Non – Splicing Function of Ubiquitin - Like Protein Hub1

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CERTIFICATE

This is to certify that the dissertation titled "Non-Splicing Function of Ubiquitin-Like Protein Hub1" submitted by Mr Deepak Saroha (Reg. No. MS09045) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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Dated: April 25, 2014

DECLARATION

The work presented in this dissertation has been carried out by me under the guidance of Dr. Shravan Kumar Mishra at the Indian Institute of Science Education and Research (IISER), Mohali. This work has not been submitted in part or in full for any degree, diploma, or fellowship to any other university or institute. Whenever contributions of others are involved, I have made every effort to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Deepak Saroha

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Dated: April 25, 2014

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr Shravan Kumar Mishra

(Supervisor)

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ABSTRACT

Ubiquitin – like protein Hub1 is an essential gene in *Schizosaccharomyces pombe* (yeast) which is required for the splice – site usage and alternative splicing. Hub1 non-covalently binds to Snu66 in yeast and mammals and to Prp38 in plants via a conserved HIND domain. Hub1's nuclear localisation is linked with RNA splicing, where it is a part of the tri-snRNP complex and in cytosol Hub1 is found to interact with Fumarase. Fumarase is an enzyme that is involved in Tricarboxylic Acid Cycle and in metabolism of amino acids. Fumarase deficiency is linked with a number of diseases. Addition of charged amino acids (aspartic acid and lysine) to the C-terminal of Hub1, leads to a loss of function in *Δhub1 S. pombe* strains, however, addition of uncharged amino acid (glycine), complements growth of *S. pombe* in *Δhub1* strains. This study indicates that which C-terminal extensions of Hub1 are tolerated by *S. pombe* and the significance of Hub1 interaction with Fumarase.

CHAPTER 1

INTRODUCTION

1.1 General Information

Ubiquitin is a small protein (8.5 kDa) which is 76 amino acid long and is found in almost all tissues of eukaryotic organisms. It was first discovered in 1975 by Goldstein (Goldstein G. *et al*, 1975) and its further characterization was done throughout the 1970s and 1980s (reviewed in Wilkinson KD, 2005). The key features of ubiquitin includes its extended C-terminal tail and seven lysine residues. The sequence of ubiquitin among eukaryotes is highly conserved as there is 96% sequence identity between yeast and human ubiquitin.

Ubiquitin is heat stable protein and upon heating it folds into a compact globular structure. It exists either in free form or a part of a complex with other proteins. When it is present as a complex with other proteins, it is conjugated to the protein through a covalent bond between the glycine on the C-terminal end of the ubiquitin and the lysine side chains present on the proteins. The conjugation process is ATP hydrolysis dependent.

A family of genes whose translation product are fusion proteins encodes ubiquitin. The ubiquitin genes usually exist in two states; they can be fused to a ribosomal protein gene resulting in a translation product that is an ubiquitin-ribosomal fusion protein or they can be present as a polyubiquitin molecule, the translation product is made of a linear chain of ubiquitin molecules fused together. Following synthesis of fusion proteins, another protein called ubiquitin-C terminal hydrolase cleaves the fusion proteins at the C-terminal end of ubiquitin. This synthesis either generates an individual ubiquitin or generates a set of ubiquitin monomers from the polyubiquitin.

1.2 Ubiquitin Function

The degradation of proteins, over time is called protein-turnover. Based on protein-turnover, proteins are either short-lived or long-lived. Short-lived proteins are typically key regulators and they are partially unfolded and are prone to degradation. The regulation of protein turnover in a cell is managed by ubiquitin and it does so by regulating the

degradation of specific proteins. A protein which is performing another function is henceforth degraded by cells via regulation of protein degradation.

Ubiquitination is a post-translational modification during which ubiquitin gets attached to a substrate protein, a process called conjugation. This conjugation of ubiquitin to a substrate can affect protein in different ways: either the protein is degraded via proteasome or protein localization is altered or protein activity is affected or protein interactions are altered (Glickman MH. *et al*, 2002).

Ubiquitin is involved in many cell processes. Such as, it plays an important role in regulating the cell cycle by conjugating to the protein cyclin during the G1 phase of mitosis. This conjugation of ubiquitin to other proteins is also involved in DNA repair, embryogenesis, the regulation of transcription, and apoptosis.

1.3 Ubiquitin – Proteasome Pathway

The conjugation of ubiquitin to lysine residues within substrate proteins and targeting them for degradation by the proteasome, a large multicatalytic proteinase complex is the most prominent function that is exhibited by the ubiquitin–proteasome system (UPS) is [Navon A. *et al*, 2009]. Ubiquitin function depends on ATP. The specificity of the target proteins that needs to be degraded depends on the utilization of ATP. The proteins are not degraded by ubiquitin itself, it merely acts only as a tag that marks proteins for degradation and then the 26S proteasome performs the degradation. In short, proteins which needs to be degraded are first tagged with ubiquitin by conjugating them and this tag serves as a signal through which proteins are then recognized and transported to the proteasome for their degradation.

