Binding Partners of Parvulin Proteins using the High-Throughput Screening Methods such as Yeast two-hybrid and Phage Display

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Abbreviations used

Ab, Antibody

ABTS, azino-bis (3-ethylbenzothiazole sulfonic acid) diammonium salt

AES, amino-terminal enhancer of split

APS, Ammonium peroxodisulphate

BM, bone marrow

cDNA, complementary DNA

cfu, colony forming unit

DMSO, Dimethyl sulfoxide

ELISA, Enzyme-linked immunosorbent assay

FKBP, FK506 binding protein

FL, fetal liver

Gal4 AD, Gal4 Transcription activation domain

Gal4 BD, Gal4 DNA Binding domain

HMG, High-Mobility Group protein

HMQC, Heteronuclear Multiple Quantum Coherence

IPTG, Lactose analog isopropyle ß-D-thiogalactoside

Kd, dissociation constant

LB medium, Luria-Bertani medium

MT2A, Metallothionein 2A

NMR, Nuclear magnetic resonance

Par14, Par17, Parvulin14, Parvulin17

PCR, polymerase chain reaction

PD, peptide

pfu, plaque forming unit

Ph.D, Phage Display

PMSF, phenylmethyl-sulphonyl fluoride

PPIases, Peptidyl prolyl cis/trans isomerases

Pre-rRNP, pre-ribosomal ribonucleoprotein

RF, replicative form

SD, Synthetic Dropout medium

SDS, Sodium dodecyl sulfate

SS DNA, single stranded DNA

Y2H, Yeast two-hybrid

Zusammenfassung

Peptidyl-Prolyl-cis/trans-Isomerasen (PPIasen) sind Enzyme, die cis/trans-Isomerisierung von Peptidyl-Prolyl-Bindungen (Xaa-Pro-Bindungen) katalysieren. Parvulin 14 (Par14) und seine Isoform Par17 gehören zu der Familie der Parvuline, einer Untergruppe der PPIasen. Für Par14 wurde gezeigt, dass es an DNA bindet. In einer anderen Studie wurde gezeigt, dass Par14 Teil des preribosomalen Ribonukleoprotein-Komplexes ist und ein RNA-Prozessierungsfaktor sei, welcher in der Ribosomenbiogenese involviert ist. Par17 kommt nur in Hominidae vor und ist in den Mitochondrien lokalisiert. Um Bindungspartner für Par14/17 (Peptide oder Proteine) zu finden, wurden Hochdurchsatz-Screening-Verfahren, das *Yeast-two-Hybrid*-System (Y2H) und das Phagen-Display (Ph.D.) verwendet. In der Y2H-Studie wurde ein *target-unrelated-protein* (TUP) gefunden. Im Ph.D. Screening gegen PinA von *Cenarchaeum symbiosum* wurde ein Peptid gefunden, das PinA mit niedriger Affinität bindet. Mithilfe des Peptids konnte ein vermutliches aktives Zentrum der PPIase PinA zugeordnet werden. Ebenfalls wurden eine 7 und 12-mer Peptidbibliothek gegen Par17 entwickelt. Die Konsensus-Sequenz XHSXVHØ wurde aus beiden Bibliotheken angereichert, wobei X eine beliebige Aminosäure und Ø eine hydrophobe Aminosäure darstellt. Die Bindung dieses Motivs an Par14/17 wurde über Phagen-ELISA und NMR-Spektroskopie untersucht, wobei gezeigt werden konnte, dass dieses Motiv an die Par14/17 PPIase Domäne bindet. Mithilfe dieser Peptide wurde das putative aktive Zentrum von Par14/17 in den NMR-Strukturen zugeordnet. Desweiteren können besagte Peptide zukünftig dazu verwendet werden, potentielle Bindungspartner von Par14/17 zu finden.

Summary

Peptidyl prolyl cis/trans isomerases (PPIases) are the enzymes that increase the rate of isomerization of the peptide bond N-terminal to the proline substrate. Parvulin14 (Par14) and its isoform Par17 belong to the Parvulin family which is the third family of PPIases. Par14 was shown to bind the DNA. Par14 was also proposed to be a part from the pre-ribosomal ribonucleoprotien complexes and an RNA processing factor that is involved in ribosome biogenesis. The longer isoform Par17 was shown to be expressed only in the Hominidae, and is targeted to the mitochondria. In order to find binding partners for Par14/17 (peptides or proteins), we applied the high through-put screening methods, the yeast two-hybrid (Y2H) and the phage display (Ph.D.). In our Y2H study we show a target-unrelated protein that may confuse a number of Y2H results. In our Ph.D. screening against PinA of *Cenarchaeum symbiosum* we selected a peptide that binds PinA with low affinity; however we used this peptide to map PinA putative PPIase active site, and to show a flexible region of PinA. The flexibility of this region is probably a general feature of the peptidyl prolyl cis/trans isomerization. Furthermore, we panned 7 and 12-mer peptide libraries against Par17. One consensus sequence was enriched from both libraries, XHSXVHØ, where X can be any amino acid and Ø is a hydrophobic amino acid. We demonstrate the binding of this motif to Par14/17 with phage ELISA and NMR spectroscopy where we could show that this motif is binding to the PPIase domain of Par14/17. Moreover, using these peptides we map the PPIase active site of Par14/17. Our peptides can be used to design peptides to study the PPIase activity of Par14/17, and to elucidate the motif that Par14/17 recognizes *in vivo*.

1. Introduction

1.1 Peptidyl Prolyl cis/trans Isomerases (PPIases)

The peptidyl prolyl cis/trans isomerases (PPIases EC number 5.2.1.8) are enzymes that are capable to increase the rate of isomerization of the peptide bond N-terminal to the proline substrate (figure 1.1). In the literature they are often called rotamases or rotomases (Fischer, 1994) due to the conformational change they catalyzed. There are 3 known families of proteins with a PPIase activity. These families are FKBPs, cyclophillins, and parvulins.

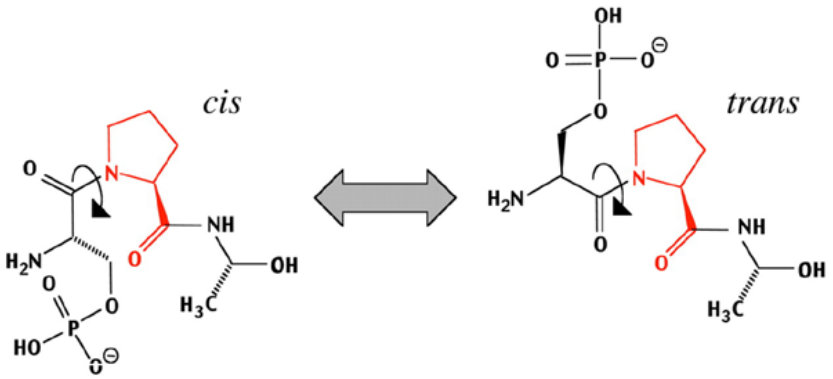

Figure1.1. The PPIases increase the rate of isomerization of the prolyl peptide bond

Isomerization of a peptide bond N-terminal to the proline (shown in red here) by a PPIase. Substrate that is shown here is phosphorylated Ser-Pro-Ala (Shaw, 2002).

The first family of PPIases are FK506 binding proteins (FKBPs) or Macrophilins. Macrophilins were reported to bind the immunosuppressive drug, FK506 and have a PPIase activity (Siekierka *et al*., 1989; Fischer, 1994).

The second family are the cyclophilins that bind the drug Cyclosporin A which was the drug of choice for auto-immune diseases before FK506. Cyclophilins bind cyclosporine A (a cyclic peptide) and possess PPIase activity that is crucial for protein folding during proteins biosynthesis (Schönbrunner *et al*., 1991). The PPIase activity of cyclophilin is strongly inhibited in the presence of low level of cyclosporin A indicating that they bind each other (Fischer *et al*., 1989; Takahashi *et al*., 1989).

The third family of PPIases are the parvulins conserved from bacteria to human. This family has been confirmed after the description of the *E. coli* parvulin, Par10 (Uchida *et al*., 1999). Par10 is localized to *Escherichia coli* cytosol, and it is the smallest parvulin protein ever described with 93 amino acid residues and a calculated molecular weight of 10101 Da. Par10

contains only a PPIase domain with neither N nor C-terminal extensions (figure 1.2). The third family of the PPIases is called Parvulin because it is small (a Latin word means very small) compared to the other well-established PPIase families (Rahfeld *et al*., 1994). The members of parvulins family are resistant against the inhibitors of cyclophilins and FKBPs.

The most important member of Parvulins family is human hPin1 (hPar18, figure 1.2) which has been identified in a yeast two-hybrid screen, as a protein interacting with the essential NIMA (in *Aspergillus nidualns*) protein kinase and suppresses its mitosis-promoting activity (Lu *et al.,* 1996). The same study reported that the depletion of Pin1/Ess1 (Ess1 is a yeast parvulin) from the yeast or Hela cells induces mitotic arrest, whereas Hela cells over-expressing Pin1 arrest in G2 phase of the cell cycle. The crystal structure of hPin1 revealed that the residues Lys63, Arg68 and Arg69 form a basic cluster at the entrance of the active site in the PPIase domain of hPin1. This basic cluster prefers an acidic group N-terminal to proline in the substrate protein (Ranganathan *et al*., 1997). Therefore, it was suggested that Pin1 is a phosphorylation dependent PPIase, that specifically recognizes phosphorylated Ser/Thr residues preceding proline in mitotic phosphoproteins (Ranganathan *et al*., 1997).

In another study, it was demonstrated that hPin1 binds to the phosphorylated Ser/Thr (Yaffe *et al*., 1997). In this report, a two-step mechanism for hPin1 mitotic regulation was proposed. The first step is the phosphorylation of Thr/Ser-Pro by mitotic kinases that creates a binding site for hPin1. In the second step, hPin1 would bind and catalyze conformational changes by isomerization of the peptide bond N-terminal to the proline in phosphoproteins such as NIMA kinase or Cdc25. Thus, the function of these phosphoproteins will be changed in terms of their degradation or their interactions with other proteins. Therefore, the isomerization catalyzed by hPin1 would affect the entire downstream mitotic pathway (Yaffe *et al*., 1997).

Recently, the NMR solution structure of Pin A from the mesosphilic archaeon, *Cenarchaeum symbiosum* (*C.symbiosum*) was solved (Jaremko *et al*., 2011). This mesophilic archaeon is closely related to the other nonthermophilic crenarchaeotes, and is able to thrive at low temperatures (8-18^{o}C). It inhabits cold water in a symbiotic relationship with the sponge *Axinella mexicana* (Preston *et al*., 1996). In contrast to hPin1, PinA of *C.symbiosum* consists of 92 amino acid residues with neither N nor C-terminal terminal domains (Jaremko *et al* 2011). Little is known about PinA of *C.symbiosum* except that it folds into ß α3 ß α ß2 like all other parvulins. Furthermore, it has a relatively large Proline binding cleft, which might be some sort of acclimatization to the cold habitat (Jaremko *et al*., 2011).

The second important member of parvulins family is parvulin14 (figure 1.2). Par14 was identified in a human lung cDNA library. It has a molecular mass of 13.676 KDa, and hence named Par14 (Uchida *et al*., 1999). From the northern blot experiments, the gene of Par14 was identified in human heart, placenta, liver, kidney, and Pancreas. Par14 has 34.5% and 34% sequence similarities to Par10 and hPin1 respectively (Uchida *et al*., 1999). Contrary to human Pin1, Par14 exhibits a preference for an arginine residue N-terminal to the Proline and has a PPIase activity 1000 times less than that of cyclophilins and FKBPs (Uchida *et al*., 1999). The finding of Par14 was confirmed by another study (Rulten *et al*., 1999). Despite its ubiquitous distribution in latter report, they concluded that it is mainly localized to the mitochondrial matrix where they have proposed its function. They have also assayed Par14 PPIase activity with the peptide Suc-Ala-Xaa-Pro-Phe-pNA, where Xaa can be Ala, Glu, Leu or Phe. They described recombinant Par14 with no PPIase activity. Weak PPIase activity of Par14 was only assayed in the case of a positively charged residue preceding Proline (Uchida *et al*., 1999; Zoldák *et al*., 2009).

An extended isoform (25 amino acids N-terminal) of Par14 was identified and named Par17 (Mueller *et al.,* 2006). The Par14 reading frame contains an additional start codon which lies 75 nucleotide upstream of the start codon that initiates Par14 translation. This leads to translation of a longer isoform (figure 1.2). Par17 is expressed in all human cells examined at that study (Mueller *et al*., 2006). It is not expressed in rodent, bovine and non-mammals. In a follow-up study, it was reported that Par17 is imported to the mitochondrial matrix where the authors proposed its function in great apes after evolution (Kessler *et al*., 2007).

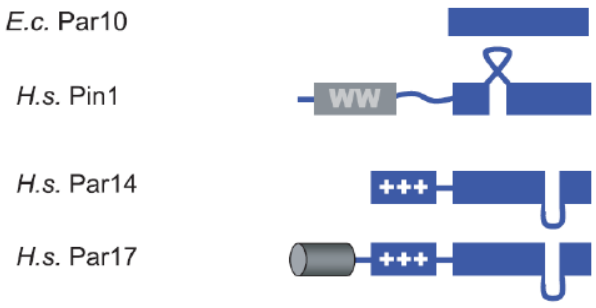

Figure1.2. Modular structures of parvulin proteins (Mueller and Bayer, 2008)

Par10 from *E. coli* with neither N nor C-terminus, hPin1 with its N-terminal WW domain, Par14 with the N-terminal basic domain, and Par17 with the 25 amino acids N-terminal extension to Par14 basic domain.

Although the N-terminal domain of Par14 does not exhibit a sequence similarity to the WW domain of hPin1, it attracted much attention. The N-terminal domain of Par14 was reported to be a prerequisite for Par14-DNA high affinity binding (Surmacz *et al*., 2002). In the latter study, it was shown that there is structural and sequence homology of Par14 to the HMGB/HMGN proteins. Par14 ability to bind the DNA and its structural and sequence homology to HMG proteins led the authors to propose a similar function of Par14 to the function of HMG proteins (Surmacz *et al*., 2002). Furthermore, the N-terminal of Par14 was also demonstrated to be phosphorylated by a casein kinase at Ser19 *in vitro* and *in vivo* (Reimer *et al*., 2003). The same study reported that Ser19 phosphorylation is responsible for the localization of Par14 to the nucleus. They have also reported that the dephosphorylated Par14 binds the DNA with higher affinity than the phosphorylated form, as the phosphorylation may masks the DNA binding surface at the N-terminal of Par14 (Reimer *et al*., 2003). Moreover, it was shown that the amino acids residues from 36-41 of Par14 are required for its binding to the pre-ribosomal ribonucleoprotein complexes (pre-rRNP), provided that the amino terminal residues (1-35) is included (Fujiyama-Nakamura *et al*., 2009). In the latter report, Par14-GST fusion protein was found to be associated with the pre-pre-rRNP compexes. These complexes contain ribosomal proteins and transacting factors involved in ribosome biogenesis. In that experiment Par14 could also pulldown non-ribosomal proteins that are expected to participate in microtubule assembly or nucleologenesis like tubulin, and fibronectin. Moreover, it was shown by knockdown of Par14 mRNA in HEK293 cells, that Par14 is required for normal cell growth (Fujiyama-Nakamura *et al*., 2009).

The NMR solution structure of Par14 was solved by two independent groups (Sekerina *et al*., 2000, Terada *et al*., 2001). Sekerina and co-workers reported that Par14 folds into a Beta strand, 3 alpha helices, Beta strand, alpha helix, 2 Beta stands (β, α3, β, α, β2, Figure1.3.A). They concluded that a positively charged amino acid N-terminal to the Proline will be preferred for the isomerization due to the negative surface potential of Par14. This conclusion is consistent with the report by (Uchida *et al.*, 1999) in which a substrate (Ala-Xaa-Pro-Phe-NH-Np) specificity study was conducted, where aa can be any amino acid. The highest specificity constant measured was with an Arginine residue just before the Proline. The same result was obtained by Zoldák and co-workers (Zoldák *et al*., 2009).

Terada and co-workers described the solution structure of Par14 (figure 1.3.B) as β 4 and α3. Moreover, NMR titration (HSQC) was performed with the peptide Suc-Ala-Leu-Pro-Phe-pNA. The residues Val91, Met90 and Phe94 showed large chemical shift changes. These residues form a hydrophobic core on the molecular surface of Par14. Based on their structural alignment, these 3 residues are conserved in Par10 and two of them in hPin1 (Met90 and Phe94). Therefore, it was suggested that these are the residues that directly interact with the substrate Proline (Terada *et al*., 2001).

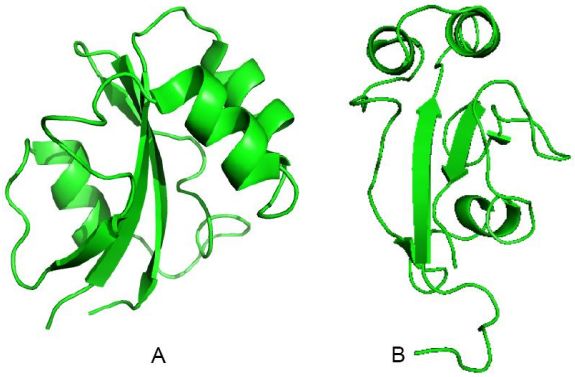

Figure1.3. NMR solution structure of parvulin14

(A**)** It folds in β, α3, β, α, β2, (PDB code 1EQ3, Sekerina *et al*., 2000). (B) It folds in β 4 and α3 (PDB code 1FJD, Terada *et al*., 2001).

Chaperone-like activity was reported for PPIases (Behrens *et al*., 2001), for instance, SurA is a periplasmic PPIase of *E. coli* and composed of an N-terminal region followed by two successive parvulin domains (only the second parvulin domain with a PPIase activity) and a C-terminal tail. SurA was demonstrated to act as a chaperone, its chaperone activity lies in its N-terminal region coupled to the C-terminal tail. Both coupled domains (N and C-termini) could prevent citrate synthase aggregation better than the full-length SurA. It was also shown that SurA N-terminal region coupled to the C-terminal tail is required for SurA outer membrane proteins recognition. Therefore, it was concluded that both parvulin domains of SurA are dispensable for its chaperone-like activity (Behrens *et al*., 2001). Another example is FKpA, also an *E. coli* PPIase. It was reported that in the presence of FKpA, the *in vitro* yield of a soluble and functional anti-fluorescein antibody single-chain Fv (scFv) fragment was increased without the PPIase activity of FKpA, as the Proline was inaccessible to the FKpA (Ramm and Plückthun 2000). The *in vivo*, FkpA was found to possess a beneficial effect on the expression of scFv displayed as fusion to gIII protein of filamentous phage when

they were co-expressed in *E. coli* from the same phagemid. FKpA also increased the expression of scFv not containing cis proline (Bothmann and Plückthun, 2000).

1.2. Using the yeast two-hybrid (Y2H) system to find interacting proteins of Par14/17

The yeast two-hybrid is a powerful genetic assay to study protein-protein interactions (Chien *et al*., 1991; Fields and Song, 1989). This system has been used in thousands of studies, which include everything from mapping the contact domains for individual pairs of proteins to identifying the entire protein interaction network, or the interactome for the whole the organism (Brent and Finely, 1997; Fields, 2005). The assay is carried out inside the nucleus of the yeast *Saccharomyces cerevisiae* where the two proteins to be tested are expressed as hybrid molecules. One is fused to the DNA binding domain (DB) of transcription factors such as Gal 4 or lex A and is called the bait or the protein of interest. The other one is fused to the transcription activation domain (AD) and is called the prey because it is often a clone among millions clones in the cDNA library. If the two proteins interact, the AD is brought to the promoter of reporter genes containing a specific binding site for the BD, and therefore the reporter genes are subsequently transcribed (figure 1.4).

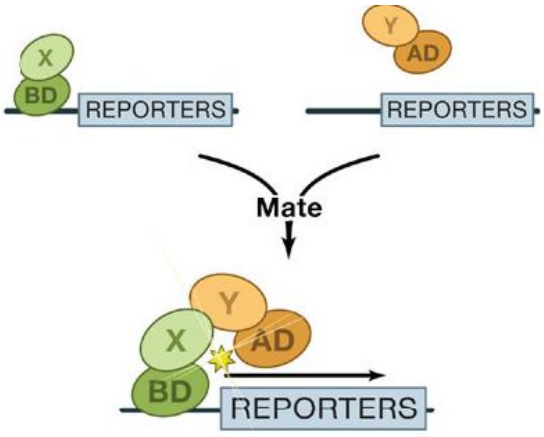

Figure1.4. The principle of yeast two-hybrid system (Finley, 2008)

If the bait and the prey proteins bind to each other, the DNA binding domain (BD) and the transcription activation domain (AD) came in proximity which leads to the transcription and translation of the yeast reporter genes.

In practice, false positives and negatives can arise in the Y2H system (von Mering *et al*., 2002; Huang and Bader, 2009). False positives are interactions that are reported by the system but biologically irrelevant. False positives are classified into technical and biological false positives (Finley, 2007). In the case of technical false positives, the reporter genes are

activated without a true interaction between the two-hybrid proteins i.e. there is no physical binding between the two-hybrid proteins. For instance, when the prey protein binds the promoter or the promoter-associated proteins (Finley, 2007). Another possibility to produce a technical false positive result, when the AD fusion protein is "sticky" and would interact with most proteins, or it interacts with the DNA-binding domain. The other type of false positive is biologic false positives. This type occurs when both proteins bind each other only in the yeast system, but this is does not happen actually, or in the cell circumstances both proteins do not meet each other since each one in a different cellular compartment or with a different expression pattern (Finley, 2007).

1.3. Metallothionein 2A (MT2A) as a clone detected many times in yeast two hybrid screenings

Metallothioneins (MTs) are ubiquitous, intracellular, and low molecular weight proteins with high cysteine content and metal binding capacity (Sutherland and Stillman, 2011).They had been studied for more than five decades since their discovery in 1957 in equine renal tissue (Margoshes and Vallee, 1957). The functions of metallothionein families are subject of speculation and remain to be challenging. It is thought that they are involved in metal storage, detoxification, control of metabolism and other various cellular processes (Karin, 1985). Previously, it was shown that there is an increase in the mRNA of MT2 in neurodegenerative diseases such as Alzheimer disease (Duguid *et al*., 1989). Moreover, metallothionein 2A seems to be the main isoform associated with cell proliferation in cancer cells (Jin *et al*., 2002; Rao *et al*., 2003; Kim *et al*., 2011).

In a number of yeast two-hybrid screens using Gal4-based system, MT2A was identified as an interaction partner of protein kinase Cμ within prostate cancer cell lines (Rao *et al*., 2003), as an interacting protein of human sex hormone-binding globulin (Pope and Lee, 2005), as an interaction partner of hepatitis B protein (Bai *et al*., 2005), and as a binding partner of transthyretin (Gonçalves *et al*., 2008).

When we screened two cDNA libraries from different tissues using Par17 (Mueller *et al*., 2006) as a bait we detected MT2A several times in several screenings as a false positive.
MT2A has been reported by other researchers (Bai *et al*., 2005*;* Pope and Lee, 2005) as a true positive though they neither complete the experiment by co-transforming pACT2-MT2A

construct with the empty pGBKT7 vector in the yeast cell, nor they confirm the interaction by performing a follow-up experiment. Furthermore, MT2A was described as a false positive in Y2H in the report by Bosch-Comas and others (Bosch-Comas *et al*., 2006), and the study by Wang and others (Wang *et al*., 2008). This accumulated occurrence of MT2A clones in the Y2H system prompted us to study these MT2A clones in detail.

1.4. Using the Phage Display (Ph.D.) technique to find binding peptides for Par17 in attempts to uncover its interaction network

Bacteriophages are the viruses that infect bacteria. The short name is phage. They are used as a vector in DNA technology. Phage display Ph.D. describes a selection technique in which a library of peptide is expressed as a fusion to the phage surface proteins or the phage coat proteins.

