Mapping the binding site of the Hof1p SH3 domain in the Bnr1p FH1 domain

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Research training 15 c, 2014
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Acknowledgements

I would like to thank Dr. Alan Munn for accepting me to join the research group and for supervising me before and during my internship on many prospects. It has been a very instructive time in my professional development.

I would also like to thank my co-supervisor Heike Mack for instructing me in the laboratory and making me feel like one in the team.

This has been my first experience of working in research and it has been a great experience in all aspects. I will be forever grateful.
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Abstract

The unusual syndrome Wiskott-Aldrich Syndrome has been found to be linked to a mutation in a gene expressing the Wiskott-Aldrich Syndrome protein (WASp). Due to the conservation of certain mechanisms and the homologous proteins from lower to higher eukaryotes, yeast has been used to study the molecular mechanisms that underlie diseases in humans. The yeast WASp homologue, Las17p (Local Anaesthetic Sensitive 17) and the yeast homologue Vrpp of its (i.e. human WASp) partner protein known as WIP (WASp-interacting protein), have been studied with the intention to investigate the phenotypes that arise in cells deficient in Vrp1p. A protein-protein interaction is believed to occur in the absence of Vrp1p, the interaction between the SH3 domain of the yeast F-Bar protein Hof1p and the FH1 domain of the yeast formin protein Bnr1p. This interaction is thought to have pathological effects, such as inhibition of cell proliferation and other phenotypes.

This study has been conducted in an attempt to map the binding site of Hof1p SH3 domain in the Bnr1p FH1 domain more specifically, based on previous studies suggesting that Fragment 1(755-905) of Bnr1p Full Interacting Fragment(755-1375), including the proline-rich FH1 domain, interacts with Hof1p SH3 domain in cells deficient in Vrp1p. Both Hof1p and Bnr1p are involved in the cytokinesis stage of the yeast cell cycle. The hypothesis implies that the excessive interaction of Hof1p SH3 domain and Bnr1p FH1 domain may interrupt the cytokinesis, which thereby can lead to the growth of defects.

There was no finding of binding site within the Bnr1p FH1 domain for the Hof1p SH3 domain during this study. This can be due to various reason as explained later. Even though, this study has cast some doubts on previously conducted studies.

Introduction

Significance

The emergence of various diseases in the human body is still unresolved. Though, studies conducted using yeast as a model organism and the studying of conserved homologue proteins is giving progress to the research. The unusual syndrome Wiskott-Aldrich Syndrome (WAS) has been found to be linked to a mutation in a gene on the short arm of the X chromosome. The protein expressed by this gene is termed the Wiskott-Aldrich Syndrome protein (WASp). The WAS Syndrome implies, among other symptoms, allergies,
low blood platelets (thrombocytes), and combined immunodeficiency. A lot of research, performed to study diseases that arise in humans, use yeast as model in which to study the molecular mechanisms that underlie disease in humans, since yeast is quick and easy to grow and because the cell cycle is very similar to the human cell cycle and regulated by homologous proteins.

The WASp protein and its partner WIP, WASp Interacting Protein, are components of the actin cytoskeleton. Actin cytoskeleton rearrangements is, among other functions, required for antigen-presenting cells to be able to present antigen, for T cells to become activated cytotoxic T cells and for the T cells to be stimulated and then turn on B cells to produce antibodies. [4] These actin rearrangements in the T cells require WASp and WIP function. Thus, WASp and its partner WIP, are of importance in the actin cytoskeleton with immunostimulatory properties. [1], [2] The actin cytoskeleton has been conserved during evolution and there is also a conserved role for actin cytoskeleton in endocytosis for higher to lower eukaryotes. [1], [2] Because of the conserved mechanisms of the actin cytoskeleton, yeast is a suitable model organism to investigate WASP and WIP function.

It has been shown [1],[2], [3] that in cells deficient in the WIP homologue Vrp1p, the F-BAR yeast protein Hof1p has pathological effects. [1], [3] It is believed that SH3 domains that normally interact with the yeast homologue of WASP, Las17p, might get misfolded and convert into prions in cells lacking Las17p protein expression, or expressing a mutated form of Las17p that cannot bind the SH3 domains.[1], [2] In this prion-like form, the SH3 domain is toxic and inhibits cell proliferation and growth phenotypes. Upon over-expression of a mammalian Hof1p homologue, PSTPIP1, in mammalian cells, similar defects are known to occur possibly via interaction with analogous partners. [1], [2]

The aim of CI Munn's research group is to gain insight into WASp and WIP protein function, by studies of their homologues in yeast. The intention is to look into more in detail on the yeast homologues of WASp and WIP protein functions and the interactions that occur in the absence of WIP, and how these interactions can be toxic.