Three enzymes i.e. the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and the ubiquitin ligase (E3) are involved in the conjugation of the ubiquitin protein to a substrate (figure 1.1). First, a thioester bond is formed with the C-terminus of ubiquitin in an ATP-dependent manner by the E1 enzyme and the ubiquitin is then transferred to E2

enzyme. Then the ubiquitin is either transferred to the E3 by E2 enzyme or E2 binds to E3 enzyme and transfers the ubiquitin directly to the substrate. Therefore, substrate specificity is determined by the E3 enzyme in either case. This process of protein-substrate conjugation is repeated several times so that multiple ubiquitin molecules can conjugate to the substrate, a key process for a protein that will be degraded by the 26S proteasome [Wilkinson K.D. *et al*, 2000].

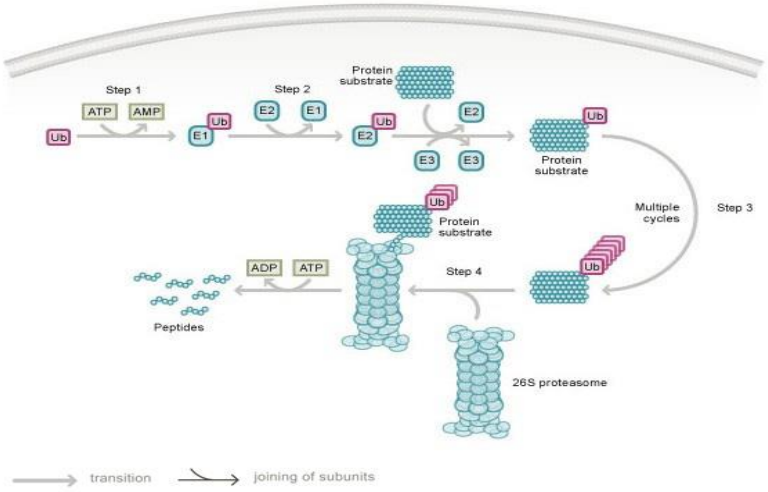


Image courtesy: http://flipper.diff.org/app/pathways/1798

Figure 1.1: The Ubiquitin Pathway

1.4 RNA Splicing

Five small nuclear RNP (snRNPs) and several non-snRNP factors constitute the large macromolecular machine called spliceosome. A specific set of interacting proteins make up the uridine-rich snRNAs, i.e. U1, U2, U4, U5 or U6. The mature mRNA by excision of the introns is produced when the snRNPs assemble dynamically on the pre-mRNA along with the several other non-snRNP protein factors and they catalyse the two transesterification reactions. First, the intron is recognized by the U1 snRNP by base-pairing of the 5′ splice site within the intron with the U1 snRNA. Second, complex A is formed by the interaction of U2 snRNP with the conserved branch-point sequence. A protruding conserved intronic adenosine residue is required for the first step of splicing called the branch-point sequence with which the U2 snRNA hybridizes with. The 5′ splice

site is attacked during the first transesterification reaction by this branch point adenosine which serves as the nucleophile. Complex B is formed when a preformed tri-snRNP complex composed of the U4, U5 and U6 snRNPs joins complex A. For the formation of the active spliceosome (B_{act}), the interaction between U4 and U6 is unwound, and the U6 snRNA forms two new associations: First it binds with the U2 snRNA and second it binds to 5′ splice site, which results in the destabilization of U1 and U4. Then the nucleophilic attack is performed by the branch-point adenosine on the first nucleotide of the intron at the 5′ splice site forming the lariat intermediate. In this manner, several rearrangements occur in the complex that results in the formation of complex C that catalyzes the second transesterification reaction, following which, the two exons are ligated and the intron gets removed between two exons [Chanarat S. *et al*, 2013].

1.5 Ubiquitin-Like Modifiers

Despite the fact that ubiquitin is the most well understood post-translation modifier, there is a growing family of ubiquitin-like proteins (UBLs) that modify cellular targets in a pathway that is parallel to but distinct from that of ubiquitin. For example, SUMO, NEDD8, FAT10 and Hub1.