In the case of M13 bacteriophage libraries, the peptide is expressed fused to the coat minor protein pIII in 5 copies. Each copy is expressing or displaying the same peptide. The Ph.D. library is applied to the target protein (antibodies, enzymes, cell-surface receptors, etc.), followed by several washing steps to remove the unbound phage. The bound phage are then eluted, amplified, and subjected to the next round. After 3 to 4 consecutive rounds of selection and enrichment, the selected phage are characterized by DNA sequencing and Phage ELISA. The application of the peptide library and selecting the binding peptides is called panning, which is some sort of affinity selection (figure1.5).

The phage can take a segment of foreign DNA, and when the phage amplifies in its bacterial cell host, the foreign DNA segment can replicate along with it. In the case of expression phage vector, the foreign DNA will be expressed as a protein. This protein will be displayed fused to the phage coat protein, creating a hybrid protein. The combinatorial phage display library is a mixture of phage each one displaying a peptide fused to the phage coat protein. The expressed foreign peptides will be exposed to the solvent and often behave as if they are free and not attached to the virion surface (As reviewed by Smith and Petrenko, 1997).

Ph.D. is a powerful method to investigate protein-protein interaction. It was successfully used to detect binding partners for a variety of target proteins. For example using Ph.D., Y2H and other methods, it was possible to identify the interaction network of the yeast SH3 domain (Tong *et al.,* 2002). Furthermore, applying Ph.D. and pulldown, the Groucho proteins, amino terminal enhancer of split (AES) was demonstrated to be a binding partner for the high mobility group 1, HMGB1 protein (Dintilhac and Bernués, 2002). In the latter study the

peptide LPLTPLP was enriched after 3 rounds of panning against HMGB1. The amino acids sequence LPLTPLP could be aligned with 100 % homology with the amino acid sequence of AES from 140-146. They showed the HMGB1-AES binding a by a pulldown experiment (Dintilhac and Bernués, 2002).

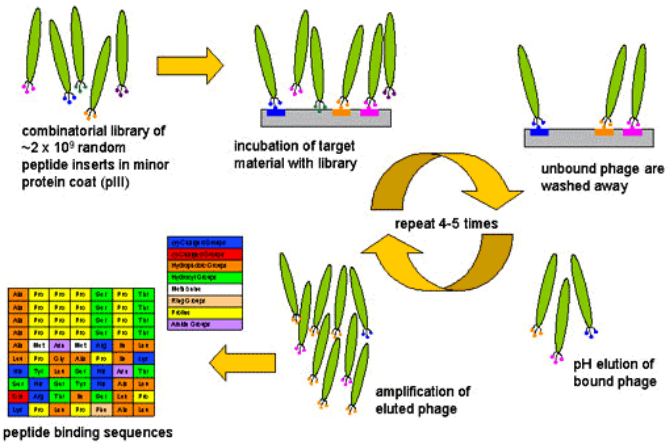

Figure 1.5. Steps of phage display

(http://www.hugroup.seas.harvard.edu/group_members/haberer/research/index.php).

In the phage display panning the target protein is first immobilized (e.g. on a polystyrene plate). After washing and blocking steps, the peptide library is applied on the coated blocked plate, followed by washing steps and elution of bound phage.

1.5. The bacteriophage M13 used as a vector in phage display Ph.D. technique

M13 is a filamentous single stranded bacteriophage (eaters of bacteria) of *E. coli.* The Genome of M13 is a single stranded DNA (SS DNA) molecule (~ 6407bp long) coated by viral proteins, which are major and minor. In order to construct phage peptide library, the peptide is expressed as fusion to the major coat protein or B protein encoded by the gene VIII (Smith and Petrenko, 1997, Sidhu, 2000). B protein is present in about 2700 copies (Simons *et al.,* 1981), and is forming a spiral around the DNA (figure1.6). The foreign peptide can also be displayed as fusion to phage minor coat protein (pIII) encoded by gene *III.* Protein pIII presents in 5 copies for each phage (Lin *et al*., 1980).

The first step of M13 replication includes conversion of the single stranded molecule to double stranded parental replicative form (RF) molecules. Since this step cannot be blocked by chloramphenicol, all the proteins needed for it are suggested to be synthesized by the host cell prior to the infection (Pratt and Erdahl, 1968). The second step is the step of progeny replicative form synthesis, and starts at 5 min of an infection at 37^{o}C. It is distinguishable by

the production of pool of RF double stranded DNA molecules in the infected cells (they reach a level of ~ 40 particles per cell after 30 min of an infection at 37^{o}C). This step is blocked by chloramphenicol, and by lethal mutation only in M13 gene II, suggesting that cellular proteins may contribute to it. The third step starts after 11 minutes of an infection. This is the step where the mature single stranded (SS) viral particles start to be released from infective cells, and some of them accumulate intra-cellular (Pratt and Erdahl, 1968).

Figure 1.6. M13 structure showing the coat proteins (Marco Antonio Arap, 2005)

Foreign peptides are displayed on either major coat protein pVIII depicted in dark violet, or minor coat protein pIII depicted here in dark green.

Objective of this study

The aim of this project was to find (a) binding partner (s) peptide (s) or protein (s) for Parvulin14/17, and the archaeal parvulin *Cenarchaeum symbiosum* PinA whose NMR solution structure had being solved during the course of this study. The Par 14 protein is almost conserved from bacteria to human, and is found in any cell tested thus far (Rulten *et al*., 1999; Uchida *et al.*, 1999). A binding protein (s) will give hint about the function of Par14 using the guilty by association method and the cellular compartment in which Par14 is functioning. The binder will help in characterizing Par14 binding sites, and if it contains proline substrate it will help to map Par14/17 PPIase active site. With the natural proline substrate Par14/17 may exhibit a moderate PPIase activity rather than the very weak activity described before (Uchida *et al*., 1999; Zoldák *et al*., 2009). Furthermore, this is going to reveal which residue should be N-terminal to the Proline substrate. To the best of our knowledge, the active site has not been shown with a natural substrate (Sekerina *et al*., 2000; Terada *et al*., 2001). Moreover, the binding peptides will probably help in prediction of Par14/17 interaction network. In order to achieve these goals we have used two approaches. The first one was the yeast two-hybrid system (Y2H) where we have screened two cDNA libraries, human fatal liver and bone marrow libraries using Par17 as a target protein. The second approach was the phage display (Ph.D.). We used Ph.D. technique to screen a 7-mer peptide library against the archaeal parvulin, *C. symbiosum* PinA. In addition, we screened the 7 and the 12-mer Ph.D. libraries against Par17. The phage display-driven peptides were subjected to phage ELISA and NMR spectroscopy.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

Adenine Hemisulfate Dehydrate MB Biomedical, Illkirch, France

Ammonium peroxodisulphate (APS), Roth, Karlsruhe, Germany

Ammonium sulphate $(NH_4)_2SO_4$ Roth, Karlsruhe, Germany

Bis-Rotiphorese gel 30%, Roth, Karlsruhe, Germany

Bovine serum albumin for Phage ELISA, Sigma-Aldrich Chemie Gmbh, Munich, Germany

Bovine serum albumin for other experiments, Roth Karlsruhe, Germany

Bromophenol blue, Roth, Karlsruhe, Germany

Calcium chloride $CaCl_2$ Roth, Karlsruhe, Germany

Chloropane (phenol-chloroform-isoamyl alcohol, Roth, Karlsruhe, Germany

Complete supplement mixture –Trp –Leu –His –Ade MB Biomedical, Illkirch, France

Disodium hydrogen phosphate Na_2HPO_4 Roth, Karlsruhe, Germany

Ethylene diaminetetraacetic acid (EDTA), Roth, Karlsruhe, Germany Dimethyl Sulfoxide DMSO, Roth, Karlsruhe, Germany

Ferric citrate, Sigma-Aldrich Chemie Gmbh, Munich, Germany

Glucose monohydrate, Roth, Karlsruhe, Germany

Glycerol 86%, Roth, Karlsruhe, Germany

Glycine, Roth, Karlsruhe, Germany

Histidine, Serva, Feinbiochemica, Heidelberg, Germany

Leucine, Roth, Karlsruhe, Germany

Lithium Acetate, Roth, Karlsruhe, Germany

Magnesium sulphate $MgSo_4$, Fluka, Germany

PMSF (phenylmethyl-sulphonyl fluoride), Serva Electrophor GmbH, Heidelberg, Germany

Potassium dihydrogen phosphate KH_2PO_4 Roth, Karlsruhe, Germany

Sodium Acetate, Roth, Karlsruhe, Germany

Sodium dodecyl sulphate SDS, Roth, Karlsruhe, Germany

Temed 99%, Roth, Karlsruhe, Germany

Thiamine chloride (Vitamin B1) Merck, Darmstadt, Germany

Tris, Roth, Karlsruhe, Germany

Tryptophan Sigma-Aldrich Chemie GmbH, Steinheim, Germany

Tween 20, Roth, Karlsruhe, Germany

Urea, Roth, Karlsruhe, Germany

X apha Gal, BIOSYNTH AG, Staad, Switzerland

Yeast nitrogen base without amino acids, MB Biomedical, Illkirch, France

β mercaptoethanol, Serva Feinbiochemica, Heidelberg, Germany

SSFPPLLD, ChemCube Bochum, Germany

KHSPVHIGGGSNH_2, JPT peptide technologies, Berlin, Germany

2.1.2. Reagents of molecular biology

Herring Sperm DNA, Promega Gmbh, Mannheim, Germany

NdeI and EcoRI, NEB, Frankfurt, Germany

Pfu Polymerase, Fermentas, Sankt-leon-Rot, Germany

T4 DNA ligase Metabion, Martinsried, Germany

Taq DNA polymerase Metabion, Martinsried, Germany

2.1.3. Complementary DNA libraries and Phage display peptide libraries

Bone marrow cDNA library, Clontech, Heidelberg, Germany

Fetal liver cDNA library, Clontech, Heidelberg, Germany

Ph.D.-12™ phage display peptide library Kit, New England Biolabs, Frankfurt, Germany

Ph.D.-7™ phage display peptide library Kit New, England Biolabs, Frankfurt, Germany

2.1.4. Plasmid vectors and yeast strains

AH109 *Saccharomyces cerevisiae*, Clontech, Heidelberg, Germany

pCL1 Control Vector, Clontech, Heidelberg, Germany

pGADT7 cloning vector, Clontech, Heidelberg, Germany

pGADT7-T Control Vector, Clontech, Heidelberg, Germany

pGBKT7 cloning vector, Clontech, Heidelberg, Germany

pGBKT7-53 Control Vector, Clontech, Heidelberg, Germany

pGBKT7-Lam Control Vector, Clontech, Heidelberg, Germany

Y187 *Saccharomyces cerevisiae*, Clontech, Heidelberg, Germany

2.1.5. Media

M9-Medium 1 L

6.81 g Na_2HPO_4, 3g KH_2PO_4, 1g NaCl, 1 g $^{15}NH_4Cl$, ddH_2O to the final volume of 1 litter and autoclaved. The following were added: $MgSO_4$ to the final concentration of ~ 0.002 M, 2ml

from TS2 (50X), freshly prepared Fr (III)-Citrate to the final concentration of 0.01mM, glucose to the final concentration of 0.4%, $CaCl_2$ to the final concentration of 0.0001 M, vitamin B1 to the final concentration of 0.00005 %., and Kanamycin to the final concentration of 40 µg / ml.

YPD medium and synthetic dropout (SD) medium

These media were prepared according to the Manufacturer's protocol (Clontech, Yeast Protocol Handbook, protocol No. PT3024-1, version No. PR742227).

LB medium

A mixture of 10g tryptone, 5 g yeast extract, 10g NaCl, ddH2O to the final volume of 1000. The mixture was autoclave at 121^{o}C for 20 minutes.

2.1.6. Antibiotics

Ampicillin, Roth, Karlsruhe, Germany

Kanamycin sulphate, Roth, Karlsruhe, Germany

2.1.7. Antibodies

Anti Gal4 AD, Labgene, NatuTec, Frankfurt, Germany

Anti-rabbit IgG HRP conjugate from Monkey, GE healthcare, Buckinghamshire, UK

Anti-mouse HRP conjugate Amersham, Freiburg, Germany

Anti-Par14 PPIase domain, Eurogenetic, Belgium

2.1.8. The SDS-PAGE

Separating gel

12.45 ml for 12% (or 15ml for 15%) Bis-Rotiphorese gel 30%

6.6 ml 1.8 M Tris-HCl pH 8.8 separating gel buffer

300 µl SDS 10%

300 µl Ammonium peroxodisulphate APS 10 %

12 µl Temed 99%

ddH2O to the final volume of 30 ml

Stacking gel

1190 µl Bis-Rotiphorese gel 30%

700 µl 1.25M Tris-HCl pH 6.8 collecting gel buffer

70 µl from SDS 10%

70 µl from APS

7 µl from Temed 99%

dd H_2O to the final volume of 7 ml.

2.1.9. Buffers and solutions

2.1.9. A. Buffers for plasmid mini-preparation from *E. coli*, alkaline extraction

S1 buffer

50 mM Tris/HCl (pH 8)

10 mM EDTA

100 µg RNAase A/ml

100µg/ml RNase were dissolved in 10mM NaAc pH 5.2, and warmed for 15 minutes at 100^{o}C, allowed to cool at room temperature and neutralized by addition of 0.1 volumes 1M Tris HCl pH 7.4

S3 buffer

2.6 M KAc/pH 5.2 CH_3COOH

S2 buffer

200 mM NaOH, 1% (w/v) SDS

2.1.9. B. Solutions for protein extraction from the yeast

Cracking buffer stock solution

48g Urea

5g SDS

4 ml from Tris-HCl pH (6.8) 1 M stock solution

20 µl EDTA 0.5 M stock solution

40 mg Bromophenol blue

dd H_2O to a final volume of 100 ml

The protease inhibitor stock solution (7X), was prepared by dissolving one tablet from complete Mini, EDTA-free (Roche, Manheim, Germany) in 1.5 ml ddH_2O.

PMSF stock solution

PMSF 0.1742g was dissolved in 10 ml isopropanol, wrapped in foil and stored at room temperature.

Cracking buffer working solution

1ml cracking buffer stock solution

10 µl β mercaptoethanol

80 µl from protease inhibitor solution,

50 µl PMSF stock solution

2.1.9. C. TS2 (50X) for M9 medium

700 mg $ZnSO_4$-7H2O

30 mg $MnCl_2$-4H2O

300 mg H_3BO_3

200 mg $CoCl_2$-$6H_2O$

200 mg $NiCl_2$-6H2O

10 mg $CuCl_2$-$2H_2O$

900 mg Na_2MoO_4

20 mg $Na2SeO_3$

1000 ml ddH_2O

2.1.10. Protein molecular weight markers

Magic Mark, Invitrogen, Darmstadt, Germany

Page ruler, Fermentas, Sankt Leon-Rot, Germany

2.1.11 Oligonucleotides used in this study

Protein	Amplified protein	Oligonucleotide	Sequence 5´—3´
Parvulin 17	Parvulin 17 full-length 1-156 aa	Jo255 Forward	AAA TTT GGG CAT ATG CCC ATG GCG GGG CTT CTA AAG
		621 Reverse	CAC ACA GAA TTC ATT ATT TTC TTC CTT CGA CCA TAA TAA TAT G
Parvulin 14	Parvulin 14 Full-length 1-131 aa	487 F	GTG TGT GTG CAT ATG CCG CCC AAA GGA AAA AGT GG
		621 R	CAC ACA GAA TTC ATT ATT TTC TTC CTT CGA CCA TAA TAA TAT G
Parvulin 14 PPIase	C-terminal of Parvulin 14	454 F	CTC TCT CAT ATG AAT GCA GTA AAG GTC AGA CAC
		621 R	CAC ACA GAA TTC ATT ATT TTC TTC CTT CGA CCA TAA TAA TAT G
Gal 4 AD fusion protein (Clontech)	Gal4 AD fused to library insert e.g. MT2A	LD amplimer F	CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CC
		LD amplimer R	GTG AAC TTG CGG GGT TTT TCA GTA TCT ACG AT
- 96 Sequencing primer (NEB)	Minor coat protein (pIII) of M13	- 96 sequencing primer (NEB)	CCC TCA TAG TTA GCG TAA CG

2.2 Methods

2.2.1 Cloning of the baits, Pa14/17 and Par14 PPIase domain used in the yeast two-hybrid screens

2.2.1.1. PCR amplification of Parvulin17 cDNA

PCR was performed by mixing the following, 5 µl from 10X pfu buffer, 4 µl 2.5mM dNTPs, 2 µl JO 255 forward primer (10 pmol/ul), 2 µl 621 reverse primer (10pmol/µl), 35.4 µl DNase / RNase free ddH_2O, 0.8 µl plasmid DNA and 0.8 µl Pfu polymerase (Fermentas, Germany). The thermal cycling profile was as follows: 2 min initial denaturation at 94^oC, followed by 35 cycles of 94^oC for 30 sec denaturation, annealing 66^oC for 30 Sec, 72^oC for 1min extension, and a final extension at 72^oC for 10 min. Lid temperature was 110^oC, block temperature was 4^oC.

2.2.1.2. PCR amplification of Parvulin14 cDNA

PCR was performed by mixing the following, 5 µl from 10X pfu buffer, 4 µl 2.5mM dNTPs, 2 µl 487 forward primer (10 pmol/ul), 2 µl 621 reverse primer (10pmol/µl), 35.4 µl DNase/RNase free ddH_2O, 0.8 µl plasmid DNA and 0.8 µl Pfu polymerase (Fermentas). The thermal cycling profiles was as follows: 2 min at 94^oC for initial denaturation, followed by 35 cycles of 94^oC for 30 sec for denaturation, annealing at 66^oC for 30 Sec, 1 min for extensions at 72^oC, and a final extension at 72^oC for 10 min. Lid temperature was 110^oC, block temperature was 4^oC.

2.2.1.3. PCR amplification of parvulin 14 PPIase

The PCR was performed by mixing the following in 1.5 microcentrifuge tube, 5 µl from (10X) pfu buffer, 4 µl 2.5mM dNTPs, 2 µl 454 forward primer (10 pmol/ul), 2 µl 621R (10pmol/ µl), 35 µl DNase/RNase free ddH_2O, 1 µl plasmid DNA and 1 µl Pfu polymerase (Fermentas). The thermal cycling profiles was as follows: 2 min at 94^oC for initial denaturation, followed by 35 cycle of 94^oC for 30 sec for denaturation, annealing at 60^oC for 30 Sec, 72^oC for 1 min for extension, and a final extension at 72^oC for 10 min. Lid temperature was 110^oC, block temperature was 4^oC.

2.2.1.4. Cloning of the baits, Pa14/17 and Par14 PPIase in the Gal 4 BD encoding vector, pGBKT7

Gels containing the PCR products (Par17, Par14, and PPIase of Par14) were excised and DNA was extracted using the Hi Yield kit (SLG, Sued-Laborbedarf GmbH, Gauting, Germany). The extracted DNA was measured using Nanodrop spectrophotometer at 260 nm (ND1000, Peqlab Biotechnologie, Germany), the concentration were as follows: Par17 was ~ 60ng/µl, Par14 was ~ 40ng/ µl, and PPIase of Par14 was ~ 40ng/µl.

The PCR products were digested with the restriction enzymes NdeI and EcoRI (NEB, Frankfurt, Germany) using the following protocol: 5 µl from NEB buffer4 (10X), Bovine serum albumin BSA 1.25 µl, 26 µl from DNA extracted from gel, 1.5 µl EcoRI, 1.5 µl NdeI, and 14.75 µl DNase / RNase free ddH_2O to the final volume of 50 µl, the 1.5 microcentrifuge tube containing the digestion mixture was incubated at 37^oC for 2 hrs.

The digestion products were then purified using the Hi Yield PCR Clean-up (SLG, Sued-Laborbedarf GmbH, Gauting, Germany). Their DNA concentrations were as follows Par17 ~ 40ng/µl, Par14 ~10ng/µl and the Par14 PPIase ~ 10ng/µl.

The pGBKT7 was also digested with the same restriction enzymes NdeI and EcoRI (NEB) by mixing the followings: 37.35 µl DNase/RNase free ddH2O, 5 µl NEB buffer 4 (10X), 1.25 BSA (NEB), 3.4 µl pGBKT7 (500ng/ul), 1.5µl EcoRI, and 1.5µl NdeI. The 1.5 microcentrifuge tube containing the digestion mixture was incubated at 37^oC for 2 hrs. The digested vector pGPKT7 was treated with alkaline phosphatase, AP to prevent vector re-ligation using the following protocol: 1 µl phosphatase (1 U/µl) in 5 µl AP buffer + 50 µl pGBKT7 (digestion product), 37^oC for 30 min, followed by inactivation of AP at 65^oC for 5 min. The digested vector pGBKT7 was run in agarose and extracted from the gel using and vector DNA purified the kit Hi Yield (SLG, Sued-Laborbedarf GmbH, Gauting, Germany).

The DNA concentration of vector digestion product was ~ 250 ng/µl. The digestion products of each of Par17, Par14 and Par14 PPIase were cloned in-frame (ligated) to the vector pGBKT7 by mixing in 1.5 microcentrifuge tube following: 5 µl from each of Par17, Pa14 or Par14 PPIase, 2.5 µl pGBKT7, 0.8 µl ATP (25mM) to the final concentration of 1mM, 2 µl ligase buffer T4, 1 µl T4 DNA Ligase, and 8.7 DNase/ RNase free H_2O to the final volume of 20 µl the ligation mixtures were then incubated at 16^oC overnight.

To verify that the cloning was successful, each of the ligation products of pGBKT7-Par17, pGBKT7-Par14 and pGBKT7-Par14 PPIase were digested with the restriction enzymes EcoRI and NdeI by mixing the following in 1.5 microcentrifuge tube: 2µl (buffer4) NEB, 1 µl BSA, 8 µl ligation product, 1 µl EcoRI, 1 µl NdeI, and 7 µl ddH_2O, the tube was incubated at 37^oC for 2 hrs. The digestion products were then run on 1.5% agarose gel stained with

ethidium bromide. The digestion products of pGBKT7-Par17, pGBKT7-Par14 and pGBKT7-Par14 PPIase gave bands at their predicted molecular weights of 471bp, 396bp, and 291bp respectively. All constructs were then verified by DNA sequencing (GATC Konstanz, Germany). For bacterial transformation, 5 µl were taken from the ligation product.

2.2.2. Bacterial chemical transformation

An aliquot of home-made chemical competent cells in 1.5 microcentrifuge tube (70-100 µl) was removed from -80^{o}C and thawed on ice. Ten µl (10-100ng of DNA) of plasmid were added to the aliquot, which was pipetted up and down to mix well. The 1.5 microcentrifuge tube was placed immediately on ice for at least 10 minutes. The cells were then heat shocked on a thermoblock for 45-50 sec at exactly 42^{o}C, and re-placed immediately on ice for 2 min. To allow outgrowth, 900 µl of room temperature Soc (preferably) or LB medium were added followed by incubation at 37^{o}C for at least 1 hr with shaking at ~ 300 rpm. The culture was centrifuged at 14000 rpm for 20-30 sec, 600-700 µl from the supernatant were discarded, and the rest was plated on an LB agar containing the appropriate antibiotic. To amplify the plasmid, O/N culture was prepared by inoculating LB medium containing the appropriate antibiotic with a bacterial colony. Then the plasmid was extracted from the bacteria using the protocol of Birnboim and Doly (1979).