**F-Bar Proteins**

F-BAR proteins are signalling proteins, in both higher and lower eukaryotes, involved in membrane remodelling during endocytosis and cell division. [1], [2] One F-BAR protein is Hof1p, Homologue Of Fifteen (Cdc5 of fission yeast Schizosaccharomyces pombe), a
protein involved in cytokinesis in S. Cerevisiae, encoded by Ymr032w. [4] Hof1p possesses a C-terminal Src Homology 3 (SH3) domain, which binds target sequences in other proteins, which typically contain proline residues. In yeast, Hof1p normally functions in cytokinesis and interacts with the WIP homologue, Vrp1p. [4], [5] One aim of CI Munn’s research group is to show that in the absence of the Vrp1p, or if the proline rich domain in WIP, the so called Hof One Trap (HOT) Domain, is mutated, the SH3 domain of the Hof1p protein is no longer able to bind to the Vrp1p and instead binds to the proline-rich proteins Las17p proteins and formin proteins. Las17p activates the Arp2/3 complex which nucleates the assembly of branched actin filaments and formin proteins are involved in the assembly of linear actin filaments. [1], [2] Binding of the SH3 to Vrp1p may inhibit the polymerisation of linear filaments. One aim of CI Munn’s research group is to analyse the possibility that the formin protein Bnr1p is a target of the Hof1p SH3 domain in the absence of the Vrp1p.

![Figure 1: The hypothesis how Hof1p SH3 inhibits cell proliferation](image)

1. Hof1p SH3 domain is bound to yeast WIP HOT Domain.

st WIP or mutated HOT Domain; Hof1p SH3 can no longer bind WIP and instead binds to proline rich sequences in Arp2/3 complex and Formins, required for the actin cytoskeleton polymerization, and inhibits them.

**Formins**

Formins, formerly known as formin homology proteins, are a group of multidomain proteins involved in interactions with a variety of proteins. For instance, they participate in the actin cytoskeleton filament polymerisation, by associating with the fast growing (+)end of linear actin filaments, and contribute to the cortical actin cytoskeleton network, which plays an important role in various cell functions as endocytosis, cellular uptake of extracellular and
Formins are characterised by the presence of three formin homology domains, FH1, FH2 and FH3. The FH1(formin homology 1) domain contains polyproline motifs that mediate binding to profilin, which in turn binds actin monomers. The FH1 domain is known to mediate interactions with a variety of proteins, including the actin-binding protein profilin and SH3 domain proteins.[3]

One formin protein is the yeast protein Bnr1p, Bni1p-Related Protein, encoded by Saccharomyces cerevisiae. Bnr1p nucleates the formation of linear actin filaments and is involved in cell processes such as budding and mitotic spindle orientation which require the formation of polarized actin cables. Formins play a key role in limb development in vertebrates during embryogenesis. [6]

The hypothesis is that the Hof1p SH3 domain targets the FH1 domain in Bnr1p, in the absence of the Vrp1p, and thereby inhibits the cell proliferation and results in other phenotypes. Even though these studies are being performed with yeast as a model organism, it is not known if the human homologue of Hof1p, PSTPIP1, binds via its SH3 domain to formin-homology proteins in humans. The interaction of the PSTPIP1 SH3 domain with human WASP has been reported though. It is not known if mutation of WASP to prevent PSTPIP1 SH3 binding would cause Wiskott-Aldrich Syndrome. What is known is that a deletion of the proline-rich domain of WASP does cause Wiskott-Aldrich Syndrome in mice. [9]

Previous studies has been performed, both [4] and in Cl Munn’s lab[1], [2]. showing that FH1 domain (768-850 aa) of Bnr1p does interact with the Hof1p SH3 domain. By dividing the so called “Bnr1p Full Interacting Fragment” (755-1375aa), which include the FH1 domain, into six overlapping sub fragments, it was also determined that Fragment 1 (755-905 aa), F1, of the Full Interacting Fragment, is the region interacting with the SH3 domain. [7] The aim of this study is to find out which part of F1 interacts with the Hof1p SH3 domain more specifically.
Figure 2: Bnr1p

Figure 3: Bnr1p Fragment 1 sub fragments F1-(F1-F5)
**Approach: Two-Hybrid System**

The approach that was used in this study to investigate the interaction between the Bnr1p FH1 domain and the Hof1p SH3 domain, was by demonstrating the interaction in a Yeast Two-Hybrid Interaction test.

