalian Assay Kit 2 User Manual from Clontech.