2.2.3. Yeast transformation with Lithium Acetate (LiAc) mediated method.

The protocol described here was used for the transformation of the yeast strain AH109. It was scaled up for library screenings or down for plasmids transformation (Clontech, Yeast Protocol Handbook, protocol No. PT3024-1, version No. PR742227). To prepare yeast overnight culture (O/N), 1 ml of YPD or SD by medium was inoculated with several colonies 2-3mm in diameter. For yeast strain that is previously transformed with another autonomously replicating plasmid, the appropriate SD medium was used to maintain the plasmid. The inoculated 1 ml was vigorously vortexed for 1-2 minutes to disperse any clumps, and transferred to a flask containing 50 ml of YPD or the appropriate SD medium. To avoid bacterial contamination, Kanamycin was added to all yeast media to the final concentration of 50µg/ml. The O/N culture was incubated at 30^{o}C for 16-18 hrs at 250 rpm to the stationary phase (OD_{600}>1.5), then 300 ml from the YPD medium was diluted with the O/N culture (10-30 ml), the OD_{600} was checked, the 300 ml were diluted until the OD_{600} was brought to 0.2-0.3. The culture was incubated at 30^{o}C for 3-4 hrs at 230 rpm. After the incubation the OD_{600}

should be 0.4-0.6, otherwise there was something wrong with the latter culture (expansion culture). The culture was removed, distributed into 50 ml falcon tubes and centrifuged at 1000g for 5 minutes at room temperature (20-21^{o}C).The supernatant was discarded and the pellets were re-suspended in sterile TE buffer or distilled water by vigorous vortexing. All cell pellets were pooled into one 50 ml falcon tubes, centrifuged as before and the supernatant was discarded. The cell pellet was re-suspended in 1.5 ml from sterile 1X TE / 1X LiAc (freshly prepared by mixing 750 µl from 2X TE buffer and 750 µl from 2X LiAc buffer). Plasmid DNA ~ 0.1 µg was mixed with 0.1mg of herring testes carrier DNA (Herring Sperm DNA Promega, Mannheim, Germany) in a fresh 1.5 ml tube, in the case of simultaneous co-transformation with two different plasmids, both plasmids were mixed with approximately equal molar ratio and then 0.1 mg carrier DNA was added. The competent cells in 1X TE/1X LiAc (0.1-0.2 ml) were added to mixture of plasmid (s) and the carrier DNA then mixed well by vortexing, 0.6 ml of sterile PEG/LiAc solution is added to each tube and mixed by vigorous vortexing for at least 20 sec. The mixtures were incubated at 30^{o}C for 30 min at 200 rpm (1.5 ml thermomixer, Eppendorf, Germany), then 70 µl of DMSO were added and mixed by gentle inversion. The cells were heat shocked at 42^{o}C for 15 min (in 1.5 ml thermomixer), chilled on ice for 1-2 min, and centrifuged at 13200 rpm for 10 sec at room temperature, the supernatant was removed. The cells were re- suspended in 1X TE buffer, and plated in the appropriate SD medium, the plates were incubated at 30^{o}C for a week.

2.2.4. Extraction of proteins from yeast

The protocol described here was employed to extract proteins from yeast Urea SDS method, Clontech, Yeast Protocol Handbook, version No PR742227. SD medium (5ml) was inoculated with colonies of the yeast strain AH109. The AH109 strain was independently transformed with pGBKT7-Par17, pGBKT7-Par14 and pGBKT7-Par14 PPIase constructs. The cultures were vigorously vortexed to disperse any cell clumps. An YPD culture (10ml) was also inoculated with untransformed yeast strain AH109 as negative control. The cultures were incubated at 30^{o}C with shaking 250-300 rpm overnight.

In next day the O/N cultures were vigorously vortexed to disperse clumps and diluted in 50 ml YPD medium (total culture volume will be 55ml) for each clone to be assayed. The diluted cultures were incubated (4-8hrs) until the OD_{600} reached 0.4-0.6.

The OD_{600} of 1 ml was multiplied by the total volume of the culture (e.g. if the OD_{600} of 1 ml is 0.5, the total OD_{600} units will be 0.5X 55= 27.5 units).

On ice, the cultures were quickly distributed in 50 ml falcon tubes and centrifuged at 1000g for 5 minutes at 4°C. The supernatants were discarded, the pellets were re-suspended in ice-cold distilled H_2O, and the pellets were recovered by re-centrifugation as before. The supernatants were removed; the pellets were quickly snap-frozen with liquid nitrogen and stored at -80°C until needed.

The freshly prepared complete cracking buffer was prepared as described in Clontech Yeast Protocol Handbook and pre-warmed to 60°C. Each cell pellet was re-suspended (to thaw them) in the complete cracking buffer, 100 µl per 7.5 OD_{600} units were added to the cell pellet, e.g. if the total OD_{600} units was 27.5, we added 366.7 µl. When the cell pellets were not thawed immediately by the pre-warmed cracking buffer, they were placed at 60°C for no longer than 2 minutes to avoid proteolysis. Because PMSF in the cracking buffer degrades quickly, additional aliquots of 100 X PMSF stock solutions were added to the samples after 15 min of starting the protocol, and approximately every 7 min thereafter until the samples were placed on ice or they were stored at - 80°C.

The cell pellet suspensions were each transferred to 1.5 microcentrifuge tube containing 80 µl of glass beads (425–600 µm) per 7.5 OD_{600} units of the cells (the volume of the glass beads could be estimated using a graduated 1.5 micro-centrifuge tube). The samples were heated at 70°C for 10 minutes, vortexed vigorously for 1 minute, and then cell were centrifuged at 14000 rpm for 5 minutes. The supernatant from each tube was transferred to a fresh 1.5 micro centrifuge tube and placed on ice. The tubes containing pellets were placed in a boiling water path for 3-5 minutes, vortexed vigorously for 1 minute, and centrifuged at 14 000 rpm for 5 min at 4°C. The supernatant from each tube transferred to its corresponding first supernatant placed on ice. The samples were snap-frozen and stored at -08°C.

2.2.5. Verification of Gal4 BD fusion proteins by SDS and western blot analysis

Proteins were extracted from the transformant yeast cells AH109 using Urea/SDS method (Yeast protocol Handbook, Clontech) prior to analysis by SDS-PAGE, transferred to a Nitrocellulose acetate membrane and probed with antibody against Par14 PPIase domain (Kessler *et al*., 2007) as a primary antibody for overnight and anti-rabbit as a secondary antibody for 2 hrs at room temperature (Amersham Bioscience, UK). The immunoreactive proteins were visualized by ECL (Amersham Bioscience).

2.2.6. Test for autonomous activity

To test the auto-activation of the bait constructs, the yeast strain AH109 was transformed simultaneously with plasmid pGBKT7 cloned with Par17, Par14, and Par14 PPIase DNA sequences as baits vector and empty library vector pGADT7. The bait vector expresses Gal4 BD fused to Par 14, Par 17 and Par14 PPIase proteins, and confers the Tryptophan (Trp). The empty library vector pGADT7 expresses Gal4 AD and confer Leucine.

The transformant yeast cells with the bait constructs (Par14.pGBKT7, Par17.pGBKT7 and PPIase.pBGKT7) as well as the positive and negative controls were suspended in 500 µl YPD medium, 50 µl were taken for measurement of the optical density OD_{600} which later was adjusted to 5. Then 200 µl were pipetted in 96 well sterile plate from each. The suspensions were then 10-fold serially diluted until the OD 0.00005. One drop from each well was transferred to SD plates selecting for the desired transformants cells, (SD –Trp –Leu) and SD plate selecting for the reporter genes activation (SD –Trp –Leu –His –Ade). The plates were incubated at 30^{o} C for 6-7 days.

2.2.7. Screenings of the cDNA libraries

Two cDNA libraries (Clontech, Heidelberg, Germany) were screened, a human fetal liver and a bone marrow library. Both of the libraries were constructed in the vector pACT2.

2.2.7.1 Fetal liver cDNA library screening using Par17 as bait

The fetal liver library was screened with Par17 as bait, using yeast transformation protocol (Matchmaker™ GAL4 Two-Hybrid System 3 & Libraries User Manual, Protocol No. PT3247-1 version no. PR742219).

2.7.1.1. A. Sequential yeast transformation

The yeast strain AH109 was transformed with bait pGPKT7-Par17, using protocol of LiAc mediated transformation. The transformant yeast cells were grown on the medium SD –Trp for 1 week at 30^{o}C. The selected transformant cells were then sequentially transformed with the fetal liver library. The transformant yeast cells were grown in the medium SD –Trp –Leu–Ade –His (high stringency selective medium). After 1 week of incubation at 30^{o} C, only 4 colonies were observed as positive colonies in the high stringency medium. The four colonies were re-streaked in the medium SD –Trp –Leu –Ade –His containing X alpha Gal (Biosynth, Staad, Switzerland) to test for the activation of the reporter gene MEL I (production of alpha

galactosidase). After incubation for 5 days at 30° C, all of them grew and turned blue. For calculation of transformation efficiency:-

$$\frac{\text{Cfu X total suspension volume (µl)}}{\text{Vol plated (µl) X dilution factor X amount of the DNA used (µg)}} = \text{cfu/µg DNA} \quad \text{(Eq.1)}$$

• 19 cfu in the plate SD –Leu –Trp.

• Total suspension volume was 10ml.

• The amount of the library plasmid = 90 µg

• Volume plate in the dilution plate = 100 µl.

• Dilution factor 1:100, 0.01

The transformation efficiency according to equation number 1

$$\frac{19 \text{ cfu } x \text{ 10ml (1000 µl/ml)}}{100 \text{ µl } x0.01 \text{ x } 90 \text{ µg}} = 2.1\ x1000 \text{ cfu/µg DNA}$$

Calculate of the number of the clones screened

cfu/ µg x µg of the library plasmid used = No. of the clones screened (Eq.2)

The number of the clones screened according to equation number 2 will be as follows:-

$2.1\ x\ 10^3$ cfu /µg x 90 µg = $1.9\ x\ 10^5$ clones screened.

To calculation of transformation efficiency for the library plasmid pACT2

• 2 cfu in the plate SD/–Leu.

• Total suspension volume or transformation mixture is 10ml.

• Volume plate in the dilution plate = 100 µl.

• Dilution factor 1:100, 0.01.

• The amount of the library plasmid = 90 µg.

For the plasmid pACT2 the transformation efficiency according to equation number 1 will be as follows:-

$$\frac{2 \text{ cfu } x \text{ 10ml (1000 µl/ml)}}{100 \text{ µl } x0.01 \text{ x } 90 \text{ µg}} = 222.2 \text{ cfu/µg DNA}$$

2.7.1.1. B. Simultaneous co-transformation

In the second screen simultaneous co-transformation was performed. In this screen the bait plasmid construct, pGBKT7-Par17 and the human fetal liver library plasmid pACT2 were introduced simultaneously in the yeast strain AH109. The transformation mixture was then plated in SD –Trp –Leu– Ade– His. After incubation at 30°C for 1 week, more than 100

colonies were observed in SD –Trp –Leu –Ade –His. To calculate of the transformation efficiency

• The amount of the library transformed was 80 µg.

• The transformation mixture or total suspension volume was 10ml.

• 15 cfu were observed in the medium SD –Trp – Leu.

• The dilution factor was 1:100.

• The volume plated is 100 µl

• Herring Sperm DNA 2mg (Promega Gmbh, Mannheim, Germany). The Herring sperm DNA was sheared by repeated passages through a hypodermic needle to reduce the viscosity of the solution, and then denatured by boiling for 20 min at 100^{o}C prior to its chilling on ice.

The transformation efficiency according to equation number 1 will be as follows:-

$$\frac{15 \text{ cfu } x \text{ 10ml (1000 µl/ml)}}{100 \text{ µl } x0.01 \text{ } x \text{ 80 µg}} = 1875 \text{ cfu/µg DNA}$$

The number of clones screened according to equation number 2 will be as follows:-

$$1875 \text{ cfu/µl } x \text{ 80 µg } = 15 \text{ x } 10{,}000 \text{ clones screened}$$

To calculate the transformation efficiency of the bait plasmid pGBKT7-Par17

• 430 cfu were observed in the medium SD –Trp

• Dilution factor was 1:100.

• The transformation mixture or total suspension volume was 10ml.

• The volume plated is 100 µl.

• The amount of the bait plasmid, pGBKT7-Par17 is 88 µg

According to equation number 1, the transformation efficiency of the bait plasmid will be as follows:-

$$\frac{430 \text{ cfu } x \text{ 10ml (1000 µl/ml)}}{100 \text{ µl } x0.01 \text{ } x \text{ 88 µg}} = 49 \text{ } x \text{ } 1000 \text{ cfu/µg DNA}$$

According to equation number 1, the transformation efficiency of the library plasmid will be as follows:-

• 49 cfu were observed in the medium SD – Leu

• Dilution factor was 1:100.

• The transformation mixture or total suspension volume was 10ml.

• The volume plated was 100 µl

• The amount of the library plasmid, pACT2 is 80 µg. the transformation efficiency of the library plasmid will be as follows:-

$$\frac{49 \text{ cfu } x \text{ 10ml (1000 µl/ml)}}{100 \text{ µl } x0.01 \text{ } x \text{ 80 µg}} = 6 \text{ } x \text{ 1000 cfu/µg DNA}$$

2.2.7.2. Screening of the human bone marrow cDNA library by yeast mating

The Bone Marrow cDNA library which was pre-transformed in the yeast strain Y187, MAT alpha. It was screened by yeast mating with the yeast strain AH109, MATa. The yeast strain AH109 was pre-transformed with the bait constructs pGBKT7-Par17. Diploids were selected in the medium SD –Trp –Leu– Ade –His. The yeast mating was done according to manufacturer´s protocol (Matchmaker™ Gold, Yeast Two-Hybrid System, User Manual, protocol No. PT4084-1, version No. PR033493). In brief, the O/N culture of AH109 was prepared by inoculating a fresh colony in 50 µl SD –Trp containing Kanamycin (50µg/ml), and incubated at 30^{o}C at 260 rpm for ~18 hrs until the OD_{600} reached 1.201. The O/N culture was centrifuged at 1000g for 5 min at room temperature. The supernatant was discarded, and the cells were re-suspended in 4 ml of SD –Trp. The cell number was 1.3X10^{8} cell/ml (counted using a Hemocytometer). The mating mixture was prepared by mixing 1 ml from the cDNA bone marrow library and 4 ml of SD –Trp containing AH109. Then 45 ml of 2X YPDA medium (containing kanamycin) were added to the mixture. The mixture was incubated at 30^{o}C for ~ 20 hours at 40 rpm. After 20 hrs, one drop of the mating mixture was examined under the microscope (40X), zygotes were present. Thereafter, the mating was stopped, the mixture was centrifuged at 1000 g for 10 min, and the supernatant was discarded. The pellet was suspended in 50 ml of 0.5X YPDA medium that were used to rinse the 2L mating flask twice. The pellet was recovered by centrifugation at 1000g for 10 min at room temperature, the supernatant was discarded. The pelleted cells were resuspended in 10 ml of 0.5X YPDA (containing kanamycin). The total volume of the cell and the medium was 12 ml, 100 µl were spread in the plate SD –Trp, SD –Leu and SD –Trp –Leu. The rest was plated in SD –Trp –Leu –His –Ade. Equation number 3 was used to calculate the number of the clones screened**.**

cfu/ml of the diploid x Re-suspension Vol. (ml) = The No. of the clones screened (Eq.3)

•Re-suspension Vol. (ml) = 12 ml.

•Plating volume 100 µl

•Number of the diploid colonies 2, 30, and 369 cfu in SD –Trp –Leu in the dilution 10^{5}, 10^{4}, and 10^{3} respectively.

•Number of the bait colonies 1024, too many (cannot be counted), and too many cfu in SD –Trp in the dilutions 10^{5}, 10^{4}, 10^{3} respectively.

•Number of the library colonies 46, 368, and too many cfu in the SD –Leu in the dilutions 10^5, 10^4, 10^3 respectively. The number of the clones screened was calculated according to equation number 3, and the mating efficiency (Percentage of diploids) was calculated using equation number 4. $30\ \text{cfu} \times 12\text{ml} \times 10 \times 10000 = 3.6 \times 10{,}000{,}000$ clones screened

$$\frac{\text{cfu/ml of diploids}}{\text{cfu/ml of limiting partner}} \times 100 \qquad \text{(Eq.4)}$$

where the limiting partner is the strain of the prey or the bait with the lower viability

• Viability of the prey or bait = No. of colonies x dilution factor (DF) $\times$ 10 (cfu in 1ml).

• 46 colonies of prey library grew on the dilution 10^5 on SD –Leu

• 1024 colonies of bait grew on the dilution 10^5 on SD –Trp

• 2 colonies of the diploid grew on the dilution 10^5 on SD –Trp –Leu

Therefore the viability of the prey library = $46 \times 10^5 \times 10 = 4.6\text{X}10^7$

Viability of the bait $1024 \times 10^5 \times 10 = 1.024 \times 10^9$

Viability of the diploid $2 \times 10^5 \times 10 = 2 \times 10^6$

The prey library is the limiting partner. Thus the mating efficiency according to equation number 4 will be

$$\frac{2 \times 10{,}000{,}0 \times 10}{46 \times 10{,}000{,}0 \times 10} \times 100 = 4.3\ \%\ Diploids$$

2.2.8. Retest of the phenotypes

In order to verify that all the clones which have grown in the medium SD –Trp –Leu –Ade– His maintained the correct phenotype, they were re-streaked in the high stringency medium SD –Trp/–Leu/– Ade/–His + X alpha Gal. They were collected in 2 master plates SD –Trp– Leu– Ade –His + X alpha Gal in a grid fashion.

2.2.9. Plasmid isolation from yeast

The plasmid isolated from yeast is always contaminated with genomic DNA, and thus, it is not pure enough to be digested with restriction enzymes. Besides, it cannot be sequenced, but it can be transformed into *E. coli* (preferably via Electroporation). Moreover, it can also be used as a template for PCR amplification. The protocol described here (Byrd and Arnaud, 2001) was successfully used to extract plasmid from yeast cells. Three ml of SD –Leu was inoculated with a well isolated colony from a plate of SD –Trp –Leu. The cultural tube was vortexed to disperse the cell clumps and incubated at 30°C until saturation at 300 rpm (the culture became milky in appearance in 2-3 days); 1.5 ml from the yeast culture was transferred to a microcentrifuge tube and centrifuged at 10000g for 0.5 minutes. The

supernatant was discarded. The latter step was repeated until the 3 ml culture is pelleted. About 0.3 g (or reasonable estimate) of acid-washed glass beads (Glass beads 425–600 µm; Sigma Cat No G-8772), 200 µl of yeast lysis solution (2% (v/v) Triton X-100, 1% (w/v) sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, in ddH_2O) and 200 µl of Chloropane were added. The mixture was vortexed for at least 2 min to ensure the cell pellet has completely suspended to a single mixture. The mixture was centrifuged at 132000 rpm for 10 min at room temperature. The top aqueous solution (containing DNA) was transferred to a new microcentrifuge tube, more 200µl of chloropane were added to the aqueous phase and vortexed briefly. The mixture was centrifuged as before. The DNA was precipitated by addition of 1/10 volume of 3 M sodium acetate pH5.2 (Roth, Karlsruhe, Germany) and 2.5 volume of absolute ethanol or (0.7 vol. of isopropanol), mixed by vortexing and centrifuged at 10,000g for 30 min at 4^{o}C. The supernatant was discarded, and the DNA pellet was washed with 750 µl of 70 % ethanol and centrifuged at 10000g for 10 min at room temperature. Extra washes step(s) was performed to improve the plasmid transformation into bacteria. The DNA pellet was dried in a vacuum centrifuge. The dried plasmid DNA pellet is glassy in appearance. The DNA pellet is resuspended in TE buffer or DNase/RNase free ddH_2O.

2.2.10. Transformation of the plasmids isolated from the yeast into electrocompetent bacteria by electroporation

The *E. coli* strain DH5alph was made electrocompetent (section 2.2.11). An aliquot of 70 µl elecrocompetent cells was thawed on ice. Then 1-2 µl from DNA plasmid were added to the electrocpmpetent cells and pipetted up and down to mix. The mixture was transferred to clean electroporation cuvette. The cuvette was placed in the cuvette holder of the electroporator (BioRad Micropulser^{TM}) adjusted to Ec1 or Ec2 according to the diameter of the cuvette used. The bottom "pulse" was pressed until a constant tone was heard meaning that the cells were successfully transformed. Immediately 1 ml of 2 X YT medium was added to the cuvette containing the electroporated cells. The bacterial cells were transferred to a fresh microcentrifuge tube and incubated at 400 rpm at 37^{o}C for 1.5 hr Meanwhile LB plate was warmed up at 37^{o}C for at least 1 hr. The bacterial culture was centrifuged at 13200 rpm for 30 Sec, ~ 800 µl were discarded from the supernatant. The pellet was re-suspended in the rest 200 µl. these 200 µl which were plated (using glass beads) in LB medium containing the appropriate antibiotic. The LB plate was placed at 37^{o}C overnight.

2.2.11. Preparation of Electrocompetent *Ecoli* (DH 5alpha)

(http://www.its.caltech.edu/~bjorker/Protocols/Prep_of_electocomp_cells.pdf)

The following materials were pre-chilled at 4°C, 1 L sterile 2 X YT medium (no antibiotics) stored at room temperature, 1L of sterile distilled H_2O, and 150 ml of sterile 10% Glycerol/H_2O. Then a single colony of *E. coli* selected from a fresh LB plate (very important to be a fresh LB plate streaked from a glycerol stock of *E. coli* competent cells, no matter, it can be either electero or chemical competent) for inoculating 10 ml 2XYT culture. The culture was incubated at 37°C at 250 rpm overnight (O/N). One litter of 2XYT medium was inoculated with the O/N culture (10 ml) and placed at 37°C shaker until the OD_{600} 0.6-0.9 (log phase growth), the culture was removed and immediately placed on ice. From hereafter all the following steps were performed on ice. The 1 L culture was distributed equally to falcon tubes. The falcon tubes were centrifuged at 4000 rpm for 25 min at 4°C. The supernatant was removed immediately and the falcon tubes were re-placed on ice. The bacterial pellet was suspended gently in 200 ml of ice-cold sterile ddH_2O. The suspension was re-centrifuged as before, and the supernatant was discarded. The falcons were placed on ice. The pellet was re-suspended gently in 100 ml of ice-cold ddH_2O and centrifuged as before. The supernatant was discarded; falcons were placed on ice, the pellets were re-suspended in 20 ml ice-cold 10% glycerol followed by centrifugation at 4000 rpm for 10 min at 4°C and discarding of the supernatant. The pellet was resuspended on 1 ml of ice-cold 10% glycerol; the suspension was aliquoted into 70 µl in 1.5 microcentrifuge tubes. The aliquots were snap-frozen with liquid nitrogen and stored at –80°C.

2.2.12. Plasmid mini-preparation from *E. coli*, alkaline extraction

The plasmid was extracted from the bacterial cells according to the protocol from (Birnboim and Doly, 1979). In brief, 1.5-2 ml was taken from the LB overnight culture containing the appropriate antibiotic. The bacteria were precipitated by centrifugation at the highest speed (14000 rpm) for 1 minute. The bacterial pellet was re-suspended in buffer I (section 2.1.9.A) either by vigorous vortexing or by pipetting up and down; no clumps should remain in the suspension. Then 250 µl of buffer II (section 2.1.9.A) were added to the suspension which was incubated at room temperature for 5 minutes, then 250 µl from buffer III (section 2.1.9.A) were added, the mixture was gently inverted several times to mix followed by incubation for 5-10 minutes on ice. The mixture was centrifuged at maximum speed at 4°C for 15 minutes to separate the cell debris and the chromosomal DNA from the plasmid DNA

which remains in the supernatant. The supernatant was taken carefully in a new microcentrifuge tube. The DNA was precipitated by addition of 0.8 volume isopropanol, followed by centrifugation for 15 minutes at 4^{o}C at maximum speed. DNA pellet was washed 2 times by 750 µl 70% ethanol (stored at -20^{o}C) and re-centrifuged. The pellet was allowed to dry, and re-suspended for 1 hr in 100 µl from free DNase/RNase ddH_2O or 1 X TE buffer.

2.2.13. Re-testing MT2A clones for the re-activation of yeast reporter genes His3, Ade2 and Mel1 with different bait proteins

The yeast strain AH109 (Clontech Heidelberg, Germany) was independently co-transformed with the two bait constructs, Par17 and DYRK3-S cloned into the vector pGBKT7, and several different MT2A clones (pACT2-MT2A). Expression of these AD fusion proteins was tested by western blotting using a Gal4 AD monoclonal antibody (Raised in mouse, Labgen/ NatuTec, Frankfurt, Germany) and their DNA sequences were aligned manually. Moreover, one MT2A clone (BM2-5 from the screen of the bone marrow) was transformed with several bait constructs pGBKT7-DYRK3-S, pGBKT7-Par17, the control bait plasmid pGBKT7–lam, and pGBKT7 without any insert (empty pGBKT7). Transformed yeast cells were selected on SD –Leu, and for double transformations on SD –Leu –Trp medium. Well separated colonies (2-3mm) for each transformation were suspended in YPD medium, 10 fold serially diluted in 96 well plate and transferred to SD –Trp –Leu and SD –Trp –Leu –His –Ade containing X alpha Gal. The SD plates were incubated at 30^{o}C for 7 days.