The Two-Hybrid System is a powerful system for accomplishing a demonstration of a protein-protein interaction. The system relies on the fact that the protein sequence of a transcription factor can be divided into two separate domains, possessing different functions. One domain is DNA-binding (BD) that binds to the promoter region, and the other domain is transcription-activating (AD) that allows activation of transcription. In order for transcription to occur, the AD and BD need to be in close proximity to each other. In this study, the Gal4p based yeast two-hybrid system was used. This system uses two expression vectors, one that encodes for the Gal4p-BD and the other for Gal4p-AD. The coding sequence of the two proteins of interest will be inserted into these vectors and will be expressed in the yeast nucleus fused to the Gal4p-BD or Gal4p-AD, respectively. Upon interaction between the proteins of interest, the AD and BD is expected to come close and transcription can start due to the binding of BD to the Upstream Activation Sequence, an enhancer to which Gal4p specifically binds to activate gene transcription.

![Figure 4: PoI1 with Gal4 Activation Domain and PoI2 with Gal4 Binding Domain.](image1)

![Figure 5: Upon interaction between PoI1 (Bnr1p FH1 domain) and PoI2 (Hof1p SH3 domain), AD and BD come close and transcription can occur.](image2)

Interaction between the proteins of interest reconstitutes the Gal4p transcription factor and enables the yeast cells to transcribe a reporter gene. This gene allows the yeast cells to grow on selective media lacking histidine.
Yeast Two-Hybrid Interaction Test of Fragment 1 sub fragments

To accomplish the aim of the study, to investigate which part of Bnr1p FH1 domain Fragment 1 interacts with Hof1p SH3 domain, F1 was divided into five overlapping sub fragments. When this study started, the respective Bnr1p FH1subfragments, F1-F1, F1-F2, F1-F3, F1-F4 and F1-F5, had already been inserted into the yeast two-hybrid vector pAS2-1 using standard cloning techniques. These plasmids were harboured in E. coli strain JM109. After transformation into yeast cells, these fragments will be expressed fused to the GAL4 BD. The Hof1p protein, containing the interacting SH3 domain, was constructed to be fused to the GAL4 AD of the pACT2 vector. This plasmid is called the pAM419. Yeast cells expressing the Hof1pSH3 domain were each transformed with a subfragment of Bnr1p in the pAS2-1 vector. Then, the Y2H test was performed with each of these five yeast strains.

Each Y2H vector carries a gene coding for one of the essential amino acids, leucine (pACT2) and tryptophan (pAS2-1), respectively.

To control that the Yeast Two-Hybrid plasmid Transformation was successful and respective yeast cell now contained a Y2H BD and AD plasmid, the transformed yeast cells were plated on selective media lacking Leucine and Tryptophan. If the transformation had been successful, the yeast cells were now able to grow on these selective plates.

The Yeast Two-Hybrid Interaction test was also performed with negative controls, to ensure the plates worked correctly. A Yeast Two-Hybrid plasmid Transformation of the combination of pAM419 plasmid and pAS2-1 empty vector as well as Bnr1p fragments in the combination of pAM419 plasmid or pACT2 vector and pAS2-1-based plasmids expressing each Bnr1p fragment or pAS2-1 vector were conducted

Yeast Two-Hybrid Interaction Test of Fragment 4 and Fragment 5

Like mentioned earlier, the whole “Bnr1p Full Interacting Fragment” (755-1375aa) has been investigated in previous studies in CI Munn's lab with the intention to explore which part interacts with SH3. The conclusion was that F1 interact with SH3, which has been investigated even further in this study to find out more specifically where the interaction occurs. Though, there was some doubts about the results obtained from Fragment 4 (F4) (1052 - 1202) and Fragment 5 (F5)(1152 - 1302). Therefore, the Yeast Two-Hybrid
Interaction test was conducted again for these fragments.

Materials and Methods

Methods

Plasmid preparation
Growth of the bacterial culture
Harvesting and lysis of the bacteria
Purification of plasmid DNA

Yeast Transformation
Lazy Bones

Yeast Two-Hybrid Interaction Test
Spotting

Materials

Colony Cracking PCR
- 10 x ThermoPol Buffer: New England Biosciences
  
  New England Biolabs: 240 County Road, Ipswich, MA 01938-2723
- 10mM dNTP mixture (dGTP, dATP, dCTP, dTTP)

Fisher Biotech: 198 Cambridge Street, Wembley WA 6014, Au
- Forward primer (20 μM)

Geneworks: GeneWorks Pty Ltd, PO Box 299, Hindmarsh SA 5007, Australia
- Reverse primer (20 μM)

Geneworks: GeneWorks Pty Ltd, PO Box 299, Hindmarsh SA 5007, Australia
- Taq Polymerase: DNA Polymerase with ThermoPol Buffer: New England Biosciences
  