2.2.14. SDS and western blot analysis for MT2A clones

Proteins were extracted from the transformant yeast cells using Urea/SDS method prior to analysis by SDS-PAGE. Proteins were then separated in 12.5 % SDS-PAGE, transferred to a nitrocellulose acetate membrane and probed with antibody against the AD protein (Raised in mouse, Labgen/NatuTec, Frankfurt, Germany) as a primary antibody for overnight at 4^{o}C and an anti-mouse HRP conjugate (Amersham, Freiburg, Germany), as a secondary antibody. The immune-reactive proteins were visualized by ECL (Western Blotting Reagents Thermo Scientific, Bonn, Germany).

2.2.15. PCR amplification of MT2A clones

The MTA insert was amplified using the forward 5' primer 5'– CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CC–3' and backward 3' primer 5'–GTG AAC TTG CGG GGT

TTT TCA GTA TCT ACG AT–3' (Matchmaker™ AD LD-Insert Screening Amplimer Set Clontech) with the following protocol: 12.25 µl PCR grade ddH2O, 2.5 µl 10x Complete Metabion Taq, 1.25 DMSO, 0.1 µl 5 LD amplimer, forward primer (100pmol/µl), 0.1 µl 3 LD amplimer reverse primer (100pmol/µl), 0.5 dNTPs (10mM), 0.3 µl Metabion Taq, 5 µl from each of MT2A templates using the following program: 94^{o}C for 2 min for initial denaturation, 94^{o}C for 15 sec for denaturation, annealing 66^{o}C for 15 Sec, 72^{o}C for 2.5 min for extension (denaturation, annealing and extension for 35 cycles) and final extension 72^{o}C for 5 min, block temperature 4^{o}C and lid temperature was 110^{o}C.

2.2.16. Site-directed mutagenesis to recover MT2A reading frame

The deletion of 2 nucleotides to bring MT2A in frame was done using Quick Change Site – Directed Mutagenesis Kit (Agilent, Stuttgart, Germany), PCR was done by mixing the following: 2.5 µl of 10X reaction buffer, 1 µl of pACT2-MT2A (70ng/ µl) pACT2-MT2A, 0.6 µl of the forward primer GCAACCTGTCCCGACTC–GCCGCCTCTTCAGCTCG, 0.6 µl of the reverse primer CGAGCTGAAGAGGCGGC– GAGTCGGGACAGGTTGC, 0.5 µl of dNTPs mix, and 19.8µl ddH_2O to the final volume of 25µl. A little deviation from manufacturer's protocol is that we added DMSO to the final concentration of 4%, to the protocol described in the kit manual.

2.2.17. Expression and purification of the target protein, Parvulin17 for Phage display and Phage ELISA

GST-tagged Par17 protein was expressed and purified as follows. The Par17 nucleotide sequence was cloned in-frame into the plasmid pET41-PreSc. The construct was chemically transformed in *E. coli* BL 21 competent cells. Protein expression from the plasmid pET41-PreSc is under the control of the T7 promoter, which is induced by Lactose analog isopropyle ß-D-thiogalactoside (IPTG). LB medium was inoculated with the transformant cells and incubated at 37^{o}C overnight. The overnight culture was diluted in 1 L of 2X YT medium containing Kanamycin (40µg/ml), followed by incubation at 37^{o}C at 200 rpm until the OD_{600} 0.93 (~3 hrs), then 200µl 1M IPTG were added to the culture which was then incubated at 24.5^{o}C at 200 rpm overnight to induce the bacterial culture to express the GST fusion protein. The O/N culture was centrifuged at 6000 rpm for 15 min at 4^{o}C. The supernatant was discarded and the pellet was re-suspended in 1X PBS (pH 7.4). Lysozyme from hen egg white (Fluka) powder (full spatula) was added to the bacterial cell suspension and mixed gently (no

air bubble should in the bacterial suspension) for ~ 40 min at 4° C. The suspension was subjected to microfluidizer, and then centrifuged at 35,000 rpm for 70 min at 4°C. The supernatant containing the GST fusion protein was taken for affinity chromatography. Par17-GST fusion was purified using (column GST Prep™ FF16/10/GE Healthcare) with phosphate buffer saline pH 7.4 used for column equilibration, high salt buffer 400mM NaCl in 1x PBS used to wash away the unbound proteins. Par17-GST fusion protein was eluted with the buffer 20 mM reduced glutathione in 1x PBS, pH adjusted to 7.4. A homemade PreSision enzyme (Jaremko *et al.*, 2011) 1 µl/6mg protein is added to the GST fusion protein to release Par17 from the GST domain. The GST fusion protein was placed at 4°C for at least 2 hrs, and concentrated using centrifugal filter units (Ultracel 3K from Millipore, Ireland) at 3000g at 4°C. The released Par17 was purified using analytical gel filtration with the column (Superdex75 prepgrade, HiLoad 16/60, GE Healthcare); the column was equilibrated with the buffer 50 mM Tris-HCl pH 8, 25mM NaCl. SDS-PAGE was run to check for the purity of Par17; the SDS showed that Par17 is released from the GST domain.

2.2.18. Expression and purification of ^{15}N-labelled Parvulin 17 for NMR titration

Overnight culture was prepared by inoculation of LB medium with *E. coli* BL21 transformed with the plasmid pET41 in which Par17-GST fusion was cloned. The culture was incubated at 37°C at 200 rpm overnight. The following day, the O/N culture was diluted (1-2%) in 400 ml LB medium containing kanamycin to the final concentration of 50µg/ml. The LB medium was incubated at 37°C at 200 rpm until the OD_{600} 0.7-0.8. The 400 ml were distributed in falcon tubes and centrifuged at 20°C, 4000 rpm for 15 min. The supernatants were discarded and the pellets were re-suspended in M9 medium (section 2.1.5.). The suspended bacterial cells were transferred to M9 medium containing Kanamycin. The culture was incubated at 37°C at 200 rpm until the OD_{600} 0.7-09. The expression of Par17-GST fusion was induced by addition of 200 µl 1M IPTG to each 1L of M9 medium containing the re-suspended bacterial pellet. The culture was allowed to express the Par17-GST at 24.5°C overnight at 200 rpm. The purification of ^{15}N Par17 was performed as exactly as for unlabelled Par17 used for phage display and Phage ELISA.

2.2.19. Phage display screening of 7 and 12-mer peptide libraries for binding peptides of immobilized Par 17 on a Petri plate

The purified target protein, Par17 was diluted in the buffer (0.1 M) $NaHCO_3$, pH 8.6 to the final concentration of 0.1mg/ml (normal concentration of target). Thereafter, 1.5 ml (150 µg) was immobilized in 60 X 15 mm Petri dish (Greiner Bio-One GmbH, Germany) by incubation overnight at 4^o C under gentle agitation. The unbound protein was removed and the plate was blocked with 0.1 M $NaHCO_3$ (pH 8.6), 5 mg/ml BSA, 0.02% NaN_3 for ~1.5 hrs at 4^oC. Phage display 7 or 12-mer peptide library (New England Biolabs, NEB, Frankfurt, Germany) were screened using the to the manufacturer's protocol (Ph.D.^{TM} Phage Display libraries instruction manual, version 1.0-6/09). The plate was then washed 6 times with TBST (TBS + 0.1% [v/v] Tween-20). For the first round approximately $2X10^{11}$ phage from the naive library were added into the coated plate and rocked gently for about 50 min at room temperature. Unbound phage were removed by washing 10 times with TBST. Bound phage were eluted with 0.2 M Glycine-HCl (pH 2.2), 1 mg/ml BSA. The eluted phage from the 1st round were amplified (section 2.7.16), and applied in the second round. The second round amplified eluate was applied in the third round. The selection stringency was increased in the 2nd and 3rd round by raising the concentration of tween 20 in TBST from 0.1 % to 0.5 %. After 3 rounds of panning, individual phage clones were collected from 3rd round tittering. The phage DNA were sequenced by GATC (Konstanz, Germany) using the –96 sequencing primer (NEB). In addition, the 7-mer library was screened against immobilized Par17, but with different strategy (reduced target concentration) where the target concentration was decreased to 10 nM in the 1st round and to 1 nM in the 2nd and the 3rd rounds. Elution was done with the target itself (Par17, 0.1 mg/ml in TBS buffer), and washing steps were performed as described above.

2.2.20. Solution phase panning of 7-mer library with affinity beads capture using Par17 as a target

This type of panning was performed according to the protocol of Ph. D. ^{TM} Phage Display libraries instruction manual, version 1.0-6/09). Briefly, The glutathione (GSH) resin (50%) was immobilized on Handee ^{TM} Spin Columns (Pierce) by pipetting 50 µl from the resin on the column and 750 µl TBST 1% tween 20 were added, and then column is capped and vortexed gently to suspend the resin. The GSH resins were pelleted by centrifugation at 1250 g for 0.5-1 min at room temperature. The supernatant was carefully discarded. The resins were suspended in 750 µl of blocking buffer (0.1 M $NaHCO_3$ (pH 8.6), 5 mg/ml BSA, 0.02%) by gentle vortexing and incubated (with occasional mixing) at 4^oC for at least 1 hr. In the first round, the target protein (2 picomoles of Par17-GST) was mixed with $2X10^{11}$ pfu from the

naive library to the final volume of 200 µl in TBST 1% tween 20. The mixture was incubated at room temperature for 20 minutes. The GSH resins were washed by addition of 750 µl of TBST 1 % tween 20 and centrifugation at 1250g for 0.5-1 min at room temperature. The supernatant was carefully discarded. The resins were washed 4 times pelleting them each time. The phage-target mixture was added to the blocked and washed resins, and incubated at room temperature for 15 min with occasional mixing. The resins were centrifuged at 1250g for 0.5-1 min at room temperature. The supernatant was carefully discarded; the resins were washed 10 times as described before. The bound phage were eluted with the 1 ml of Glycine elution buffer (0.2 M Glycine-HCl, pH 2.2, 1 mg/ml BSA), and immediately neutralized with 150 µl of 1 M Tris-HCl, pH 9.1. The eluted phage were amplified, tittered and applied in the 2nd round. The 2nd round amplified eluate was applied in the 3rd round. Starting from the 2nd round, a negative selection was performed were we incubated the phage with the resins (in the absence of target). The supernatant from this negative selection was then incubated with the target in a positive selection. Stringency was increased by raising the tween 20 concentration from 0.1% to 0.5 % in the 2nd and the 3rd rounds.

2.2.21. Phage Display screening for binding peptides for *C. symbiosum* PinA

A polystyrene Petri plate (the same used for Par17 panning) was coated with recombinant PinA of *C. symbiosum* 1.5 ml with concentration 0.1mg/ml in the buffer 0.1M $NaHCO_3$ pH 8.6. The plate was placed at 4^{o}C overnight with gentle agitation. The following day, the coating solution was removed. The plate was blocked as mentioned above in the panning of Par17. About 2 X 10^{11} pfu from the naive library were applied into the coated and blocked plate followed by washings steps and elution of the bound phage also as mentioned above for the panning of Par17 immobilized on a Petri plate. The stringency of the panning was increased using the same strategy used for the panning of Par17 mentioned above. About 20 sequencing templates from the a 3rd round tittering plate (containing <100 pfu) were purified by the protocol described below. The phage DNA were sequenced in GATC (Konstanz, Germany) using the primer –96 sequencing primer NEB.

2.2.22. Amplification of the phage eluted from the panning

The eluted phage from the panning were stored at 4^{o}C overnight. The following day, the phage were added to an overnight culture of *E. coli* strain ER2738 (NEB, Frankfurt, Germany) diluted 1:100 in 20 mL of LB medium (containing tetracycline) in 250 mL

Erlenmeyer flask. Incubated for 4.5-5 hrs at 250 rpm at 37^{o}C to allow the phage to amplify, then the culture was transferred to a sterile tube and centrifuged at 12000g for 20 min at 4^{o}C. The supernatant was transferred to a fresh sterile tube and re-centrifuged. The upper 18 ml were pipetted in a fresh sterile tube prior to addition of 4ml of sterile 20% PEG/2.5 M NaCl, and incubated at 4^{o}C overnight to precipitate the phage.

2.2.23. Phage precipitation

On the following day for amplification, the PEG-precipitated phage were centrifuged at 12000g at 4^{o}C for 30 min. The supernatant was discarded and phage pellet was re-centrifuged briefly to discard any supernatant. The pellet was suspended in 1 mL of sterile TBS and transferred to 1.5 microcentrifuge tube and centrifuged at 13200 rpm for 7 min at 4^{o}C to pellet any remaining bacterial cells. The supernatant was then transferred to a new 1.5 microcentrifuge tube and re-precipitated with 200 µl of 20 % PEG/2.5 M NaCl, incubated at 4^{o}C for ~ 1 hr. The PEG precipitation was centrifuged at 13200 rpm for 35 min at 4^{o}C, the supernatant was discarded. The phage pellet was re-suspended in 200 µl of sterile TBS, centrifuged at 13200 rpm for 7 min at 4^{o}C. The supernatant was transferred to a fresh 1.5 microcentrifuge tube, these were the amplified phage. The amplified phage were tittered and taken for the following round. The rest were stored at –25^{o}C after addition of an equal volume of sterile 86 % glycerol.

2.2.24. Phage tittering

Ten ml of LB medium containing tetracycline was inoculated with a colony of *E. coli* ER2738 and incubated at 37^{o}C for 4-8 hours (until the mid-log phase was reached). Agarose top was melted using microwave, and 3ml were distributed into sterile cultural tubes (These cultural tubes were kept at 45^{o}C on a water bath) each tube per phage dilution. Phage pool was then 10-fold serially diluted up to the dilution 10^{4} for unamplified phage, or 10^{11} for amplified phage in LB medium. Two hundred µl from the bacterial culture were distributed into 1.5 mico-centrifuge tubes each per phage dilution. Infection was done by addition of 10 µl form each phage dilution to a corresponding 200 µl bacterial culture. The infected bacterial cultures were incubated at room temperature for 1-5 min. The dilutions that were taken from 10^{1}-10^{4} for unamplified phage, and 10^{8}-$10^{11}$for the amplified phage. Very quickly, the phage-infected bacterial culture was transferred to the cultural tube containing the 3 ml melted agarose, mixed by vortexing and immediately poured on a Petri plate (containing LB medium with

tetracycline and X Gal) each per dilution. The Petri plate was tilted several times to distribute the melted agarose; the plate was then placed at 37^{o}C overnight.

2.2.25. Rapid purification of phage clones sequencing templates

Firstly, the plaques were amplified as follows: an overnight culture of *E. coli* ER2738 was diluted 100 times in LB medium containing tetracycline (0.02 mg/ml). For each phage clone to be purified 1ml was distributed in a cultural tube. Plaque forming units (pfu) were picked from an LB plate with <100 cfu and incubated for less than 18 hrs at 37^{o}C, each pfu in a different cultural tube. Cultural tubes were incubated at 250 rpm, 37^{o}C for 4.5-5 hrs. The cultures were each transferred to 1.5 micrcentrifuge tube, and centrifuged at 14000 rpm for 1 min, at this step, 600-700 µl were taken from the supernatant for purification of sequencing template. The remaining supernatant was transferred to a fresh 1.5 microcentrifuge tube and re-centrifuged as before. The upper 80% supernatant was pipetted in a fresh microcentrifuge tube. An equal volume of 86% sterile glycerol is added to the upper 80% supernatant to prepare glycerol stocks which were stored at –25^{o}C. Secondly, the phage in 600-700 µl were precipitated by 280µl of PEG/NaCl, the tube was inverted several times and left at room temperature for 20 min. The PEG precipitates were centrifuged at 4^{o}C, 13200 rpm for 20 min, and then the supernatant was discarded. The tubes were centrifuged briefly, and the remaining supernatant was carefully discarded. The phage pellet was thoroughly suspended in 100 ml of Iodine buffer followed by 250 µl of absolute ethanol. This mixture was incubated at room temperature for 15min to preferentially precipitate phage DNA leaving most proteins in solution. The mixture was centrifuged at 13200 rpm for 20 min at 4^{o}C, the supernatant was discarded. The pellet was washed with 0.6 ml 70% ethanol (stored at –25^{o}C) and re-centrifuged. The DNA pellet was dried under the vacuum (dried phage DNA pellet is white). M13 phage ssDNA templates were quantitated by running on agarose gel, 5µl of the suspended template should give a band of comparable intensity to 0.5 µg of purified single-stranded M13mp18 DNA (NEB, Frankfurt, Germany).

2.2.26. Phage ELISA

An ELISA 96 well plate (immulon 4HBX, Thermoscientific, USA) was coated with 100 µl from Par17 (10-20 µg/ml) in the buffer 0.1M $NaHCO_3$ pH 6.8 overnight. The following day, the target was removed by tapping the ELISA plate several times. The plate was blocked at 4^{o}C for at least 1 hr with 0.1 M $NaHCO_3$ (pH 8.6), 5 mg/ml BSA, 0.02 % NaN_3. Then the

plate was washed 6 times with TBST 5% tween 20 (Sigma-Aldrich, Munich, Germany) followed by addition of 10^9 pfu of phage in 100 µl to each well (10 wells), 2 blocked wells left without phage. The plate was incubated at room temperature for 1-2 hrs with agitation. The unbound phage were removed by washing the plate 6 times with TBST 5% tween 20. HRP conjugated anti-M13 phage Antibody (GE Healthcare) was diluted 1/5000 in the blocking buffer as recommended by the manufacturer, 200 µl were applied to each well followed by incubation at room temperature for 1 hr with agitation. Unbound antibody was removed by washing the plate 6 times with TBST 5% tween 20. The HRP substrate was prepared in advance by dissolving 22mg azino-bis (3-ethylbenzothiazole sulfonic acid) diammonium salt (ABTS, Sigma-Aldrich, Munich, Germany) in 100ml 50mM sodium citrate pH 4, the solution is filtered and stored at 4°C. Prior to the detection step, H_2O_2 was added to the HRP substrate solution. Then 200µl from the HRP substrate were applied to each well followed by incubation at room temperate (20-60 min) with gentle agitation. The plate was then read at 405nm.

2.2.27. NMR titration of ^{15}N-labelded Parvulin 17 with phage display-selected peptides SSFPPLLD and KHSPVHIGGGS-NH_2

The peptide SSFPPLLD was synthesized by ChemCube (Bochum, Germany). Aspartic acid (D) was added to the C-terminus to increase the peptide solubility. The peptide KHSPVHI was synthesized by JPT (Berlin, Germany). In the phage display (Ph.D.) peptide library, the C-terminus of the peptide is fused to pIII (minor coat protein of the phage) through the linker sequence GGGS. Therefore, we have added the sequence GGGS to the designed peptide, and we amidated the C-terminus to exactly mimic the situation in Ph.D. The ^{15}N SOFAST-HMQC titration experiments were performed at 300 K on a 700 MHz Ultrashield NMR spectrometer (Bruker Biospin, Rheinstetten, Germany). For the NMR HMQC titration experiment (table 2.1), peptide KHSPVHIGGGS-NH_2 was dissolved to the final concentration of 30 mM in the buffer 50 mM Tris-HCl pH 7.5, 25mM NaCl. The peptide SSFPPLLD was dissolved in the buffer 50 mM potassium phosphate pH 6.5.

Each peptide was titrated to Par17 (table 2.1) in 600 µl (550 µl ^{15}N-labeled Par17, 0.40 mM and 50 µl Deuterium Oxide 99.9 atom % D Sigma Aldrich, Munich, Germany). ^{15}N-labeled Par17 was dissolved in the same buffer in which each peptide was dissolved for the NMR titration. The total chemical shift differences upon addition of certain amount of peptide were calculated according to equation number 5.

$$\Delta\delta_{total} = \sqrt{\Delta\delta_N * 0.154^{\,2} + (\Delta\delta_H)^2} \qquad \text{(Eq. 5)}$$

where $\Delta\delta_N$ is the chemical shift differences of N nucleus (ppm) and $\Delta\delta_H$ is the chemical shift changes of H nucleus, and 0.154 is the weight factor of N (Ayed *et al*., 2001).

To calculate the dissociation constant (Kd), the total chemical shift differences $\Delta\delta_{total}$ of each amino acid was plotted against the peptide concentration according to equation number 6 (Sinnen, Ph.D thesis 2008).

$$\Delta\delta_{total} = S_{max} \; x \; \frac{Eo+Lo+Kd}{2} - \frac{\sqrt{\frac{Eo+Lo+Kd^{\,2}}{2} - (Lo \; x \; Eo)}}{Eo} \qquad \text{(Eq.6)}$$

where $\Delta\delta_{total}$ is the total shift differences, S_{max} is the hypothetical total shift reached at saturation, Eo is Par17 concentration, Lo is the ligand concentration, and Kd is the dissociation constant.

Table 2.1. HMQC Titration steps of Phage display-selected peptides with ^{15}N-labelled Par17.

Titration step	KHSPVHIGGGS-NH_2 (mM)	SSFPPLLD (mM)
1	0	0
2	0.59	0.50
3	0.97	0.97
4	1.88	2.73
5	4.1	4.29

3. Results

3.1. The target proteins (Par17, Par14, and Par14 PPIase) for the Y2H were well-expressed inside the yeast cell

The bait proteins (Par14/17 or Par14 PPIase) for the Y2H were cloned in-frame to the vector pGBKT7. DNA bands were observed at ~ 396 bp and at ~ 291 bp corresponded to DNA of Par14 and Par14 PPIase respectively (figure 3.1). For Par17, bands were observed at ~ 471 bp corresponded to Par17 DNA (figure 3.2). We also observed bands at ~ 7.3 kbp matched the digested pGBKT7 (figures 3.1 and 3.2).

In order to check the actual expression of the bait proteins fused to Gal4 DNA binding domain (Gal4 BD) inside the yeast cell, western blot analysis was performed. The yeast lysates were prepared using Urea/SDS method. Using antibody against Par14 PPIase domain as a first antibody and anti-rabbit as a second antibody, we could detect the bait proteins expressed inside the yeast cells fused to Gal 4 BD (figure 3.3).

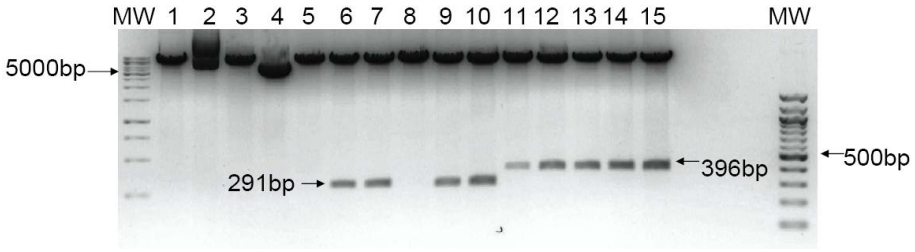

Figure 3.1. Digestion of the constructs pGBKT7-Par14 and pGBKT7-Par14 PPIase
Agarose gel 1.5% stained with ethidium bromide showing the digestion products of the constructs pGBKT7-Par14 (lanes 11-15) and pGBKT7-Par14 PPIase (lanes 6-10) with the restriction enzymes, EcoRI and NdeI.

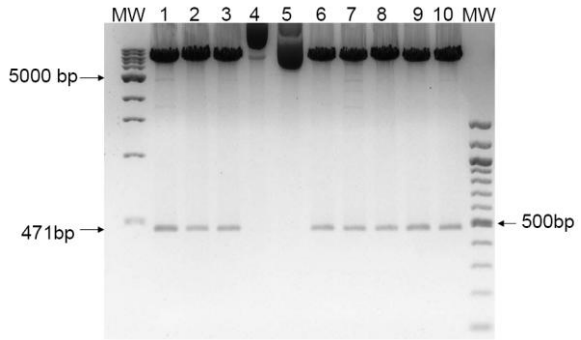

Figure 3.2. Digestion of the constructs pGBKT7-Par14 and pGBKT7-Par14 PPIase
Agarose gel 1.5% stained with ethidium bromide showing the digestion products of the construct pGBKT7-Par17 (lanes 1-10) with the restriction enzymes EcoRI and NdeI.

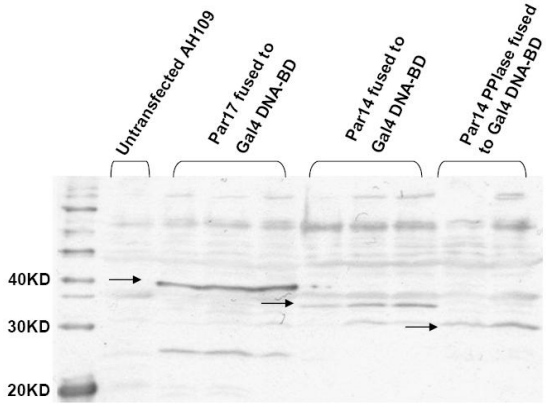

Figure 3.3. Western blot analysis for Gal4 BD fused to the bait proteins used in the Y2H screen

From left to right, lane 1 is a molecular weight marker (Magic Mark, Invitrogen, Darmstadt, Germany), lane 2 is un-transfected AH109, lane 3-5 are Par17 fused to Gal4 BD, lane 6-8 are Par14 fused to the Gal4 BD, lane 9 and 10 are Par14 PPIase fused to the Gal4 BD.

3.2. The bait proteins cannot activate the yeast reporter genes on their own

To test for the autonomous activity of the Par14/17 and the Par14 PPIase domain fused to Gal4 BD, these target constructs were transformed independently with the empty library plasmid, pGADT7 which will express Gal4 AD only. The transformation mixtures were plated in SD –Trp –Leu to test for the success of transformation, and in SD –Trp –Leu –His –Ade to check for the yeast reporter genes activation. After the incubation time (~1 week at 30^{o}C), the baits as well as positive controls (pGBKT7/pCL1 and pGBKT7-53/pGADT7-T) and negative control (pGBKT7-Lam/pGADT7-T) could grow in the medium selecting for the transformants (SD –Trp –Leu). Only the positive controls could grow in the medium selecting for the reporter genes activation (figure 3.4). This result indicates that the target constructs encoded by pGBKT7-Par14, pGBKT7-Par17, and pGBKT7-Par14 PPIase are autonomously inactive.

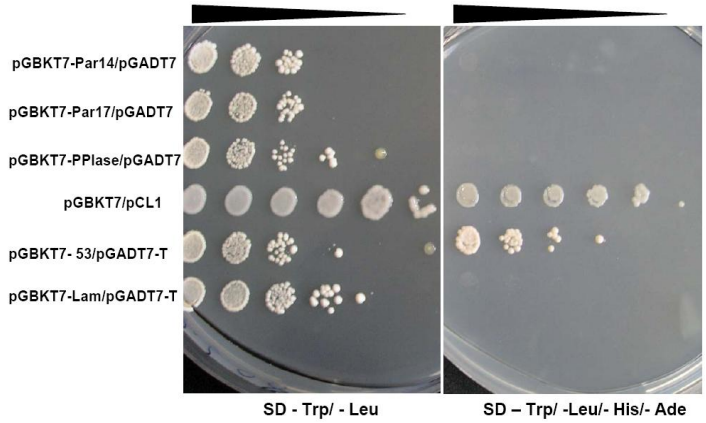

Figure 3.4. Colonies of yeast strain AH109 simultaneously co-transformed with target constructs and empty library plasmid

Yeast colonies were observed in the medium SD –Trp –Leu, but not in the medium SD –Trp –Leu –His –Ade, indicating that the transformation was successful, and that Par17 and Par14 and Par14 PPIase fused to Gal4 BD (target constructs) cannot activate the yeast reporter genes on their own. The plasmids pGBKT7/pCL1 encodes the full length wild type Gal4 protein. The plasmids pGBKT7-53 and pGADT7-T encode p53 and large T-antigen proteins respectively. It is known that p53 and large T antigen bind each other. The plasmids, pGBKT7-Lam and pGADT7-T encode human lamin C and large T-antigen proteins respectively; lamin C neither forms complexes nor interacts with most other proteins.

3.3. Screening of the cDNA fetal liver and bone marrow libraries resulted in more than 100 putative positive clones

In order to find binding partners for Par17 protein we screened a fetal liver library using sequential yeast transformation and simultaneous co-transformation. More than 100 yeast colonies were observed in the high stringency medium SD –Trp –Leu –His –Ade. The bone marrow cDNA library was screened against Par17 by yeast mating (figure 3.5). The positive yeast colonies from both libraries were re-streaked in 2 Petri dishes of high stringency medium containing X alpha Gal. All the colonies grew and turned blue (figure 3.6) indicating an interaction between the two-hybrid proteins. The library plasmids were rescued from the yeast cells using the protocol of (Byrd and Arnaud, 2001), and transformed by electroporation into *E. coli* DH5 alpha.

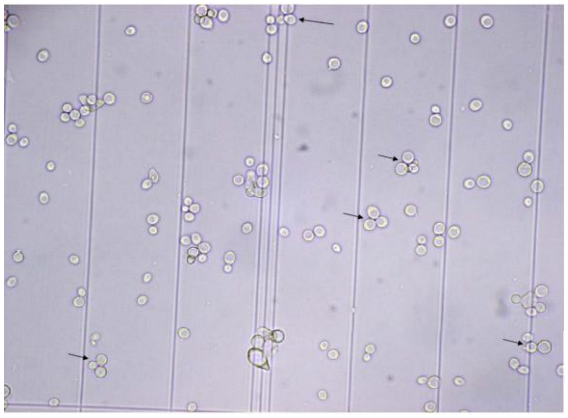

Figure 3.5. Yeast zygotes (indicated with black arrows) after mating of the yeast strain AH109 harbours the bait protein (Par17) and yeast strain Y187 harbours the bone marrow cDNA library

After ~ 20 hrs of mating, the zygotes were evident. In this field (figure 3.5), at least 3 zygotes were observed each one consisted of 3 lobes indicating a successful mating.

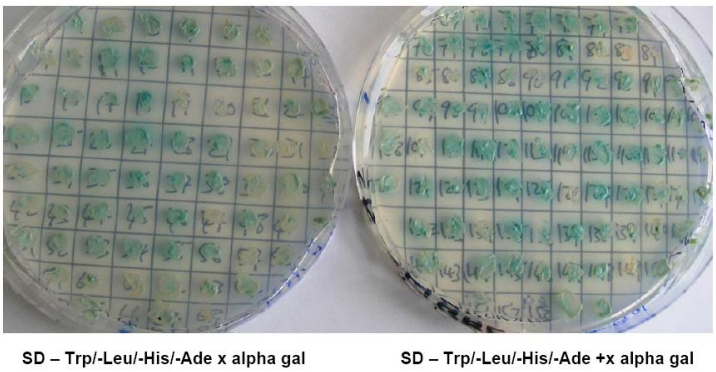

Figure 3.6. More than 100 putative positive yeast colonies grew in the high stringency medium from screening of both libraries

The yeast colonies grew and turned blue in the high stringency medium (these colonies may be harbouring candidate clones). The entire yeasts reporter genes (Ade2, His and Mel1) were activated indicating a physical binding between the two-hybrid proteins.

3.4. All of metallothionein 2A clones obtained from the screenings of two libraries can activate the yeast reporter genes in the presence of their respective bait protein

In search for interacting partners for the Par17 (Mueller *et al*., 2006) we obtained several independent clones with significant similarity to human metallothionein 2A (MT2A) on the nucleotide level. To examine the interaction of these clones, we performed a series of yeast

re-transformations. Four independent MT2A clones (FL-53, FL-127, FL-24, BM-2.5) from the libraries screening with Par17 were simultaneously co-transformed with the constructs pGBKT7-Par17 in the yeast strain AH109, and 2 different MT2 clones (BM-1, BM-8) from a previous screening done in our group, were simultaneously co-transformed with the construct pGBKT7-DYRK3-S in the AH109. These re-transformations were done to test for the ability of MT2A clones to re-activate the yeast reporter genes when they were independently re-transformed with 2 different bait constructs. After incubation for 1 week at 30^{o}C, yeast colonies were observed in the high stringency medium SD –Trp –Leu –His–Ade (SD–WLHA). This result shows that all MT2A clones can activate the yeast reporter genes in the presence of their respective baits (figure 3.7).

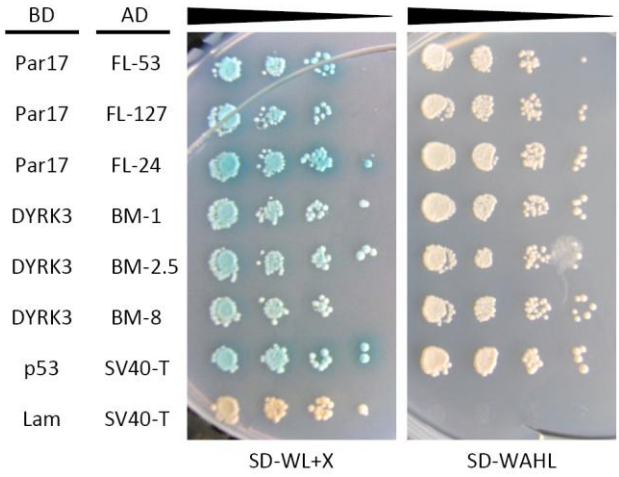

Figure 3.7. Colonies of yeast strain AH109 independently co-transformed with different MT2A clones and their respective target proteins

Each horizontal row represents yeast strain AH109 colonies co-transformed with one of different constructs of pACT2-MT2A from fetal liver library clones (FL) FL-53, 127 or 24, and BM-1, BM-2.5, BM-8 from bone marrow library with either pGBKT7-Par17 or pGBKT7-DYRK3. The suffix behind FL and BM refers to the number of positive colony obtained in screenings. pGBKT7-53 expresses fusion of Gal 4 BD with murine p53, whereas pGADT7-T encode fusions of the Gal4 BD with SV40 large T-antigen. p53 and large T-antigen interact in Y2H. pGBKT7-lam encodes a fusion of Gal4 BD with human lamin C that does not interact with most other proteins. All transformation mixtures were 10-fold serially diluted in 96 well plate and transferred to SD –Leu –Trp (SD –WL) medium containing X alpha Gal (left) and SD –Trp –Leu –His –Ade (SD –WLHA, right) and incubated at 30^{o} C for 6-7 days.

3.5. The MT2A mRNA is represented by many independent clones in the two cDNA libraries

To compare the MT2A clones obtained from the human fetal liver and human bone marrow cDNA libraries, we aligned the nucleotide sequences of eight different clones (figure 3.8). Judging from PCR screening and restriction patterns, many more MT2A clones were obtained in our Y2H screens with the bait Par17. These aligned clones differ in the entire numbers of triplets between the 5´ end of the MT2A insert and linker sequence (GGCCGCGTCGAC) of the vector. In addition, they contain polyA tails of different lengths ranging from of 28 to 103 nucleotides (figure 3.8). Due to the different lengths of their polyA tails and the nucleotides number, these eight MT2A clones cannot be a single redundant clone, but rather independent clones that resulted from different ligation processes. All aligned clones represent the same open reading frame (ORF), just differing by maximum of 10 amino acids C-terminal to linker sequence of the vector pACT2. The complete ORF of human MT2A as defined in the GenBank Ref. Seq. entry NM-005953 is contained within these clones. However, this sequence is not in-frame relative to Gal4 AD domain (+1 frame-shifted relative to the MT2A frame). Regarding the complexity of hybrid cDNA libraries, only ~16.6 % of the inserts will be in the correct reading frame relative to the AD domain (Two-Hybrid cDNA Library Construction Kit User Manual, protocol PT1113-1, version PR71194). Therefore, it is most probably that all clones analyzed in this study encode the same out-of-frame protein that is not MT2A. This protein is not rich in cysteine as it is known for metallothioneins, but rich in proline and alanine (figure 3.9).

Figure 3.8. Alignment of the DNA sequence of different MT2A clones obtained from the libraries screening

Distinct metallothionein 2A clones originated independently within 2 different cDNA libraries and differ in their 5' ends and polyA tails. DNA sequences of several MT2A clones (BM-1, -2 and -8 from the screens with DYRK3-S (DY3) as bait, and BM2-5, FL-24, FL-53, FL-67, FL-106 and FL-127 as candidate clones from the screen with Par17 (P17) as a bait. The clones were aligned starting 5′end within their vector and EcoRI/NotI/SalI linker sequences (part of NotI and SalI sites underlined). The reading frame coming from the vector pACT2 is given below in single letter code. The point mutation in clone FL-67 results in a Val to Phe exchange. This clone also differs from the others by an extensive 3' sequence downstream of the polyA tail. However, encoding a very similar AD fusion as the BM-8 clone (This figure has been prepared by Dr. Jonathan W. Mueller).

```
BM-8   GRVDQVPANPRATCPDSSRLFSSPWIPTAPAPPVTPAPAPAPANAKSANAPPARKAAAPA 60
BM-1   GRVDVP-ANPRATCPDSSRFFSSPWIPTAPAPPVTPAPAPAPANAKSANAPPARKAAAPA 59
BM2-5  GRVDVP-ANPRATCPDSSRLFSSPWIPTAPAPPVTPAPAPAPANAKSANAPPARKAAAPA 59
FL-127 GRVD---ANPRATCPDSSRLFSSPWIPTAPAPPVTPAPAPAPANAKSANAPPARKAAAPA 57
FL-53  GRVD---ANPRATCPDSSRLFSSPWIPTAPAPPVTPAPAPAPANAKSANAPPARKAAAPA 57
FL-24  GRVD---ANPRATCPDSSRLFSSPWIPTAPAPPVTPAPAPAPANAKSANAPPARKAAAPA 57
FL-106 GRVD----------PDSSRLFSSPWIPTAPAPPVTPAPAPAPANAKSANAPPARKAAAPA 50
BM-2   GRVD----------PDSSRLFSSPWIPTAPAPPVTPAPAPAPANAKSANAPPARKAAAPA 50
       ****          *****:****************************************

BM-8   ALWAVPSVPRAASAKGRRTSAAAAPDAGTAPLPDVKNATSTNLDFLCTTLTVTVCYIPFS 120
BM-1   ALWAVPSVPRAASAKGRRTSAAAAPDAGTAPLPDVKNATSTNLDFLCTTLTVTVCYIPFS 119
BM2-5  ALWAVPSVPRAASAKGRRTSAAAAPDAGTAPLPDVKNATSTNLDFLCTTLTVTVCYIPFS 119
FL-127 ALWAVPSVPRAASAKGRRTSAAAAPDAGTAPLPDVKNATSTNLDFLCTTLTLTVCYIPFS 117
FL-53  ALWAVPSVPRAASAKGRRTSAAAAPDAGTAPLPDVKNATSTNLDFLCTTLTVTVCYIPFS 117
FL-24  ALWAVPSVPRAASAKGRRTSAAAAPDAGTAPLPDVKNATSTNLDFLCTTLTLTVCYIPFS 117
FL-106 ALWAVPSVPRAASAKGRRTSAAAAPDAGTAPLPDVKNATSTNLDFLCTTLTVTVCYIPFS 110
BM-2   ALWAVPSVPRAASAKGRRTSAAAAPDAGTAPLPDVKNATSTNLDFLCTTLTLTVCYIPFS 110
       **************************************************:********

BM-8   MK 122
BM-1   MK 121
BM2-5  MK 121
FL-127 MK 119
FL-53  MK 119
FL-24  MK 119
FL-106 MK 112
BM-2   MK 112
       **
```

Figure 3.9. Alignment of the protein expressed by MT2A clones obtained by the screenings of the 2 cDNA libraries

The out-of-frame MT2A proteins differ in length due to different in the number of the nucleotides between the adapter sequence GRVD and 5′ end of the MT2A inserts, which is not in-frame relative to Gal 4 AD. There is point mutation in FL-127, FL-24, and BM-2

resulted in exchange of Val to Leu, and another one in BM-1 resulted in exchange of Leu to Phe.

3.6. The protein encoded by the MT2A clones can activate the yeast reporter genes irrespective of the nature of the bait protein

In order to test for the specificity of interactions of the fusion protein encoded by the pACT2-MT2A constructs, one MT2A clone (BM.2-5) was independently co-transformed with pGBKT7-Par17, pGBKT7-DYRK3, pGBKT7-lam or with the empty bait vector (pGBKT7). Yeast colonies were observed in the high stringency medium (SD –Leu –His –Ade containing X alpha Gal), meaning that all the reporter genes His3, Ade2 and Mel1 have been activated (figure 3.10 upper panel). When we co-transformed pACT2-MT2A with the empty plasmid (pGBKT7) expressing the un-fused Gal4 BD, the transformants yeast could also grow in high stringency medium. Next, we asked whether the fusion protein encoded by the pACT2-MT2A binds the promoter or the promoter-associated proteins, to address this, we have transformed pACT2-MT2A alone into AH109. The transformant cells could grow in SD –Leu but not in SD –Leu –His –Ade containing X alpha Gal (figure 3.10 lower panel), meaning that the reporter genes have not been activated. This result gives 2 evidences; firstly, the protein encoded by the MT2A clones activates the yeast reporter genes regardless of the bait protein and even without bait protein. Secondly, the protein encoded by the MT2A clones does not bind the promoter or the promoter-associated proteins (figure 3.10).

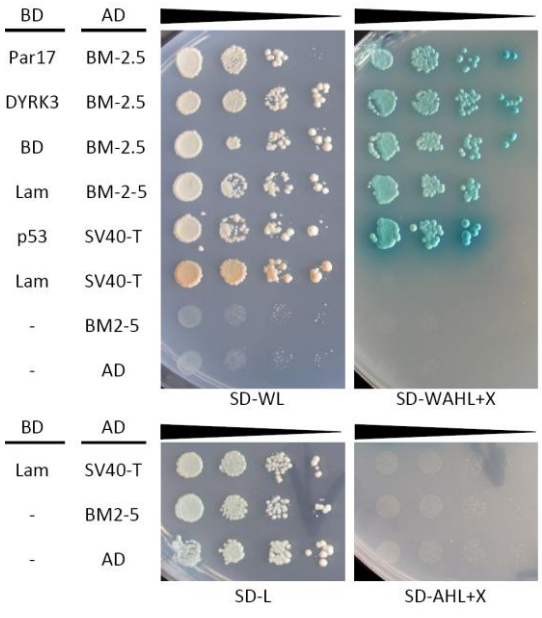

Figure 3.10. Colonies of yeast strain AH109 simultaneously co-transformed with the pACT2-MT2A clones and the control plasmids in SD media

Each horizontal row represents yeast strain AH109 colonies co-transformed with the pACT2-MT2A construct (from bone marrow library, second screening, positive clone No.5, BM2-5) with either pGBKT7-Par17, pGBKT7-DYRK3, pGBKT7-lam or an empty bait vector pGBKT7-BD.Yeast colonies could grow in the high stringency medium whenever MT2A clone met Gal4 BD indicating an interaction between the 2 hybrid proteins. The last two rows are pACT2-BM2-5 and empty pGADT7 both of them were transformed in yeast cells independently, yeast colonies were not observed in the high stringency medium, indicating that the protein encoded by MT2A clones does not bind to the promoter or to the promoter-associated proteins. pGBKT7-53 expresses a fusion of Gal4 BD with murine p53, whereas pGADT7-T encodes a fusion of the Gal4 AD with SV40 large T-antigen. p53 and large T-antigen interact in Y2H. pGBKT7-lam encodes a fusion of the Gal4 BD with human lamin C which does not interact with most other proteins. All transformation mixtures were then 10-fold serially diluted in 96 well plate and transferred to SD medium –Trp –Leu (–WL, left upper panel) and SD –Trp –Leu –His –Ade containing X alpha Gal (SD –WLHA+X, right upper panel), and SD –Leu containing X alpha Gal (SD –L, left lower panel) and SD –Ade–His–Leu containing X alpha Gal (SD –AHL+X, right lower panel) all plates were incubated at 30^{o} C for 6-7 days.

3.7. The expressed proteins from MT2A clones are different in molecular weights

To learn more about the AD fusion protein encoded by MT2A clones, we performed SDS - PAGE and western blot analysis. As described in materials and methods, the AD fusion proteins were probed using a primary antibody against Gal4 AD and anti-mouse as a secondary antibody. Bands of different sizes were observed ranging between 26-32 KD (figure 3.11 upper panel), indicating that the MT2A clones encode proteins of different molecular weights. In addition, the PCR amplification of the MT2A clones reveals that their molecular weights are ranging between 600-650 base pair (figure 3.12).

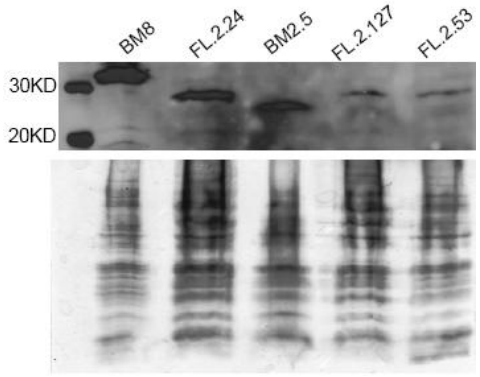

Figure 3.11. Western blot analysis and SDS-PAGE for the protein encoded by MT2A clones

Upper panel is a western blot analysis of the protein encoded MT2A clones either from bone marrow (BM) or fetal liver (FL) library followed by the No. of the clone detected as positive in the library screen. Different bands were observed on the blot. The number on the left indicates the molecular weight marker. The lower panel is the transferred SDS gel after electrophoresis to show that almost equal amounts of the yeast lysates had been loaded.

3.8. In-frame MT2A can no longer activate the yeast reporter genes on its own

Next, we asked whether in-frame MT2A can activate the yeast reporter genes on its own. The MT2A was brought in-frame relative to Gal4 AD (figure 3.13). The yeast strain AH109 was simultaneously co-transformed with empty pGBKT7 and in-frame pACT2-MT2A. We also co-transformed empty pGBKT7 and out-of-frame pACT2-MT2A. Positive control was also co-transformed in parallel. The yeast AH109 cells that were co-transformed with empty pGBKT7 and out-of-frame MT2A as well as positive control could grow and turned blue in high stringency medium, SD –Trp –Leu –His –Ade containing X alpha Gal. Whereas, the yeast AH109 cells that were co-transformed with in-frame MT2A and empty pGBKT7 fail to grow in the high stringency medium, indicating that the AD fusion protein is no longer autonomously active (figure 3.14). This result shows that the protein encoded by the out-of-frame MT2A clones and bind non-specifically in our Y2H system is not MT2A, but a 1+ frame-shifted MT2A, and that the MT2A does not bind non-specifically in Y2H system.

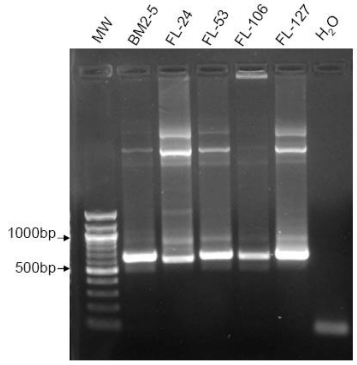

Figure 3.12. Agarose gel stained with ethidium bromide containing the PCR amplified MT2A clones

From left to right**,** MW is 100 bp DNA ladder, BM2-5 is MT2A clone fished from the bone marrow library. FL-24, FL-53, FL-106, FL127 are MT2A clones from the screening of the fetal liver library. H_2O is DNA-free water as a negative control.

ggaaaaaatcgagatctctatgtgcttacccatacgatgtatcccgattacgctagcttg
G K N R D L Y V L T H T M Y P D Y A S L
ggtggtcatatggccatggaggccccggggatccgaattcgcggccgcgtcgacgtccca
G G H M A M E A P G I R I R G R V D V P
gcgaacccgcgtgcaacctgtcccgactcgccgcctcttcagctcgccatggatcccaac
A N P R A T C P D S P P L Q L A M D P N
tgctcctgcgccgccggtgactcctgcacctgcgccggctcctgcaaatgcaaagagtgc
C S C A A G D S C T C A G S C K C K E C
aaatgcacctcctgcaagaaaagctgctgctcctgctgccctgtgggctgtgccaagtgt
K C T S C K K S C C S C C P V G C A K C
gcccagggctgcatctgcaaaggggcgtcggacaagtgcagctgctgcgcc
A Q G C I C K G A S D K C S C C A

Figure 3.13. The DNA sequence of MT2A clone brought in-frame relative to Gal4 AD, and the amino acid sequence (one letter symbol) of the translated protein

The in-frame MT2A clones encode the correct MT2A (GenBank Ref Seq entry NM-005953), the vector reading frame is highlighted in light blue, and the primer-annealing site is highlighted in green.