  New England Biolabs: 240 County Road, Ipswich, MA 01938-2723

Agarose Gel Electrophoresis
- DNA Grade Agarose

PROGEN Biosciences: Unit 3/121 Kerry Rd Archerfield, QLD 4108
- Ethidium Bromide  
  Sigma-Aldrich: PO Box 14508, St. Louis, MO 63178, US
- TAE Buffer  
  - Tris base; Bio-Rad Laboratories Headquarters: 1000 Alfred Nobel Drive, Hercules, CA 4547, US
  - Acetic Acid; Chem Supply: 38 - 50 Bedford Street GILLMAN, SA 5013
  - EDTA:
- Orange G Dye  
  Sigma-Aldrich: PO Box 14508, St. Louis, MO 63178, US
- Loading Buffer
- 1Kb Plus DNA Ladder  
  Invitrogen: 11 Sandringham Ave, Thornton, NSW 2322
- Alpha Innotech, Fluour Chem FC2  
  Protein Simple: 3040 Oakmead Village Drive, Santa Clara, California, 95051 US

**Overnight culture**
- LB Media  
  Tryptone and Yeast Extract: Oxoid Limited: Wade Road, Basingstoke Hampshire, RG24 8PW, UK
- Ampicilin  
  Amresco LLC: 86681 Cochran Road, Solon, OH, 44139 US

**DNA Mini Preparation**
Promega Corporation: 2800 Woods Hollow Road, Madison, WI 53711 USA

**Check Plasmid Yield**
NanoDrop ND-1000 Spectrophotometer  
Thermo Fisher Scientific: NanoDrop products, 3411 Silverside Rd, Bancroft Building, Wilmington, DE 19810, USA

**Preparation for DNA Sequencing:** DNA-PCR and DNA-PCR Clean-Up  
**DNA-PCR**
GUDSF Sequencing Protocol
DNA-PCR Clean-Up
GUDSF Sequencing Protocol: Ethanol/EDTA Precipitation

DNA Sequencing
Applied Biosystems 3130xl Genetic Analyser

Bioinformatic Analysis
Finch TV
Geospiza, Inc.: 100 West Harrison, North Tower, Suite #330, Seattle, WA 98119

Restriction enzymes
- EcoRI-HF
  New England Biolabs: 240 County Road, Ipswich, MA 01938-2723
- Ncol-HF
  New England Biolabs: 240 County Road, Ipswich, MA 01938-2723

Spotting
1 x PBS (Phosphate-Buffered Saline)

Other
Milli-Q water (purified water by filtration and deionization)
Millipore Corporation: EMD Millipore Headquarters, 290 Concord Road, Billerica, MA 01821

Nutrient supplements which is by leaving them out used for selection
Uracil, Methionine, Leucin, Tryptophan, Adenine, Histidine.
Results

The first step in the research process was to determine which bacteria carried the correct plasmid. The aim was to test if the DNA inserts had been cloned into the desired vector (pAS2-1). This was performed by lysing the E. coli bacteria containing (or transformed with) the plasmids generated in the ligation reaction. This was done using a procedure called 'Colony Cracking'. See the 'Colony Cracking for PCR' in the Methods section.

Colony Cracking PCR was performed on the crude bacterial lysates prepared as described above using the same primers used initially to amplify the desired fragments by PCR (i.e. prior to the ligation reaction). See the 'Colony Cracking PCR' in the Methods section. Those bacterial transformants used to prepare those crude lysates from which it was possible to amplify the desired DNA fragments were determined to be the transformants harbouring plasmids with the desired insert.

To control that the PCR reaction had occurred and the correct fragments had been amplified, the PCR reaction product was subjected to agarose electrophoresis gel, which separates different DNA fragments according to size. See the 'Agarose Gel Electrophoresis' in the Methods section.

A 2% agarose gel containing 0.5ug/ml ethidium bromide (a DNA stain) was used, in order to provide optimal separation of the expected DNA fragments of about 100 base pairs. Plasmid clones expressing F1-(F1-F5) subfragments were chosen by subjecting the colony cracking PCR amplification products to agarose gel electrophoresis. The Bnr1p Full Interacting Fragment” (755-1375aa) was divided into six overlapping fragments, around 100 amino acids in size. Fragment 1 was thereafter divided up into five overlapping subfragments of about 30 amino acids, each with around 20 amino acids of unique sequence. This corresponds to a DNA insert of about 90 base pairs. Together with oligonucleotide primers and restriction enzyme cleavage sites, the gel electrophoresis bands corresponding to DNA sequences encoding each of the Bnr1p subfragments were predicted to be around 100 base pairs in size. Those bacterial transformants used to prepare those crude lysates from which it was possible to amplify these positive bands of around 100 base pairs were picked for extraction of purified plasmid DNA. The purified plasmid DNA from those transformants was subjected to DNA sequence analysis in order to further confirm the sequence of the insert DNA carried by these plasmids.
To obtain a larger culture of bacterial cells harbouring the plasmid of interest and therefore a better yield of the plasmid, the E. coli bacteria used to prepare those crude lysates that after PCR amplification produced positive bands on the agarose electrophoresis gel, were picked from the agar plates and cultured overnight. See the “Overnight Culture of positive isolates” in the Methods section.

**DNA PCR and Clean-Up**

**Plasmid DNA Mini Preparation**
A so called “DNA Mini Preparation” procedure was performed to extract plasmid DNA from the E.coli overnight culture, using a Promega Wizard Mini Prep Plasmid DNA Purification kit. The protocol has four steps: Production of Cleared Lysate, Binding of Plasmid DNA, Washing and Elution. The cells were lysed under alkaline conditions, which denatures both nucleic acids and proteins. When the solution was then neutralized, chromosomal DNA and proteins precipitated because it is impossible for them to renature correctly.

**Check Plasmid DNA Yield**
To ensure the plasmid DNA yield was sufficient for use as a template in PCR reactions, the plasmid DNA yield was checked using a 'Nanodrop Spectrophotometer' from Thermo Fisher Scientific.

**Purification of plasmid DNA**

**Plasmid DNA preparation for DNA Sequencing**

**Sequencing PCR reactions**
In the 'DNA Mini Preparation' procedure, the plasmid DNA is purified according to a protocol chosen based on the desired plasmid DNA yield. In this case, the desired yield should be between 150 and 300 ng. This is 2X the amount of template DNA needed for one sequencing PCR reaction

The individual single strands of the template DNA were amplified by performing separate PCR reactions using the forward and reverse oligonucleotide primers. This enhanced the upcoming sequencing and also facilitated the later bioinformatic analysis. When later analysing the DNA sequences, comparison between the published sequence and the sequence determined here is performed. Possessing the sequence of both strands of the insert DNA in the template instead of only the sequence of one strand for comparison with
the published sequence facilitates the analysis and makes verification of the insert DNA sequence more reliable.

**DNA Clean-up after PCR**

An alcohol precipitation procedure was used to concentrate the DNA and to remove any unincorporated dye-conjugated deoxynucleotides after the enzymatic reactions. The ethanol causes the DNA to precipitate and the waste material can be removed with the supernatant before the sample gets sent away for sequencing.

**DNA Sequence Determination**

Separation and analysis of the DNA sequencing reaction products was performed by Griffith University DNA Sequencing Facility, using a 16-capillary, automated electrophoresis instrument.

**Bioinformatic Analysis**

In order to ensure that the BNR1 fragments had been inserted into the vectors, a bioinformatic analysis was performed. The published sequence of the BNR1 Coding DNA sequence (CDS) was obtained online from the Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org).

The restriction enzyme sites used for cloning into the pAS2-1 vector multiple cloning site (MCS) were first identified in the determined sequence as they represent the boundaries of the insert DNA. Then the insert nucleotide and the predicted translated amino acid sequences were aligned to and compared with the published BNR1 and Bnr1p sequences using the BLAST algorithm. Also, the reading frame was checked to ensure the sequence of GAL4 in the pAS2-1 vector (encoding the Gal4p DNA-binding domain) read in-frame into the insert BNR1 sequence such that upon translation a Gal4-Bnr1p fusion protein would be produced.

**Yeast Transformation with plasmid DNA**

**Lazy Bones**

Lithium acetate transformation using the 'Lazy Bones' protocol. After transformation, the yeast cells will be able to grow on SD Leu-, Trp- plates, which verifies that the transformation
has occurred, since the pACT2 vector contains a gene for producing leucine (LEU2) and the pAS2-1 vector contains a gene to produce tryptophan (TRP1).

**Yeast Two-Hybrid Interaction Test**

**Spotting**
To determine if any of the plasmid pairs conferred protein interaction, a yeast two-hybrid test was performed. The test was executed by preparing serial dilutions of each yeast [or PJ69-4a] double transformant strain and thereafter applying 5uL [or 7uL] of each of these dilutions to the surface of SD His- plates for testing growth in the absence of histidine.

The yeast two-hybrid reporter strain, PJ69-4a, containing one member of each of two different sets of plasmids, one based on the vector pACT2 and one based on the vector pAS2-1, was used. Three different control yeast strains were made, two negative control strains and one positive control strain.

**Control 1: pAM419 + empty vector pAS2-1**
Transfer the pAM419 plasmid encoding the Gal4p transcription activation domain fused to the Hof1p SH3 domain (residues 501-669/end), into a yeast strain already transformed with the pAS2-1 empty vector, encoding the Gal4p DNA-binding domain.

Growth of this double transformant on SD His- plates is not expected, since the Bnr1p FH1 domain is not encoded by the pAS2-1 empty vector for the Hof1p SH3 domain encoded by pAM419 to interact with, and therefore the transcription activation and DNA-binding domains will not come together to initiate transcription of the HIS3 two-hybrid reporter gene. Growth will only occur when histidine is present in the medium as nutrition for the yeast cells.