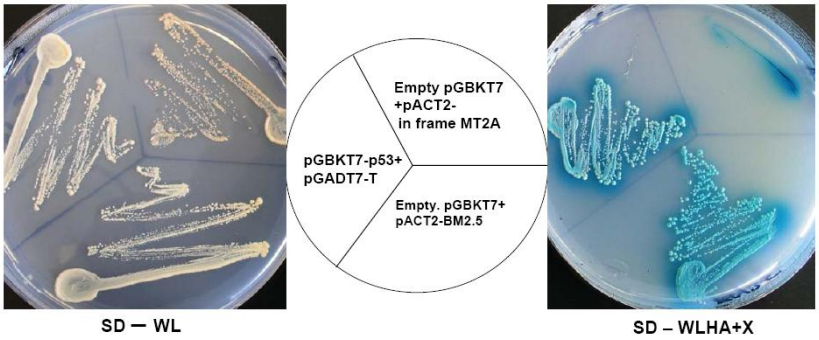

Figure 3.14. Colonies of yeast strain AH109 independently co-transformed with in-frame or out-of-frame MT2A clone and the empty bait plasmid (pGBKT7) grew in SD media

All the yeast transformants grew in SD –Trp –Leu (SD –WL). In-frame MT2A transformants failed to grow in SD –Trp –Leu –His –Ade + X alpha Gal (SD-WLHA+X), whereas out of-frame MT2A and positive control transformants could grow.

3.9. The panning of the 7 and 12-mer phage display (Ph.D.) libraries against Par17 enriched for the same consensus peptide sequence

Recombinant Par17 (figures 3.15, 3.16 and 3.17) was used to screen M13 Ph.D. peptide libraries for potential peptide binders. For this purpose, 100µg/ml (normal concentration) of recombinant Par17 in 0.1 M $NaHCO_3$ (pH 8.6) was immobilized on a Petri dish through hydrophobic interactions and subjected to three rounds of panning. The bound phage were eluted with the general buffer for nonspecific disruption of binding interactions (0.2M Glycine-HCl (pH 2.2), 1 mg/ml BSA). Unbound phage were washed with Tris buffer saline TBST 0.1 % Tween 20 in the 1st round. The tween 20 concentration was raised to TBST 0.5 % Tween 20 in the 2nd and the 3rd rounds to increase stringency in a step-wise manner. Table 3.1 below is showing the titer of each round of panning of both Ph.D. libraries. Sequence analysis of enriched phage clones after the third panning yielded the consensus sequence X H S/T X V H Ø (where X can be any amino acid and Ø is a hydrophobic amino acid). This consensus sequence was found in 17 out of 24 clones sequenced from the 7-mer library panning, and 10 out of 16 sequenced clones from the 12-mer library panning (table 3.2).

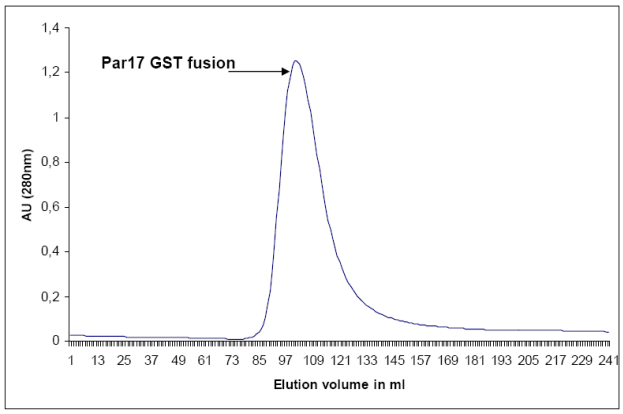

Figure 3.15. Affinity chromatography of Par17-GST

Elution profile of the recombinant protein followed by absorption at 280 nm showing a peak of Par17-GST fusion protein eluted from the GST column using reduced glutathione 20mM in 1x PBS pH 7.4. A large Par17-GST fusion peak was observed in the chromatogram.

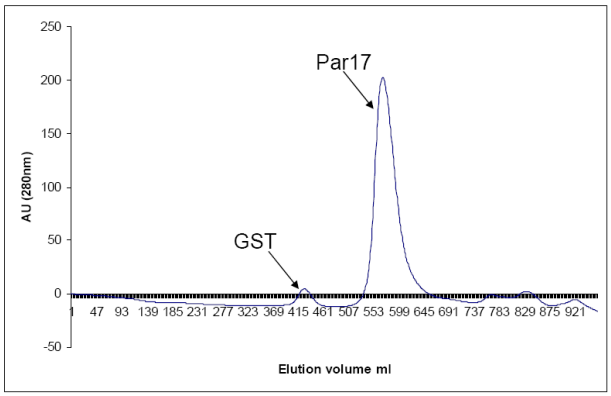

Figure 3.16. Gel filtration of recombinant Par17 and GST protein

Elution profile of analytical gel filtration run followed by absorption at 280 nm showing a small peak of GST and a large peak of Par17 as observed in SDS gel.

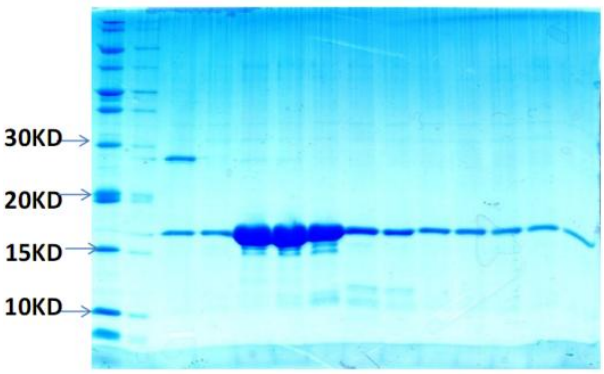

Figure 3.17. SDS gel for purification of Par17

SDS gel (15%) stained with Commassie brilliant blue showing the proteins content in some collected fractions from the gel filtration showing that Par17 (band at ~17 KD) is of appropriate purity, and therefore could be used for further assays.

Table 3.1. Titers of panning of the peptide libraries against Par17 with normal concentration

Approximate titer values of bound phage eluted from each round of panning of the 7 and 12-mer libraries against immobilized Par17 on the Petri plate.

Number of round	Titers of 7-mer library (pfu/10µl)	Titers of 12-mer library (pfu/10 µl)
1st round unamplified eluate	$9.1X10^4$	$1X10^4$
1st round amplified eluate	$1.1X10^{11}$	$1X10^{11}$
2nd round amplified eluate	$8.3X10^{10}$	$8.7X10^{10}$
3rd round unamplified eluate	$8.7X10^4$	$2.6\ X10^5$

Table 3.2. Peptide sequences deduced from the phage clones eluted after the 3rd round of panning against Par17 with normal concentration The 7-mer library (upper table), and 12-mer library (lower table) were screened against Par17 with normal concentration. The conserved similar and homologous amino acid residues between peptide sequences are shown in bold.

7-mer Ph.D library 3rd round

N	P	L	R	V	S	**L**	1X
N	Q	D	V	P	L	**F**	1X
H	D	**T**	P	A	P	**P**	1X
L	P	L	T	P	L	**P**	1X
M	P	**S**	N	T	V	R	1X
S	Y	**S**	S	F	**H**	R	1X
S	P	**T**	H	G	**H**	D	1X
T	**H**	**S**	I	**V**	**H**	**P**	1X
L	**H**	**S**	P	**V**	**H**	**L**	1X
I	**H**	**S**	P	**V**	**H**	**I**	4X
K	**H**	**S**	P	**V**	**H**	**I**	4X
K	**H**	**S**	V	**V**	**H**	**V**	1X
Y	**H**	**S**	V	**V**	**H**	**I**	4X
H	**H**	**T**	G	**V**	**H**	**L**	2X

12-mer Ph.D library 3rd round

T	**H**	**S**	P	**V**	**H**	**V**	L	A	E	H	I	5X
Y	**H**	**S**	D	**V**	**H**	C	A	N	T	C	N	5X
S	**H**	**S**	V	**V**	**H**	**V**	T	K	N	Q	Y	3X
W	**H**	**T**	G	**V**	**H**	**I**	S	A	K	P	G	2X
E	L	**T**	N	A	Q	**I**	K	L	L	W	E	1X

Consensus sequence

X H S / T X V H Ø

3.10. Solution-phase panning against Par17-GST, and panning against immobilized Par17 (with reduced concentration) enriched for the same phage clones

To find peptides that bind with high affinity to Par17, Par17 concentration was decreased to 10 nM in the 1st round and to 1nM in the 2nd and 3rd rounds. The elution was performed with Par17 itself. Three rounds of panning (table 3.3) were performed as described in the material and methods. After the 3rd round, 14 phage clones were purified and sequenced (Table 3.4).

In a solution-phase panning experiment, Par17-GST fusion protein was immobilized in glutathione resins to avoid partial denaturation of the target protein, and to make all binding sites accessible to ligands. After the 3rd round of panning (table 3.5), 15 phage clones were purified and sequenced (table 3.6). From both types of panning mentioned above we found the same phage-borne peptides, SSFPPLL (the most frequented), LPLTPLP, and GKPMPPM (tables 3.4 and 3.6).

Table 3.3. Titers of 7-mer library panning against Par17 with reduced concentration

Approximate titer values of bound phage eluted from each round of panning of the 7-mer library against immobilized Par17 (with reduced concentration) on a Petri plate.

Number of round	Titre (Pfu/10)
1st round unamplified eluate	$2.7X10^4$
1st round amplified eluate	$2.5X10^{11}$
2nd round amplified eluate	$9X10^{10}$
3rd round unamplified eluate	$2.8X10^4$

Table 3.4. The amino acid sequences deduced from the phage eluted after the 3rd round of panning against Par17 with reduced concentration

In this panning the target concentration was reduced to 10 nM in the 1st round and to 1 nM in the 2nd and the 3rd rounds.

L	P	L	T	P	L	P	5 X
S	S	F	P	P	L	L	8 X
G	K	P	M	P	P	M	1 X

Table 3.5. Titers of solution-phase panning with Par17-GST

Approximate titre values of bound phage eluted from each round of panning of the 7-mer library against Par17-GST immobilized on glutathione resins.

Number of round	Titre (pfu/10)
1st round unamplified eluate	$3X10^4$
1st round amplified eluate	$2X10^{11}$
2nd round amplified eluate	$1.7X10^{10}$
3rd round unamplified eluate	$7X10^4$

Table 3.6. The amino acid sequences deduced from the phage eluted after the 3rd round of solution-phase panning with Par17-GST

In this panning, the target protein (GST-Par17) was immobilized on glutathione resins.

L	P	L	T	P	L	P	1 X
S	S	F	P	P	L	L	13X
G	K	P	M	P	P	M	1 X

3.11. The enriched phage-borne peptides bind Par17 and neither the plastic nor Par17-related protein

In order to test the phage-borne peptides enriched in the phage display panning in term of affinity and specificity, phage ELISA experiments were performed. In the first ELISA experiment (figure 3.18), the phage-borne peptides were independently (10^9pfu from each) incubated with the target (Par17) protein, or with a target-related protein, hPin1. The phage bindings were assessed using M13 antibody (Ab), this Ab was Horse Radish Peroxidase HRP conjugated. We used azino-bis (3-ethylbenzothiazole sulfonic acid) diammonium salt (ABTS, Sigma) as an HRP substrate. This substrate produces a soluble end product that is green in color and can be read spectrophotometrically at 405 nm. Our phage ELISA suggests that the phage borne-peptide KHSPVHI binds Par17 with the highest affinity and specificity than the other phage-borne peptides (figure 3.18). The phage-borne peptide SSFPPLL binds Par17 with a relatively higher affinity than to Pin1 (figure 3.18). In another ELISA experiment (figure 3.19). The phage-borne peptide KHSPVHI was compared with phage-borne 12-mer

peptides all of them containing the motif X H S/T X V H Ø. Here, the phage-borne peptides were independently (10^9pfu from each) incubated with Par17 coated or uncoated wells.

This ELISA experiment indicates that the phage-borne peptide sequence XHSXPVHØ (where X can be any amino acid and Ø is a hydrophobic amino acid) binds Par17 with a moderate affinity, and it binds the BSA-blocked plastic with a minimal affinity (figures 3.18 and 3.19).

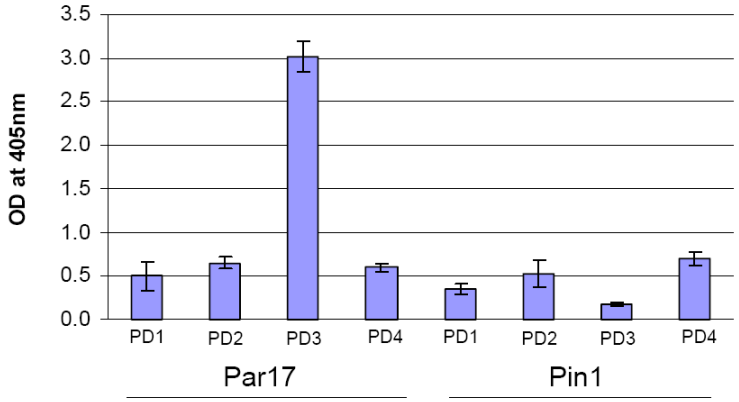

Figure 3.18. Phage ELISA showing the ability of the phage-borne 7 and 12-mer peptides to bind Par17

Peptide1 **(PD1)** is **SSFPPLL, PD2** is **LPLTPLP, PD3** is **KHSPVHI,** and **PD4** is **NRPDSAQFWLHH**. ELISA 96 well plate was coated with Par17 or hPin1 (10-20µg/ml). One raw (10 wells) for each phage to be tested, 10^9 pfu were applied in each well. The ELISA plate was read at 405nm, mean values and standard deviations were taken for the 10 coated wells.

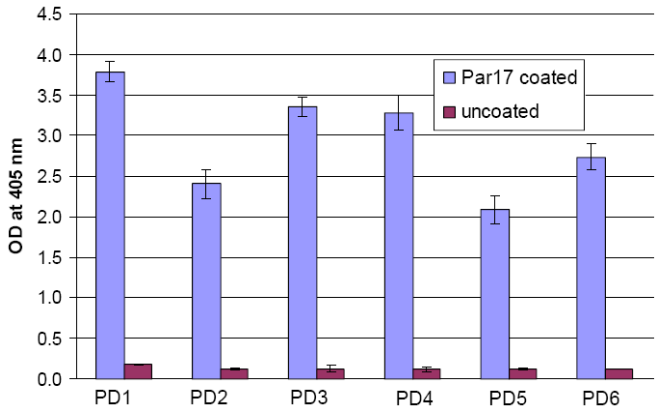

Figure 3.19. Phage ELISA showing the ability of the phage-borne 7 and 12-mer peptides to bind Par17 (blue bars) and not BSA-blocked plastic (red bars)

Peptide 1 (PD) 1 is **WHTGVHISAKPG**, PD2 is **SHSVVHVTKNQY**, PD3 is **YHSDVHCANTCN,** PD 4 is **THSPVHVLAEHI**, PD 5 is **ELTNAQIKLLWE**, and PD 6 is

KHSPVHI, ELISA 96 well plate was coated with Par17 (10-20µg/ml). One raw was taken for each phage to be tested. Ten wells were coated and 2 wells were left uncoated. Phage 10^9 pfu were applied in each well. Mean values and standard deviations were taken for the 10 coated wells, and for the 2 uncoated wells. Since the peptides are not rich in aromatic amino acids, we assumed that they are not plastic binders. Therefore, only 2 wells were left uncoated to test the ability of the peptides to bind the polystyrene plastic plate.

3.12. The peptide KHSPVHIGGGS-NH_2 binds in a hydrophobic pocket in the PPIase domain of Par17

To confirm binding of the phage display-selected peptide KHSPVHIGGGS-NH_2 to Par17 (table 3.2), and to map the binding surface of Par17, NMR titration experiments were performed (figure 3.20). The peptide KHSPVHIGGGS-NH_2 binds Par17 with low binding affinity or binding affinity in high micromolar range (table 3.7). The dissociation constant (Kd) was estimated using NMR chemical shift changes (figure 3.21), using the program SigmaPlot 11. The peptide KHSPVHIGGGS-NH_2 binds Par17 with a Kd of ~ 0.7 mM. Judging from the region of residues exhibiting significant chemical shift changes upon HMQC titration (figure 3.21), the binding occurs in the loop between β sheet 2, and α helix 3. The helix 3 is also involved in binding (figure 3. 23). The Par14/17 residues showed significant chemical shift changes were Val40, Met52, Ala76, Asp81, Met90, Val91, Phe94, Gln95, Glu96, and Ala98 (figures 3.21and 3.22).

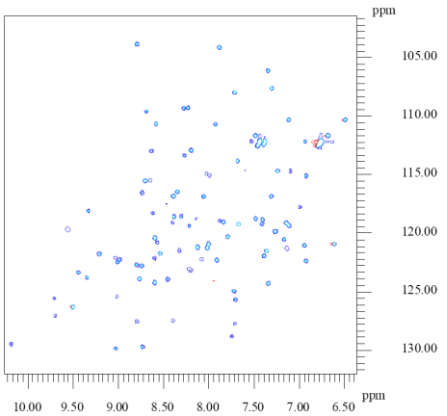

Figure 3.20. ^{15}N-SOFAST-HMQC of spectrum of ^{15} N-labeled Par17

Full-length Par17 was expressed in *E. coli* and purified (as described in materials and methods, section 2.2.18).

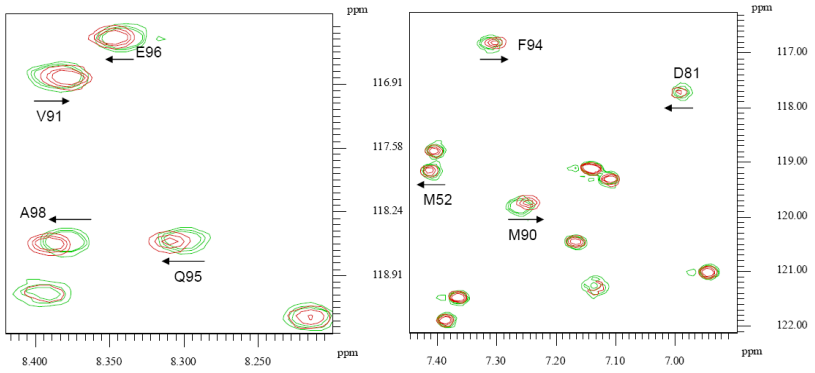

Figure 3.21. Parts of ^{15}N-SOFAST-HMQC spectra of ~ 0.4 mM ^{15}N-labeled Par17 alone (green contour), and Par17 with ~ 4 mM KHSPVHIGGGS-NH_2, (red contour)

The amino acid residues with significant chemical shift perturbations are indicated with one-letter symbol, the number of residue, and the direction of the chemical shift.

Table 3.7. Kd values of Par17 amino acid residues exhibiting significant chemical shift changes upon titration with the peptide KHSPVHIGGGS-NH_2

Approximate Kd values and standard (Std) errors of the amino acids (and their mean) with significant chemical shift changes upon titration of ^{15}N-labeled Par17 with the peptide KHSPVHIGGGS-NH_2.

Amino acid (aa) residue of Par17	Kd (mM)	Std Error
Glutamine (Q95)	0.8	0.3
Glutamic acids (E96)	0.7	0.06
Alanine (A98)	1.7	0.9
Aspartic acid (D81)	0.3	0.18
Methionine (M52)	0.04	0.02
Mean of Kd of all the aa	0.7	0.30

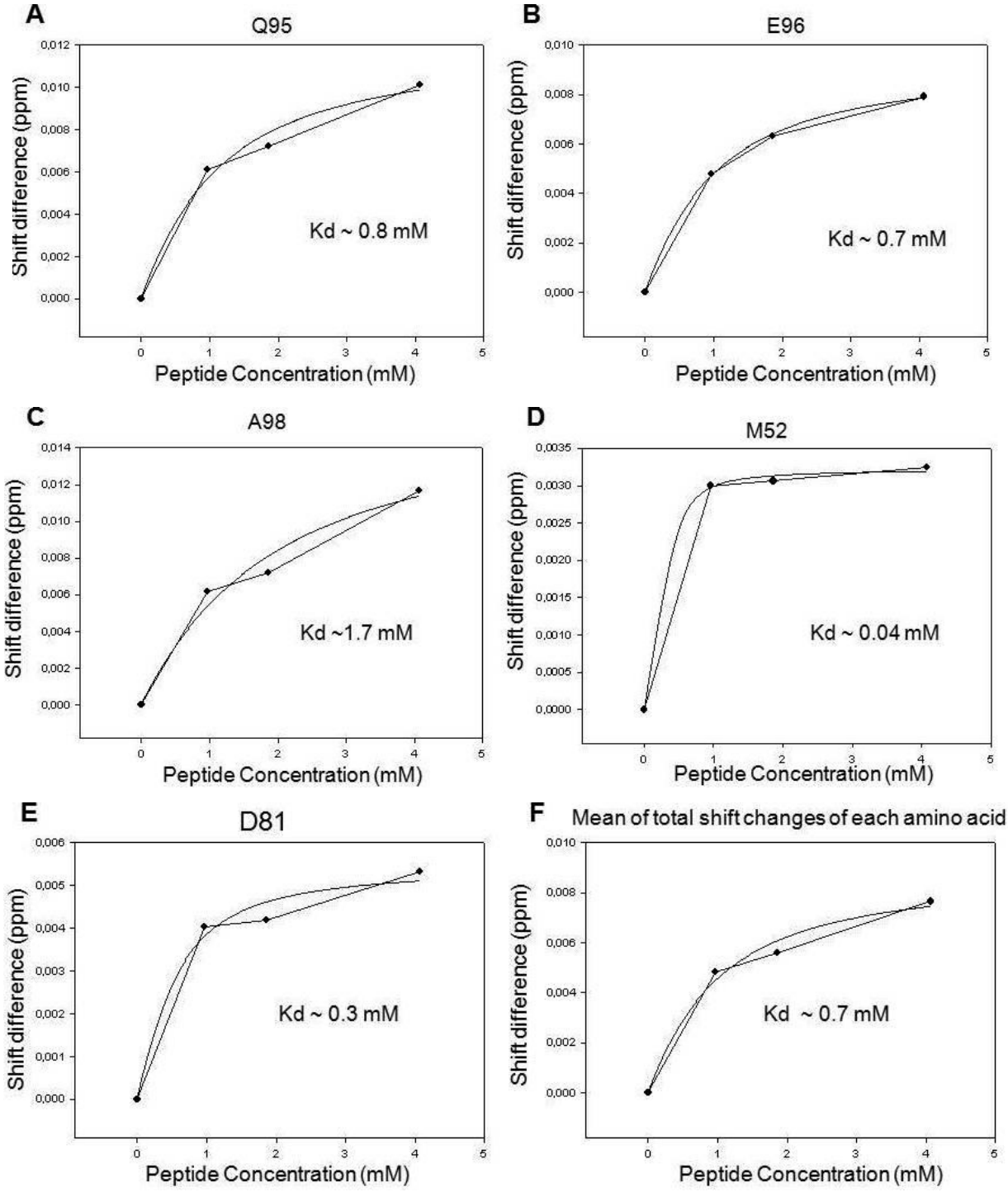

Figure 3.22. Curves of some ^{15}N-labelled Par17 amino acid residues showing significant chemical shift changes (and their mean value) upon titration with the peptide KHSPVHIGGGS-NH_2

The program (SigmaPlot11) was used for curves-fitting and Kd calculation. Total chemical shift changes (ppm) of each of the residues and their mean are plotted against the peptide concentration (protein: peptide, 0.4mM: 4mM).