**Control 2: pAS2-1 + empty vector pACT2**
Transfer the pAS2-1 plasmid encoding the Gal4p DNA-binding domain fused to respective Bnr1p fragment F1 subfragments F1-F1, F1-F2, F1-F3, F1-F4, F1-F5, into a yeast strain already transformed with the pACT2 empty vector, encoding the Gal4p transcription activation domain.

Growth on SD His- plates is not expected either for this control strain, since the Hof1p SH3 domain is not encoded by the pACT2 empty vector for the Bnr1p fragment F1 subfragments to interact with.
Positive Control: pAS2-1 clone expressing the Bnr1p [full interacting region] + pAM419 encoding the Hof1p SH3 domain (residues 501-669/end)

Transfer the pAS2-1 clone expressing the Full Interacting Region of Bnr1p into a yeast strain already transformed with the pAM419 plasmid encoding the Hof1p SH3 domain (residues 501-669/end). Upon interaction between the Bnr1p Full Interacting Region and the Hof1p SH3 domain, the DNA-binding domain (BD) and transcription activation domain (AD) will come close and initiate transcription of the HIS3 two-hybrid interaction reporter gene. Now, the yeast cells will grow on SD His- plates which indicates that interaction between Bnr1p and Hof1p SH3 domain has occurred, which was the aim of the project to demonstrate.

Gel Electrophoresis

The gel images below show more than one PCR-positive isolate for each plasmid being constructed. The arrows show the PCR products (around 100bp) the presence of which identifies the colony as harbouring a potential positive plasmid clone to be purified and subjected to confirmation by DNA sequence analysis.

Colony Cracking PCR was performed on several independent E.coli transformant colonies picked at random from the LB Amp plate following electroporation with the pAS2-1-BNR1 F1-F1 ligation mix. Samples of 10 micro litres of the PCR reaction mix were made up in gel loading buffer and loaded on a 2% agarose gel containing 0.5ug/ml ethidium bromide (fluorescent DNA stain) together with 1kb Plus ladder in a separate well. The DNA fragments in each sample were then separated by electrophoresis at 90 V for 40 minutes. Then the ethidium bromide stained DNA fragments were visualised using an Alpha Innotech Fluor Chem FC2 UV transilluminator and image capture system.
Colony Cracking PCR was performed on several independent E. coli transformant colonies picked at random from the LB Amp plate following electroporation with the pAS2-1-BNR1 F1-F2 and F1-F4 ligation mixes. Samples of 10 micro litres of the PCR reaction mix were made up in gel loading buffer and loaded on a 2% agarose gel containing 0.5μg/ml ethidium bromide (fluorescent DNA stain) together with 1kb Plus ladder in a separate well. The DNA fragments in each sample were then separated by electrophoresis at 90 V for 40 minutes. Then the ethidium bromide stained DNA fragments were visualised using an Alpha Innotech Fluor Chem FC2 UV transilluminator and image capture system.

Figure 6: F1-F1 (isolate 4)

Figure 7: F1-F2(isolate 4) and F1-F4(isolate 3)
Colony Cracking PCR was performed on several independent E. coli transformant colonies picked at random from the LB Amp plate following electroporation with the pAS2-1-BNR1 F1-F5 ligation mix. Samples of 10 micro litres of the PCR reaction mix were made up in gel loading buffer and loaded on a 2% agarose gel containing 0.5ug/ml ethidium bromide (fluorescent DNA stain) together with 1kb Plus ladder in a separate well. The DNA fragments in each sample were then separated by electrophoresis at 90 V for 40 minutes. Then the ethidium bromide stained DNA fragments were visualised using an Alpha Innotech Fluor Chem FC2 UV transilluminator and image capture system.
Yeast Two-Hybrid Interaction Test

The following figures show the result from the yeast two-hybrid interaction test performed by spotting yeast double transformants carrying one pAS2-1-based plasmid and one pACT2-based plasmid on SD His+ and SD His- plates. If interaction occurs, the yeast cells are expected to grow on SD His- plates. The SD His+ plates are a control to ensure that any lack of growth of the same double transformant on SD His- is due to an inability to synthesise histidine (due to lack of expression of the HIS3 two-hybrid interaction reporter gene).

The first figure below (Fig. 11) shows the yeast two-hybrid interaction spotting result of three control samples. Row 1 shows pAM419 + empty pACT2 and does not show any interaction, which is the expected result. Rows 2 and 3 demonstrate the results for positive controls provided by the manufacturer which are both expected to give a positive result, which they do.

The first illustration below shows the spotting result of three control samples. Row 1 shows pAM419 + pACT2 and do not show any interaction, which is according to expected result. Row 2 and 3 demonstrates positive control samples which were both expected to interact, which they do.
Empty pACT2 vector + pAS2-1 clones expressing Bnr1p F1 fragment F1-F5 subfragments

The spotting of the yeast double transformants harbouring the pACT2 empty vector and the pAS2-1-based plasmids encoding Bnr1p fragment F1 subfragments shows no interaction on the SD His- plate as expected.

Figure 10: SD His
Panel A: His-  Panel B: His+
Row 1: pAM419 + empty pACT2
Row 2: pTD1 (human tumour suppressor protein p53) + pVA3 (SV40 [simian virus 40] large T antigen)
Row 3: pCL1 (full-length Gal4p) + pGBT9 (empty vector)

Figure 11: Empty pACT2 vector + pAS2-1 clones expressing Bnr1p F1 fragment F1-F5 subfragments
Panel A: His-  Panel B: His+
Empty pACT2 vector + pAS2-1-based plasmids encoding Bnr1p Fragment 1 F1-F5 subfragments
pAM419 encoding Hof1p SH3 domain (residues 501-669/end) + pAS2-1-based plasmids encoding Bnr1p Fragment 1 F1-F5 subfragments

The figures below demonstrate the results of two-hybrid interaction spotting of yeast double transformants harbouring the pAM419 plasmid encoding the Hof1p SH3 domain (residues 501-669/end) and each of the pAS2-1-based plasmids encoding Bnr1p Fragment 1 subfragments F1-F5. This is the yeast two-hybrid interaction final test in this study aiming to map the interaction site for the Hof1p SH3 domain within the Bnr1p FH1 domain. However, the yeast double transformants did not show any growth on the SD His- plate.

**Figure 12:** pAM419 encoding Hof1p SH3 domain (residues 501-669/end) + pAS2-1-based plasmids encoding Bnr1p Fragment 1 F1-F5 subfragments

Panel A: His-  Panel B: His+

pAM419 encoding Hof1p SH3 domain (residues 501-669/end) + pAS2-1-based plasmids encoding Bnr1p Fragment 1 F1-F5 subfragments

Control: Empty pACT2 vector, pAS2-1-based plasmid encoding Bnr1p Full Interacting Region and pAS2-1-based plasmid encoding Bnr1p Fragment 1

Since the Bnr1p Fragment 1 subfragments F1-F5 did not interact with the Hof1p SH3 domain, a control experiment was performed to confirm that the Bnr1p Full Interacting Region (Fig. 2) and Fragment 1 (Fig. 2) do interact with the Hof1p SH3 domain as previously shown [7]. However, yeast double transformants expressing these fragments do not show significant growth on SD His- and therefore do not demonstrate interaction of the Hof1p SH3 domain with the Bnr1p Full Interacting Region or the Bnr1p Fragment F1.
Figure 13: Empty pACT2 vector, pAS2-1-based plasmid encoding Bnr1p Full Interacting Region and pAS2-1-based plasmid encoding Bnr1p Fragment 1

Panel A: His-  Panel B: His+

Row 1: empty pACT2 + empty pAS2-1

Row 2: empty pACT2 + pAS2-1-based plasmid encoding Bnr1p Full Interacting Region

Row 3: empty pACT2 + pAS2-1-based plasmid encoding Bnr1p Fragment 1

Row 4: pAM419 encoding Hof1p SH3 domain (residues 501-669/end) + empty pAS2-1

Row 5: pAM419 encoding Hof1p SH3 domain (residues 501-669/end) + pAS2-1-based plasmid encoding Bnr1p Full Interacting Region

Row 6: pAM419 encoding Hof1p SH3 domain (residues 501-669/end) + pAS2-1-based plasmid encoding Bnr1p Fragment 1
Discussion

The hypothesis says the interaction of Hof1p SH3 with Bnr1p becomes inhibitory to cell growth when the WIP homologue VRP1 is not expressed. However, the yeast two-hybrid strain that was used in this study does express Vrp1p. So any interaction seen in the yeast two-hybrid strain would occur in the presence of Vrp1p. This interaction might not inhibit the growth of the yeast two-hybrid strain though. If the VRP1 gene, that encodes Vrp1p in the yeast two-hybrid strain, was deleted, the yeast two-hybrid interaction between the Hof1p SH3 domain and Bnr1p may get stronger and we may see inhibition of cell growth.