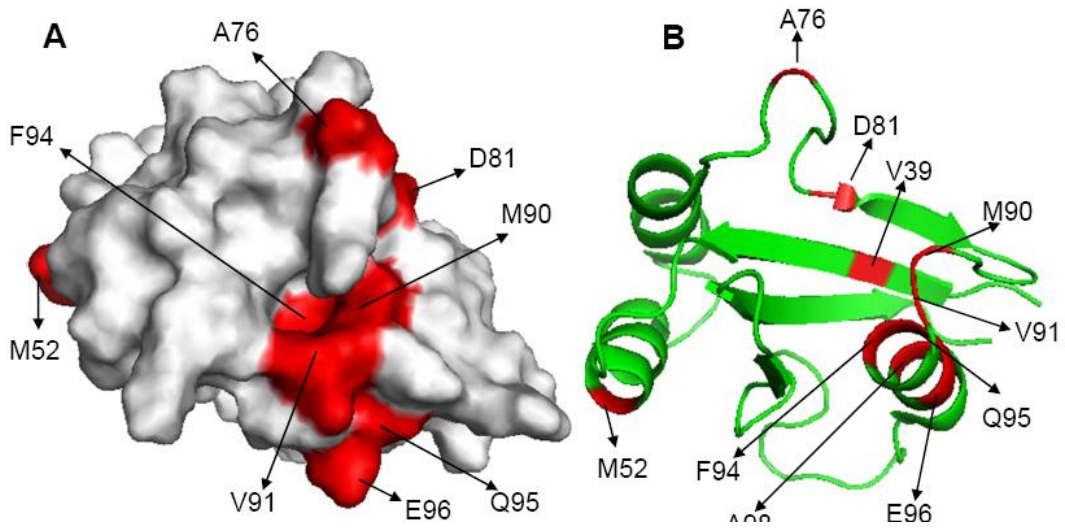

Figure 3.23. Mapping of the binding surface of the peptide KHSPVHIGGGS-NH_2 on the molecular surface of Par17

The amino acid residues showing significant chemical shift changes upon peptide titration are sown in red, and indicated with numbers and arrows in (A) 3 dimensional surface structure of Par14 (PDB. ID 1EQ3). (B) Par14 cartoon structure. This figure was generated using PyMol1.3.

3.13. The peptide SSFPPLLD binds Par17 PPIase domain at the postulated binding pocket

To map the binding surface of Par17 with the peptide SSFPPLLD selected from the Phage display (tables 3.4 and 3.6), and to calculate the binding constant (Kd) we performed NMR titration studies. Our NMR data suggest that the peptide SSFPPLLD binds Par17 with a Kd of ~ 1.6 (table 3.8 and figure 3.25) also at its PPIase domain (figures 3.24 and 3.26). Par17 amino acid residues exhibited significant chemical shift changes were His48, Gly49, Met52, Thr86, Met90, Phe94, Glu96, Val91, and Phe111 and may be Phe120 (figures 3.24 and 3.25).

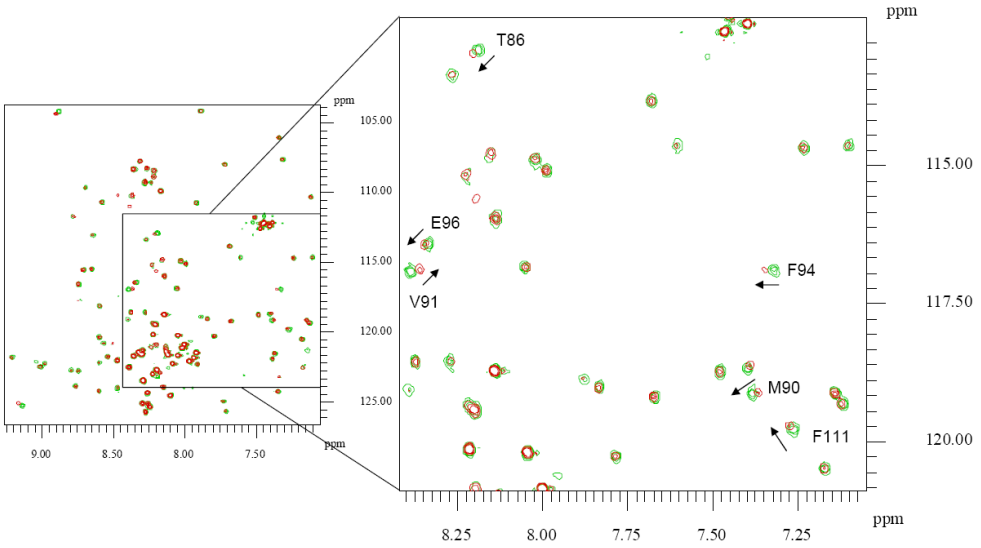

Figure 3.24. Part of ^{15}N-SOFAST-HMQC spectra of ~ 0.4 mM ^{15}N-labelled Par17 alone (green contour), and Par17 with ~ 4.3 mM the peptide SSFPPLLD (red contour)

The amino acid residues with significant chemical shift perturbation are indicated with one-letter symbol, the number of residue and the direction of the chemical shift.

Table 3.8. Kd values of Par 17 amino acid residues exhibiting significant chemical shift changes upon titration with the peptide SSFPPLLD

Approximate Kd values and standard (Std) errors of the amino acids (and their mean) with significant chemical shift changes upon titration of ^{15}N-labeled Par17 with the peptide SSFPPLLD.

Amino acid (aa) residue of Par17	Kd (mM)	Std Error
Threonine (T86)	1.6	1.5
Methionine (M90)	0.71	0.55
Phenylalanine (94)	3.1	2.3
Glutamic acid (E96)	1	1.65
Mean of Kd of all the aa	1.6	1.4

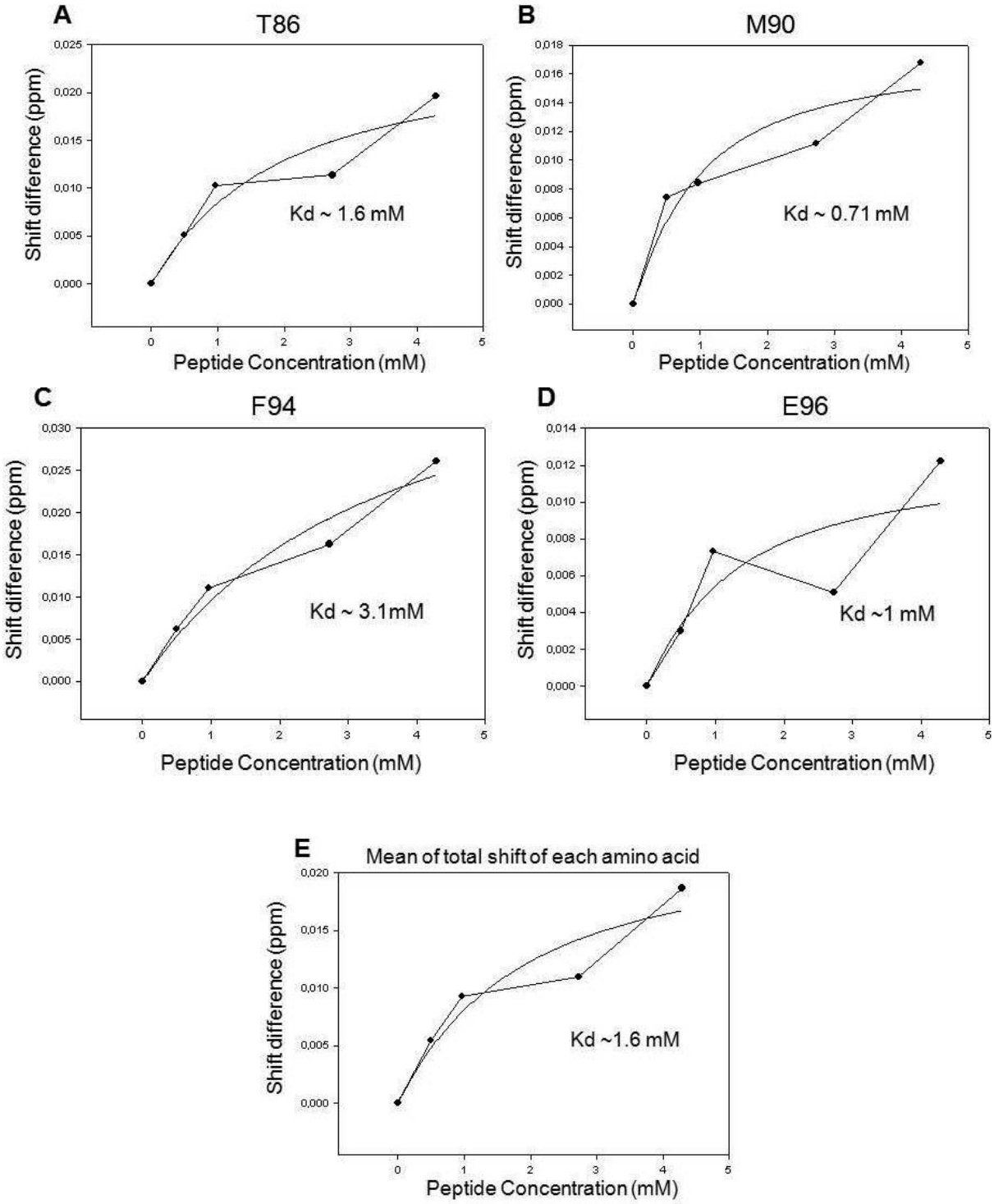

Figure 3.25. Curves of some ^{15}N-labelled Par17 amino acid residues showing significant chemical shift changes (and their mean value) upon titration with the peptide SSFPPLLD

The program (SigmaPlot11) was used for curves-fitting and Kd calculation. Total chemical shift changes (ppm) of each of the residues (and their mean value) are plotted against the peptide concentration (protein: peptide, 0.4mM: 4.3mM).

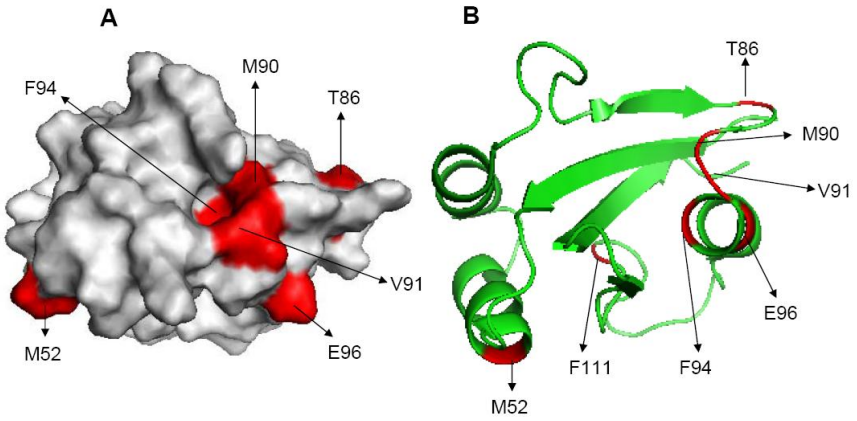

Figure 3.26. Mapping of the binding surface between Par17 and the peptide SSFPPLLD
The residues exhibiting chemical shift changes are shown in red and indicated with No. and arrows in (A): Three dimensional surface structure of Par14, PDB. ID 1EQ3 (B): Par14 cartoon structure. This figure was generated using PyMol1.3.

3.14. The panning of the 7-mer library against *C. symbiosum* PinA enriched for 2 peptides, HKRPRNN and HQSPWHH

In order find peptide binding partners for *C. symbiosum* PinA, we screened a 7-mer phage display library from NEB. *C.symbiosum* PinA (0.1 mg/ml) was immobilized on a polystyrene plate. After 3 consecutive rounds of panning, elution and amplification (Table 3.9), two peptides HKRPRNN and HQSPWHH were enriched (table 3.10). The stringency of panning was increased in washing steps by raising the tween 20 concentration of TBST from 1% in the 1st round to 5% in the 2nd and the 3rd rounds. Washings steps were followed by elution of the bound phage with 0.2 M Glycine-HCl (pH 2.2), 1 mg/ml BSA.

We also found the peptide HAIYPRH, a dominating target-unrelated peptide (Brammer *et al*., 2008), in our 3rd round elaute. Next, we performed phage ELISA to learn more about the phage-borne peptides enriched in the panning in term of affinity and specificity for *C. symbiosum* PinA. The phage-borne peptides were independently (10^9pfu from each) incubated with the target protein *C. symbiosum* PinA. The phage bindings were assessed with HRP-conjugated M13 antibody. Azino-bis (3-ethylbenzothiazole sulfonic acid) diammonium salt (ABTS) was used as an HRP substrate, the products for this substrate is green and can be read at 405 nm. Our phage ELISA suggests that the phage borne-peptides HKRPRNN and HQSPWHH bind *C. symbiosum* PinA with moderate affinity (figure 3.27). Therefore, these peptides were taken for NMR titration to study the binding sites of *C. symbiosum* PinA.

Our NMR data indicate that the peptide HQSPWHH binds in a big hydrophobic cleft formed from helix II, β sheet II and helix III. The amino acids residues that exhibited chemical shift changes were Phe31, Gly32, Gly42, Ser44, Gly53, Gly55, Gly57, Lys58, Val60, Phe83, Gly84, and Lys89 (figure 3.28).

Table 3.9. Rounds of *C. symbiosum* PinA panning

Approximate titre values of rounds of the 7-mer library screening against the Pin A of *C. symbiosum.*

Number of round	Titre (Pfu/10 µl)
1st round unamplified eluate	4×10^4
1st round amplified eluate	1.24×10^{11}
2nd round amplified eluate	3.2×10^{10}
3rd round unamplified eluate	5.1×10^4

Table 3.10. The amino acids sequences that were deduced from the phage DNA selected from the 3rd round of screening of the 7-mer library (NEB) against PinA of *C. symbiosum*

The peptides HKRPRNN and HQSPWHH were selected 5 and 3 times respectively from 20 sequenced phage clones. All other peptides were detected once. Conserved residues between phage clones shown in bold. The phage-borne peptide HAIYPRH was found in the 3rd round phage pool.

H	K	R	**P**	R	N	N	5X
H	Q	S	**P**	W	H	H	3X
A	P	S	**P**	M	I	W	1X
W	D	P	S	Q	M	R	1X
S	L	H	S	R	P	N	1X
T	I	E	Q	H	P	P	1X
V	Y	L	T	G	P	S	1X
L	D	R	A	N	V	F	1X
N	Q	L	T	T	L	N	1X
S	H	T	I	R	M	L	1X
Y	V	H	Q	Q	R	H	1X
T	M	C	I	Y	C	T	1X
G	L	C	C	S	R	L	1X
H	A	I	Y	P	R	H	1X

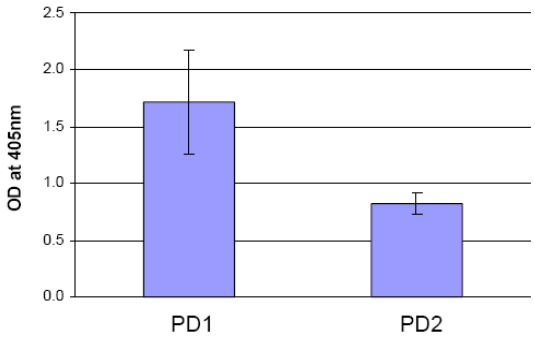

Figure 3.27. Phage ELISA for the peptides (PD1 is HKRPRNN and PD 2 is HQSPWHH) enriched in the screening of the 7-mer library with *C. symbiosum* PinA as a target protein. An ELISA 96 well plate was coated with *C. symbiosum* PinA (0.1mg/ml). One raw for each phage to be tested, 8 wells were coated with *C. symbiosum* PinA. Phage (10^9 pfu) were applied to each well. The mean values and standard deviation were taken for the 8 coated wells of each phage.

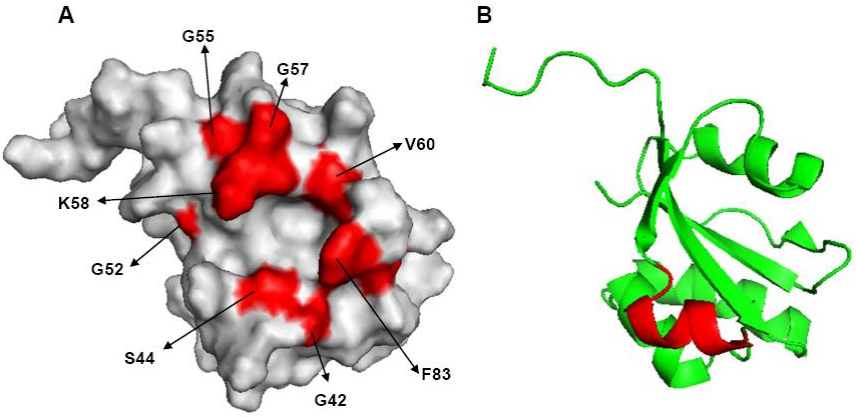

Figure 3.28. *Cenarchaeum symbiosum* PinA binding pocket and flexible region

Cenarchaeum symbiosum PinA PDB. ID 2RQS, (A) The amino acid residues (shown in red here) showing chemical shift changes when *C. symbiosum* PinA titrated with the peptide HQSPWHH. (B) The flexible region shown in red from Ser44 to Gly49. This figure was generated using PyMol 1.3.

4. Discussion

4.1 MT2A is a target unrelated or a specific binder in the Y2H system

During the screening of two cDNA libraries (figures 3.5 and 3.6) from different tissues with Par17 (Mueller *et al.,* 2006), MT2A (figure 3.8) has been detected as false positive several times in several screenings in the Y2H Gal4 system (figures 3.7 and 3.10). It is not unusual to detect false positives in the screening, but Metallothionein has been reported by other researchers as genuine interaction partner, and they confirm the interaction with different alternative methods (Rao *et al*., 2003; Gonçalves *et al*., 2008). Furthermore, MT2A was described as a binding partner for different bait proteins using only the Y2H, and without checking the correct reading frame of MT2A (Bai *et al*., 2005; Pope and Lee, 2005, Thijssen *et al*., 2006). On the other hand MT2A has been reported as a false positive in Y2H, therefore it was excluded from the list of candidate clones (Bosch-Comas *et al*., 2006; Wang *et al*., 2008).

It was reported that a large ORF not in-frame relative to Gal4 AD will correspond to the expressed protein (Matchmaker™ Library Construction & Screening Kits User Manual, Clontech, Protocol No. PT3955-1, Version No. PR792376, published 2007). In agreement with this, the length of the fusion protein expressed inside the yeasts as determined by western blotting (figure 3.11) is between 25 and 32 kDa; larger than the predicted molecular weight of the Gal4 AD-MT2A fusion (~ 24 kDa). Yeasts can sometimes correct not in-frame clones by frame-shifted translation (Fromont-Racine *et al.,* 1997). But this is unlikely to be in our case where we have different protein sizes from MT2A clones, and the out-of-frame MT2A translated to a protein that is rich in proline (figure 3.9), and interact non-specifically in Y2H (figures 3.7 and 3.10 upper panel), whereas in-frame MT2A do not (figures 3.13 and 3.14).

We show that MT2A clones, which are +1 out-of-frame relative to Gal4 AD (figure 3.8) can activate the reporter genes of the yeast strains AH109 and Y187 when they were co-transformed with pGBKT7-Lam or with empty pGBKT7 and as long as Gal 4 BD existed (figure 3.10 upper panel).

In the cDNA libraries, only ~ 16.6% of the inserts will be in the correct reading frame relative to Gal4 AD (Two-Hybrid cDNA Library Construction Kit User Manual, Protocol PT1113-1, version PR71194). The protein that is expressed inside the yeast from the out-of-frame MT2A clones is not MT2A, but a +1 frame-shifted MT2A (Elfaki *et al.,* 2011). We show that MT2A

in the correct reading frame relative to Gal4 AD domain (figure 3.13) can no longer activate the yeast reporter genes, but the +1 frame-shifted MT2A did (figure 3.14), this results indicate that MT2A is not a sticky protein (Elfaki *et al.,* 2011). It is most probably that the protein that was described as false positive (Bosch-Comas *et al.,* 2006, Wang *et al*., 2008) is not MT2A, but a +1 frame-shifted MT2A.

When pACT2-MT2A was co-transformed with pGBKT7-lam, the yeast cells could grow in the high stringency medium (figure 3.10 upper panel) that means, the entire reporter genes were activated, which is inconsistent with (Bai *et al*., 2005). Moreover, when we co-transformed pACT2-MT2A with empty plasmid (pGBKT7) expressing the un-fused Gal4 BD, the transformant yeast cells could grow in high stringency medium, meaning that pACT2-MT2A construct encodes a fusion protein alone can activate the yeast reporter genes. These data indicate that MT2A clones (which are not in-frame relative to Gal4 AD) generate a technical false positive in Y2H. It was reported that in rare cases the AD fusion protein can activate the reporter genes on its own, possibly by binding to the promoter or to the promoter-associated proteins (Finley, 2007). To address this issue, we have transformed it alone into AH109. The transformant cells could grow in SD –Leu but not in SD –Leu –His –Ade containing X-alpha-Gal (figure 3.10 lower panel) indicating that the reporter genes were not activated, and the encoded protein does not bind the promoter or the promoter-associated protein. This is in agreement with (Pope and Lee, 2005). It is therefore, the studies which have proposed that MT2A as a binding partner for their bait proteins using only the Y2H, and without checking the correct reading frame of MT2A (Bai *et al*., 2005; Pope and Lee, 2005, Thijssen *et al*., 2006), further verifications of the interaction of their bait protein with MT2A are needed.

Perhaps the +1 frame-shifted MT2A fused to Gal4 AD produces a promiscuous protein. Since this protein is rich in proline, it can form sticky stiff arms as reported for the proline rich regions that bind other proteins rapidly and reversibly (Williamson, 1994). However, a coincidental specific protein–protein interaction with Gal4 BD cannot be excluded.

Among the other clones that we have obtained in our screenings is the zink finger protein which is known to be a technical false positive (Hengen, 1997). We also found vitronectin within our screens. It did not bind Gal4 BD when it was transformed with the empty pGBKT7, but it binds another construct of pGBKT7 (pGBKT7-DIRK3) indicating that its interaction with Par17 “in our Y2H screenings” is rather nonspecific.

4.2. Two peptide sequences were enriched in the panning of 7 and 12-mer Ph.D. libraries against Par17, a consensus peptide sequence and a phage pool-dominating peptide

Our screening of Ph.D. 12 and the 7-mer peptide libraries against Par17 (with a normal concentration) enriched one consensus sequence, XHSXVHØ (table 3.2), where X can be any amino acid, and Ø is a hydrophobic amino acid. This motif was present in many different peptides in the 3rd round from both Ph.D. libraries (table 3.2). It represents ~ 70 % of the phage-borne peptides of the 3rd round from the 7-mer library panning. Similarly, it is found in ~ 62% of the phage-borne peptides of the 3rd round from the 12-mer library panning (table 3.2).

To obtain peptides bind with a high affinity, we increased the stringency of panning by reducing the target concentration to the minimal. Using this strategy we obtained the phage-borne peptide SSFPPLL (table 3.4). This peptide was also enriched when we applied the solution-phase panning against Par17-GST (table 3.6), where it was almost dominating the phage pool of the 3rd round. We propose that the phage-borne SSFPPLL peptide is a "sib" clone binds Par17, but with low affinity. It has the ability to amplify and dominate the phage pool, probably because it has developed an amplification advantage over other phage of the library (Troubleshooting Forum, Molecular Biology Techniques Q&A, Bio-Techniques, doi 10.2144/000113537, 2010, 49: 789-791).

With phage ELISAs, we demonstrated that the phage-borne peptides containing the motif (XHSXVHØ), and the phage-borne peptide SSFPPLL bind Par17 (figures 3.18 and 3.19). However, the phage-borne peptides containing the motif (XHSXVHØ) bind Par17 with a relatively higher affinity than the phage-borne peptide SSFPPLL. For the motif, XHSXVHØ, our phage ELISAs suggest that Pro at position i 0 can be replaced by most amino acids. His at position i –2 is required for the binding, but it can be replaced by Leu with a decrease in ELISA OD at 405 nm. Ser at position i –1 is also required for the peptide binding, however, it can be substituted by Thr. Val and His at position i +1 and i +2 respectively must be present for a good binding, while i +3 must contain a hydrophobic amino acid (figures 3.18 and 3.19).