This study was based on previous studies [4], demonstrating an interaction between the Hof1p SH3 domain and Fragment 1 of Bnr1p Full Interacting Fragment. The spotting of yeast double transformants expressing the Bnr1p Full Interacting Fragment together with the Hof1p SH3 domain and Bnr1p Fragment 1 together with the Hof1p SH3 domain (Fig. 13), do not show any interaction between the proteins. This can be due to some time constraints of the growth. If the plates are left for some additional incubation time, they may show some interaction. The lack of growth of the yeast double transformants expressing the Bnr1p Full Interacting Region together with the Hof1p SH3 domain and Bnr1p Fragment 1 together with the Hof1p SH3 domain do raise some doubts about earlier studies.

The aim of this study was to map more specifically the potential binding site for the SH3 domain within the Bnr1p FH1 domain. The investigation of Fragment 1 performed in this study is also based on earlier studies, conducted in Dr. Alan Munn’s laboratory. The Yeast Two Hybrid Interaction Test of pAM419 (encoding Hof1p SH3 domain (residues 501-669/end) and Bnr1p F1-(F1-F5) (Fig. 13) does not show any growth of the yeast double transformants expressing the Hof1p SH3 domain in combination with any of the Bnr1p Fragment 1 subfragments F1-F5. The lack of growth can be due to various reasons. It can be due to some factors, affecting the growth of the cells, such as lack of nutrition media for example. Also these plates have been left for further incubation to see if growth becomes apparent after longer times of incubation.

Another reason for the lack of growth can be that the inserts F1-(F1-F5) are too short. The inserts, which are only around 20 amino acids (see Gel Electrophoresis in Methods), are fused to the GAL4-BD(5505-5944) [8] with a large protein fold. Potentially, this protein fold
could have been folded the Bnr1p subfragments, causing the binding site of Bnr1p not to be exposed to the SH3 from a sterical point of view.

A possible alternative strategy to detect an interaction between the proteins of interest would be to permutate the vectors by inserting the sequences encoding the Bnr1p fragments into the pACT2 vector and sequences encoding the Hof1p SH3 domain into the pAS2-1 vector. The reason for this is that an interaction is sometimes only detected in the yeast two-hybrid system when one fragment is fused to the Gal4p DNA-binding domain and the other fragment is fused to the Gal4p transcription activation domain, and not vice versa. The reasons for this are not usually understood.

Also, a different reporter gene can be used to detect the protein interaction. In this study, the PJ69-4A yeast strain [10] expressing the Gal4p-BD and Gal4p-AD and the histidine (HIS3) two-hybrid interaction reporter gene has been used. The histidine reporter gene is more sensitive to detect interaction. Instead, the adenine two-hybrid interaction reporter gene (ADE2) could be used, which is more stringent in its activation. Activation of the adenine reporter gene requires higher Gal4p activity than the histidine reporter gene.

Another option to possibly be able to detect an interaction, could be to redo the spotting with some of the other yeast transformants. This would address the possibility that the specific double transformant colonies tested for interaction may not have been representative of the double transformants as a whole.

There are some limitations of this study. The protein interaction was only tested using one assay, the yeast two-hybrid. Also, the interactions were only tested in one orientation, i.e. permuted vectors were not used. Only one double transformant colony tested for each combination of plasmids. As well, the experiment, e.g. the transformations, were only done once, no replicates were made.

The future outcome knowledge of this research project will hopefully be able to be applied in order to develop gene therapeutic treatment for Wiskott-Aldrichs syndrome among other diseases, that are caused by the pathological protein interaction.
One therapeutic method would for example be to add more WIP to a patient who are at risk for developing these diseases, due to it is believed that interaction occur in the absence of WIP.

Even though the outcome of this study and the yeast two-hybrid interaction test of the Hof1p SH3 domain and respective Bnr1p subfragments was not as hoped for (i.e. that interaction occurred) this study has still been helpful in CI Munn's research project. This study is questioning previous published studies.
References


[9] (Badour et al. 2003)

required for coupling T cell antigen receptor engagement to WASp effector function and T cell activation. Mount Sinai Hospital, Toronto, Ontario, Canada. 199:99-112

Appendix – Description of workplace

This research internship was conducted at Dr. Alan Munn’s laboratory at the School of Medical Science at Griffith University, Gold Coast, Australia. We were seven persons included in the research group, Professor Alan Munn, the PhD students Heike Mach and Caroline, the honour students Anh and Luca, Michael and myself.

The group is working at different aspects of the research project, with the overall aim to gain insight of the WASp and WIP function.

During my research training I was supervised by Dr. Alan Munn and co-supervised in the laboratory by the PhD student Heike Mach.

I got to learn various biological laboratory techniques to extract and amplify DNA, and also the technique to investigate protein-protein interaction.

My practical and theoretical knowledge about yeast cells and approaches to explore interactions has improved significantly. I have also gained experience in an administrative perspective, how to arrange and plan this type of recruitment and also how to function in a team at a workplace. The internship has been a great experience, both on a personal and professional level.