Our NMR results indicate the peptide KHSPVHIGGGS-NH_2 binds Par17 with a Kd of ~ 0.7 mM, (STDEV ~ 0.6) whereas, the peptide SSFPPLL binds Par17 with a Kd of 1.5 mM (STDEV ~ 1.06). However, from our Ph.D. and Phage ELISA results we would have expected higher binding affinities (lower micromolar range). Probably in this case such short peptides are not sufficient for a strong binding (Landgraf *et al*., 2004).

Our NMR data suggest that the peptide KHSPVHIGGGS-NH_2 binds Par14/17 PPIase domain (figures 3.22 and 3.23). The Par14/17 residues showed significant chemical shift changes were Val40, Met52, Ala76, Asp81, Met90, Val91, Phe94, Gln95, Glu96, and Ala98 (figures 3.21 and 3.22). For the peptide SSFPPLLD which also binds Par14/17 PPIase domain, Par17 amino acid residues exhibited significant chemical shift changes were His48, Gly49, Met52, Thr86, Met90, Phe94, Glu96, Val91, and Phe111 (figures 3.24, 3.25 and 3.26). Phe120 also probably exhibited small chemical shift changes.

The amino acid M52 is not a round the PPIase active site (figure 3.23). Perhaps it exhibited chemical shift changes due to Par14/17 conformational changes or structural rearrangement.

The residues Met90, Val91 and Gln95 are forming the rim of a hydrophobic core in the molecular surface of Par14/17, whereas, Phe94 is lying at the bottom of this core (figures 3.23 and 3.26). Met90, Phe94, Val91 were proposed to be among the residues of Par14 active site (Sekerina *et al*., 2000; Terada *et al*., 2001; Jaremko *et al*., 2011). Our data suggest that this hydrophobic core is the PPIase active site of Par14/17.

The phage display-derived peptide sequence (XHSXPVHØ) can be aligned with significant homology in many proteins. If this peptide is available for interaction in the native protein structural context, we can expect an interaction between XHSXPVHØ-containing protein and Par14/17. For instance mixed lineage kinase 4, MLK4 (mitogen activated protein kinase kinase kinase, Ref. Seq NP_115811.2) can be a good candidate. The peptide sequence XHSXPVHØ can be aligned two times with the amino acid sequence of MLK4. In MLK4 amino acid sequences from 935 to 941 there is the peptide KHSTVHI, and from 115 to 119 there is sequence SPVHV. Both of these peptides KHSTVHI and SPVHV from MLK4 contain the Ph.D. selected motif (XHSXPVHØ). Weak protein-protein interactions occur *in vivo* (Vaynberg and Qin, 2006). However, other amino acid residues from both proteins may contribute to the interaction, and thereby increasing the binding affinity. Therefore Par14/17 can bind this motif *in vivo* if it is available for interaction and solvent-exposed in the protein natural context. In addition our peptide can be used to design a substrate or an inhibitor for Par14/17 PPIase catalytic assay.

We also found the peptide LPLTPLP in our screening for peptide binders for Par17 as 1 clone among 15 sequenced clones from the 3rd round (table 3.2). This panning was done by immobilizing 100µg/ml of Par17 protein, eluting with the general buffer for nonspecific disruption of binding interactions (0.2 M Glycine-HCl (pH 2.2). The peptide LPLTPLP was also enriched when we reduced the target (Par17) concentration to the minimal (table 3.4), and when we performed the solution-phase panning against Par17-GST (table 3.6).

In a Ph.D. panning against the high mobility group 1 (HMGB1), the peptide LPLTPLP was selected several times after 3 rounds of panning (Dintilhac and Bernués, 2002). The amino acids sequence LPLTPLP could be aligned with 100 % homology with the amino acid sequence of the Groucho protein, amino terminal enhancer of split (AES) from 140-146. The interaction of HMGB1-AES was shown by a pulldown experiment (Dintilhac and Bernue´s, 2002).

It has been shown that Par14 binds the DNA and shares some sequence similarity with HMG proteins (Surmacz *et al*., 2002). Therefore, it is intriguing to imagine an interaction between Par14 and AES. However, it seems that the peptide LPLTPLP is a target-unrelated peptide. Apart from the binding to HMG proteins reported by (Dintilhac and Bernués, 2002), the peptide LPLTPLP was also described as a binder for a variety of target proteins. For instance, it was selected in panning of a 7-mer library against the aminoacyle- tRNA site of 16 rRNA, but they have suggested that it binds RNA non-specifically (Li *et al*., 2009). Moreover, the same peptide was identified as a binder for human endothelium (Maruta *et al*., 2003). It was also enriched in a panning intended to search for cardiac targeting peptides when they panned the 7-mer Ph.D. library against WKY rat heart (Nicol *et al*., 2009). Recently, this peptide was proposed to be with unknown specificity (Vodnik *et al*., 2011).

The peptide GKPMPPM was also enriched in the 3rd round of our Ph.D. library screening (tables 3.4; 3.6). This peptide (GKPMPPM) was also selected in other Ph.D. screens. It has been selected in panning against Polymer-Film Surfaces (Serizawa et al., 2007), and in a panning against the surface protein of the acute respiratory syndrome associated corona virus (Hüttinger, PhD thesis 2009). This peptide may be over-represented in the library, or probably a sticky peptide as proposed for proline rich regions (Williamson, 1994).

4.3. Peptides that bind Pin A from *Cinarchaeum symbiosum*

The peptides that were enriched from the phage peptide library when it was screened against the PPIase PinA of *C. symbiosum*, HKRPRNN and HQSPWHH were used for NMR study in peptide-protein interaction to get more insight about its PPIase active site. In NMR ^{1}H ^{15}N HSQC titration, the peptide HQSPWHH binds PinA of *C. symbiosum* with low affinity (high micromolar / low millmolar range). Therefore, it could be used to demonstrate binding sites of *C. symbiosum* PinA whose NMR solution structure was recently solved (Jaremko *et al*., 2011). It binds in a hydrophobic core formed from helix II, β sheet II and helix III. The amino acids residues that exhibited chemical shift changes in NMR titration namely are Phe31, Gly32, Gly42, Ser44, Gly53, Gly55, Gly57, Lys58, Val60, Phe83, Gly84, and Lys89. All these amino acid residues were around the postulated Proline binding cleft (figure 3.28. A). Furthermore, the peptide HQSPWHH was also used to demonstrate a flexible region in *C. symbiosum* PinA (from Ser44-Gly49). Since a glycine and two positively charged residues are conserved in all parvulins in this region, it was proposed that the flexibility of this region (figure 3.28.B,) might perform a general function in peptidyl prolyl cis/trans isomerization (Jaremko *et al*., 2011).

We also found the peptide HAIYPRH in the 3rd round pool. This peptide was thought to be a binder for different targets until Brammer and others could demonstrate that it is a target-unrelated peptide (Brammer *et al*., 2008). The phage clone expressing or "displaying" the peptide HAIYPRH has developed a mutation in the Shine-Dalgarno (SD) sequence for gIIp. This mutation enables the SD of gIIp to fit better in 16 S ribosome, and thereby it gives the phage the advantage of faster amplification than all of the phage of the library. Therefore, it amplifies without being selected in the panning (Brammer *et al*., 2008).

References

Arap, M. A., (2005). Phage display technology - Applications and innovations, Genetic and molecular Biology. 28 (1) 1-9.

Ayed, A., Mulder, F.A., Yi, G.S., Lu, Y., Kay, L.E. and Arrowsmith, C.H. (2001). Latent and active p53 are identical in conformation. Nat Struct Biol., 8 (9), 756-760.

Bai, G.Q., Cheng, J., Zhang, S.L., Huang, Y.P., Wang, L., Liu, Y., Lin, S.M. (2005). Screening of hepatocyte proteins binding to complete S protein of hepatitis B virus by yeast-two hybrid system. World J Gastroenterol. 11(25):3899-904.

Behrens, S., Maier, R., de Cock, H., Schmid, F.X., Gross, C.A. (2001). The SurA periplasmic PPIase lacking its parvulin domains functions in vivo and has chaperone activity. EMBO J. (1-2):285-94.

Birnboim, H.C., Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7(6), 1513-1523.

Bosch-Comas, A., Lindsten, K., Gonzàlez-Duarte, R., Masucci, M.G., Marfany, G. (2006). The ubiquitin-specific protease USP25 interacts with three sarcomeric proteins. Cell. Mol. Life Sci. 723–734.

Bothmann, H., Plückthun, A. (2000). The periplasmic *Escherichia coli* peptidylprolyl cis/trans isomerase FkpA. I. Increased functional expression of antibody fragments with and without cis-prolines. J Biol Chem. 275(22):17100-5.

Brammer, L.A., Bolduc, B., Kass, J.L., Felice, K.M., Noren, C.J., and Hall, M.F. (2008). Target-unrelated peptide in an M13 phage display library traced to an advantageous mutation in the gene II ribosome-binding site. Anal Biochem. 373 (1): 88-98.

Brent, R., and Finley, R.L. Jr. (1997). Understanding gene and allele function with two-hybrid methods. Ann. Rev. Gen. 31, 663-704.

Byrd, A., and St-Arnaud, R. (2001). Strategies for rescuing plasmid DNA from yeast two-hybrid colonies. Methods Mol Biol.177:107-19.

Chien, C.,T., Bartel, P.L., Sternglanz, R., Fields, S. (1991). The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc Natl Acad Sci U S A. .88(21):9578-82.

Dintilhac, A., and Bernués, J. (2002). HMGB1 interacts with Many Apparently Unrelated Proteins by Recognizing Short amino Acid Sequences. JBC. 1; 277(9):7021-8.

Duguid, J.R., Bohmont, C.W., Liu, N.G., Tourtellotte, W.W. (1989). Changes in brain gene expression shared by scrapie and Alzheimer disease. Proc Natl Acad Sci U S A. 86(18):7260-4.

Elfaki, I., Bayer, P., and Mueller, W.J. (2011). A potential transcriptional regulator is out-of-frame translated from Metallothionein 2A messenger RNA. Anal. Biochem. 409 (1) 159-161.

Fields, S. (2005). High-throughput two-hybrid analysis. The promise and the peril. FEBS J. 272(21):5391-9.

Fields, S., Song, O. (1989). A novel genetic system to detect protein-protein interactions. Nature. 340 (6230):245-6.

Finley, R.L., Jr. (2007). A Guide to Yeast Two-Hybrid Experiments In Evaluating techniques in Biochemical Research. D. Zuk, ed. (Cambridge, MA: Cell Press).

Fischer, G.(1994). Peptidyl-Prolyl Cis/trans Isomerases and Their Effectors. Angewandte Chemie international Edition in English, 33; 1415-1436.

Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber. T, Schmid, F.X. (1989). Cyclophilin and peptidyle-prolyl cis–Trans isomerase are probably identical proteins. Nature, 2; 337 (6206); 476-478.

Fromont-Racine, M., Rain, J.C., Legrain, P. (1997). Toward a functional analysis of the yeast genome through exhaustive two-hybrid screens. Nat Genet. (3):277-82.

Fujiyama-Nakamura, S., Yoshikawa, H., Homma, K., Hayano, T., Tsujimura-Takahashi, T., Izumikawa, K., Ishikawa, H., Miyazawa, N., Yanagida, M., Miura, Y., Shinkawa, T., Yamauchi, Y., Isobe, T., Takahashi, N. (2009). Parvulin (Par14), a peptidyl-prolyl cis-

trans isomerase, is a novel rRNA processing factor that evolved in the metazoan lineage. Mol Cell Proteomics. (7):1552-65.

Gonçalves, I., Quintela, T., Baltazar, G., Almeida, M.R., Saraiva, M.J., Santos, C.R. (2008). Transthyretin interacts with metallothionein2. Biochemistry. 47(8):2244-51.

Hengen, H.P. (1997). Methods and reagents: False positives from the yeast two-hybrid system, Trends in Biochemical Sciences, 22 (1), 33-34.

Huang, H. and Bader S.J (2009). Precision and recall estimates for two-hybrid screens. Bioinformatics, 25 (3) 372–378.

Hüttinger, C. (2009). Isolation and Characterisation of High-Affinity Peptides Directed Against Surface Glycoproteins of Pathogenic Viruses. PhD thesis, University of Veterinary Medicine Hannover, Germany.

Jaremko, L., Jaremko, M., Elfaki, I., Mueller. J.W., Ejchart, A., Bayer, P., Zhukov, I. (2011). Structure and dynamics of the first archaeal parvulin reveal a new functionally important loop in parvulin-type prolyl isomerases. J Biol Chem. 286(8):6554-65.

Jin, R., Chow, V.T., Tan, P.H., Dheen, S.T., Duan, W., Bay, B.H. (2002). Metallothionein 2A expression is associated with cell proliferation in breast cancer. Carcinogenesis, 23 (1), 81-86.

Karin, M. (1985). Metallothioneins: proteins in search of function. Cell, 41(1):9-10.

Kessler, D., Papatheodorou, P., Stratmann, T., Dian, E.A., Hartmann-Fatu, C., Rassow, J., Bayer, P., Mueller. J.W. (2007). The DNA binding parvulin Par17 is targeted to the mitochondrial matrix by a recently evolved prepeptide uniquely present in Hominidae. BMC Biol. 17, 5-37.

Kim, H.G., Kim, J.Y., Han, E.H., Hwang, Y.P., Choi, J.H., Park, B.H., and Jeong, H.G (2011). Metallothionein2A overexpression increases the expression of matrix metalloproteinase -9 and invasion of breast cancer cells, FEBS Lett. 585(2):421-8.

Landgraf, C., Panni, S., Montecchi-Palazzi, L., Castagnoli, L., Schneider-Mergener, J., Volkmer-Engert, R., Cesareni, G. (2004). Protein interaction networks by proteome peptide scanning. PLoS Biol. (1):E14.

Li, M., Duc, A.C., Klosi, E., Pattabiraman, S., Spaller, M.R., Chow, C.S. (2009). Selection of Peptides That Target the Aminoacyl-tRNA Site of Bacterial 16S Ribosomal RNA. Biochemistry. 48(35):8299-311.

Lin, T.C., Webster, R.E., Konigsberg, W. (1980). Isolation and characterization of the C and D proteins coded by gene IX and gene VI in the filamentous bacteriophage fl and fd. J Biol Chem. 255(21):10331-7.

Lu, K.P., Hanes, S.D., Hunter, T. (1996). A human peptidyl-prolyl isomerase essential for regulation of mitosis. Nature. 380(6574):544-7.

Margoshes, M. & Vallee, B.L. (1957). A cadmium protein from equine kidney cortex. J. Am. Chem. Soc. 79, 4813–4818.

Maruta, F., Parker, A.L., Fisher, K.D., Murray, P.G., Kerr, D.J., Seymour, L.W. (2003). Use of a phage display library to identify oligopeptides binding to the lumenal surface of polarized endothelium by ex vivo perfusion of human umbilical veins. J. Drug Target. 1, 53-9.

Matchmaker™ GAL4 Two-Hybrid System 3 & Libraries User Manual, Clontech, Protocol No. Protocol No. PT3247-1, Version No. PR742219, published 2007.

Matchmaker™ Library Construction & Screening Kits User Manual, Clontech, Protocol No. PT3955-1, Version No. PR792376, published 2007.

Matchmaker™ Pretransformed Libraries User Manual, Clontech, Protocol No. PT3183-1 Version No PR6Y2132 A, published 2007.

Mueller, J.W. and Bayer, P., (2008). Small Family with Key Contacts: Par14 and Par17 Parvulin Proteins, Relatives of Pin1, Now Emerge in Biomedical Research. Perspectives in Medicinal Chemistry 2, 11-20.

Mueller, J.W., Kessler, D., Neumann, D., Stratmann, T., Papatheodorou, P., Hartmann-Fatu C., and Bayer, P. (2006). Characterization of novel elongated Parvulin isoforms that are ubiquitously expressed in human tissues and originate from alternative transcription initiation. BMC Mol Biol. 7; 7:9.

Nicol, C.G., Denby, L., Lopez-Franco, O., Masson, R., Halliday, C.A., Nicklin, S.A., Kritz, A., Work, L.M., and Baker, A.H. (2009). Use of in vivo phage display to engineer novel adenoviruses for targeted delivery to the cardiac vasculature. FEBS Lett. 583(12):2100-7.

Pope, S.N., Lee, I.R. (2005). Yeast two-hybrid identification of prostatic proteins interacting with human sex hormone-binding globulin. J. Steroid Biochem. Mol. Biol. 94 (1–3): 203-8.

Pratt, D., and Erdahl, W.S. (1968). Genetic control of bacteriophage M13 DNA synthesis. JMB, 37 (1), 181-200.

Preston, C.M., Wu, K.Y., Molinski, T.F., DeLong, E.F. (1996). A psychrophilic crenarchaeon inhabits a marine sponge: *Cenarchaeum symbiosum* gen. nov., sp. nov. Proc. Natl. Acad. Sci. USA, (93)6241-6246.

Rahfeld, J.U., Rücknagel, K.P., Schelbert, B., Ludwig, B., Hacker, J., Mann, K., Fischer, G. (1994). Confirmation of the existence of a third family among peptidyl-prolyl cis/trans isomerases. Amino acid sequence and recombinant production of parvulin. FEBS Lett. 352(2):180-4.

Ramm, K., Plückthun, A., (2000). The periplasmic Escherichia coli peptidylprolyl cis,trans-isomerase FkpA. II. Isomerase-independent chaperone activity in vitro. J Biol Chem. 275(22):17106-13.

Ranganathan, R., Lu, K.P., Hunter, T., Noel, J.P. (1997). Structural and functional analysis of the mitotic rotamase Pin1 suggests substrate recognition is phosphorylation dependent Cell. 89(6):875-86.

Rao, P.S., Jaggi, M., Smith, D.J., Hemstreet, G.P., Balaji, K.C. (2003). Metallothionein 2A interacts with the kinase domain of PKCmu in prostate cancer. Biochem Biophys Res Commun. 310(3):1032-8.

Reimer, T., Weiwad, M., Schierhorn, A., Ruecknagel, P.K., Rahfeld, J.U., Bayer, P., Fischer, G. (2003). Phosphorylation of the N-terminal domain regulates subcellular localization and DNA binding properties of the peptidyl-prolyl cis/trans isomerase hPar14. J Mol Biol. 330(5):955-66.

Rulten, S., Thorpe, J., Kay, J. (1999). Identification of eukaryotic parvulin homologues: a new subfamily of peptidylprolyl cis-trans isomerases. Biochem Biophys Res Commun. 259(3):557-62.

Schönbrunner, E.R., Mayer, S., Tropschug, M., Fischer, G., Takahashi, N., and Schmid, F.X. (1991). Catalysis of Protein Folding by Cyclophilins from Different Species. 266 (6):3630-5.

Sekerina, E., Rahfeld, J.U., Müller, J., Fanghänel, J., Rascher, C., Fischer, G., Bayer, P. (2000). NMR solution structure of hPar14 reveals similarity to the peptidyl prolyl cis/trans isomerase domain of the mitotic regulator hPin1 but indicates a different functionality of the protein. J Mol Biol. .301(4):1003-17.

Serizawa, T., Sawada, T., Kitayama, T. (2007). Peptide motifs that recognize differences in polymer-film surfaces. Angew Chem Int Ed Engl. 46(5):723-6.

Shaw, P.E. (2002). Peptidyl-prolyl isomerases: a new twist to transcription. EMBO Rep. (6):521-6.

Sidhu, S.S.(2000). Phage display in pharmaceutical biotechnology, Curr Opin Biotechnol. 11(6):610-6.

Siekierka, J.J., Hung, S.H., Poe, M., Lin, C.S., Sigal, N.H. (1989). A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. Nature. 341(6244):755-7.

Simons, G.F., Konings, R.N., Schoenmakers, J.G.(1981). Genes VI, VII, and IX of phage M13 code for minor capsid proteins of the virion, Proc Natl Acad Sci U S A. 78(7):4194-8.

Sinnen, C. (2008). Untersuchungen zur Domäneninteraktion von hPin1 mit NMR. Phd Thesis, University of Duisburg-Essen, Germany.

Smith, G.P., Petrenko, V.A. (1997). Phage Diplay, Chem Rev. 97(2):391-410.

Surmacz, T.A., Bayer, E., Rahfeld, J.U., Fischer, G., Bayer, P. (2002). The N-terminal basic domain of human parvulin hPar14 is responsible for the entry to the nucleus and high-affinity DNA-binding. J Mol Biol. 321(2):235-47.

Sutherland, D.E., Stillman, M.J. (2011). The "magic numbers" of metallothionein. Metallomics. 3(5):444-63. Epub 2011 Mar 15.

Takahashi, N., Hayano, T., Suzuki, M. (1989). Peptidyl-prolyl cis-trans isomerase is the cyclosporin A-binding protein cyclophilin. Nature. 337(6206):473-5.

Terada, T., Shirouzu, M., Fukumori, Y., Fujimori, F., Ito, Y., Kigawa, T., Yokoyama, S., Uchida, T. (2001). Solution structure of the human parvulin-like peptidyl prolyl cis/trans isomerase, hPar14. J Mol Biol. 305(4):917-26.

Thijssen, V.L., Postel, R., Brandwijk, R.J., Dings, R.P., Nesmelova, I., Satijn, S., Verhofstad, N., Nakabeppu, Y., Baum, L.G., Bakkers, J., Mayo, K.H., Poirier, F., Griffioen, A.W. (2006). Galectin-1 is essential in tumor angiogenesis and is a target for antiangiogenesis therapy. Proc Natl Acad Sci U S A. ,103(43):15975-80

Tocci, M.J., Matkovich, D.A., Collier, K.A., Kwok, P., Dumont, F., Lin, S., Degudicibus, S., Siekierka, J.J., Chin, J., Hutchinson, N.I. (1989). The immunosuppressant FK506 selectively inhibits expression of early T cell activation genes. J Immunol. .143(2):718-26.

Tong, A.H., Drees, B., Nardelli, G., Bader, G.D., Brannetti, B, Castagnoli, L., Evangelista, M., Ferracuti, S., Nelson, B., Paoluzi, S., Quondam, M., Zucconi, A., Hogue, C.W., Fields, S., Boone, C. and Cesareni, G. (2002). A Combined Experimental and Computational Strategy to Define Protein Interaction Networks for Peptide Recognition Modules. Science, 295, 321-324.

Uchida, T., Fujimori, F., Tradler, T., Fischer, G., Rahfeld, J.U., (1999). Identification and characterization of a 14 kDa human protein as a novel parvulin-like peptidyl prolyl cis/trans isomerase. FEBS Lett. .446(2-3):278-82.

Vaynberg, J., Qin, J. (2006) Weak protein-protein interactions as probed by NMR spectroscopy. Trends Biotechnol. (1):22-7.

Vodnik, M., Zager, U., Strukelj, B., Lunder, M. (2011). Phage display: selecting straws instead of a needle from a haystack. Molecules. 16(1):790-817.

von Mering, C., Krause, R., Snel, B., Cornell, M., Oliver, S.G., Fields, S., Bork, P. (2002). Comparative assessment of large-scale data sets of protein-protein interactions. Nature. .417(6887):399-403.

Wang, J., Yuan, Y., Zhou, Y., Guo, L., Zhang, L., Kuai, X., Deng, B., Pan, Z., Li, D., He, F. (2008). Protein interaction data set highlighted with human Ras-MAPK/PI3K signaling pathways. J Proteome Res. (9):3879-89.

Williamson, M.P. (1994). The structure and function of proline-rich regions in proteins, Biochem.J. 297, 249-260.

Yaffe, M.B., Schutkowski, M., Shen, M., Zhou, X.Z., Stukenberg, P.T., Rahfeld, J.U., Xu, J., Kuang, J., Kirschner, M.W., Fischer, G., Cantley, L.C., and Lu, K.P. (1997). Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. Science. 278(5345):1957-60.

Yeast Protocols Handbook, Clontech, Protocol No. PT3024-1, 2 Version No PR742227, published 2008.

Zoldák, G., Aumüller, T., Lücke, C., Hritz, J., Oostenbrink, C., Fischer, G., Schmid, F.X. (2009). A Library of Fluorescent Peptides for Exploring the Substrate Specificities of Prolyl Isomerases. Biochemistry, 48, 10423-10436.

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