The ubiquitin-like protein modifiers (UBLs) are related to ubiquitin by a common 3D structure, the ubiquitin fold, and a C-terminal glycine residue, whose carboxyl group is the site of attachment to the lysine residue of substrates via isopeptide bond formation not so by their sequence homology. Hence, their conjugation to proteins is similar to ubiquitin and they function in “ubiquitin-like” manner. Since some proteins can become modified by more than one UBL, and sometimes even at the same lysine residue, there is cross-regulation between the various conjugation pathways. This may enhance or inhibit the binding of the protein to another molecule, can affect enzymatic activity or subcellular localization, and this ultimately determine the half-life of the protein. For instance, SUMO (Small Ubiquitin-like Modifier), often serves to stabilize protein substrates, which is completely opposite to ubiquitination. (Herrmann J. *et al*, 2007)

1.6 Ubiquitin-like protein Hub1 and its Role in Alternative RNA Splicing

Hub1 (Homologous to Ubiquitin) is an evolutionary conserved ubiquitin-like modifier (UBL), whose tertiary structure is similar to that of ubiquitin; however, the C-terminal extension which is a unique feature related to ubiquitin, which is required for covalent modification of target proteins is absent in Hub1. It does not have a C-terminal GG motif, instead, it has a very unique YY motif, followed by a non-conserved amino acid residue. Cells deficient in Hub1 are viable in *S. cerevisiae* with minor growth defects as opposed to *S. pombe* where cells deficient in Hub1 are lethal. It also binds to proteins non-covalently in an ATP independent process as compared to other ubiquitin which binds to proteins covalently in an ATP dependent manner [Luders. *et al*, 2003].

The alternative splicing of *S. cerevisiae SRC1* pre-mRNA is promoted by Hub1. Hub1 binds non-covalently to a conserved HIND domain, present on the spliceosomal protein Snu66 (part of tri-snRNP complex) in yeast and mammals, and Prp38 in plants. Hub1 is necessary for the nuclear localization of Snu66 in *S. cerevisiae* but how Snu66-Hub1 interaction affects splicing is not clear. Hub1 modifies the spliceosome by binding to Snu66 in such a way that allows it to use certain non-canonical 5' splice sites. (Mishra SK. *et al*, 2011)

When the dityrosine residues of the C-terminus of Hub1 (-LEMYYS) was mutated with different mutations like -LEMDD-, -LEMKK- and –LEMGG-, only glycine (G) was able to rescue the of *Δhub1* strain in *S. pombe*, whereas growth in aspartic acid (D) mutation was slower and there was no growth for lysine (K) mutation (Wilkinson RM. *et al*, 2004).

1.7 Fumarase (Fum1) and its Deficiency Related Diseases

Fumarase is an important enzyme of the TCA Cycle (Tricarboxylic Acid Cycle) which catalyzes the reversible hydration/dehydration of fumarate to malate. It is present in mitochondria as well as in cytosol and its mitochondrial localisation is linked with TCA cycle (Tricarboxylic Acid Cycle), whereas in cytosol it is involved in metabolism of amino acids and fumarate.

Fumarase deficiency is known to be the cause for a number of diseases, like renal cell cancer. Fumarase deficiency (Polygamist Down's), is an autosomal recessive metabolic disorder characterized by a deficiency of the enzyme fumarate hydratase, which is indicated by an increase in the fumaric acid in urine. Fumarase was surprisingly shown to underlie a tumor susceptibility syndrome, Hereditary Leiomyomatosis and Renal Cell Cancer (HLRCC) [Yogev. O. et al, 2010].

Point mutations in fumarase also contribute to a large number of diseases. The most common type of mutation is missense (57%), followed by frameshifts & nonsense (27%), and diverse deletions, insertions and duplications (Bayley JP. *et al*, 2008). Mutations in the Fumarase gene leads to the disruption in the enzyme's ability to convert fumarate to malate, interfering with the function of this reaction in the citric acid cycle.

CHAPTER 2

MATERIALS & METHODS

2.1 Polymerase Chain Reaction

PCR (Polymerase Chain Reaction) is a revolutionary technique which is used to amplify particular DNA fragment *in vitro*. PCR relies on thermal cycling, a principle which is based on cycles of repeated heating and cooling events of the reaction for DNA melting and enzymatic replication of the DNA.

PCR has three major steps, which are generally repeated for 30 or 40 cycles:

Initialization Step: This step is carried out for 1-9 minutes, in which the reaction mixture is heated to a temperature of 94-96 °C. This step is essential for the heat activation of DNA polymerase.

Denaturation Step: The reaction mixture is heated at 94-98 °C for 20-30 seconds. It is the first step of regular cycling event and it causes melting of DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

Annealing Step: The temperature of the reaction mixture is lowered to 50-65 °C for 20-40 seconds. This allows the annealing of the primers via stable DNA-DNA hydrogen bonds to the single-stranded DNA template.

Extension/Elongation Step: In this step a new DNA strand is synthesized by DNA polymerase which is complementary to the DNA template. DNA polymerase synthesizes new strand by adding dNTPs that are complementary to the template in 5’ to 3’ direction. Generally, at its optimum temperature DNA polymerase synthesizes a thousand bases per minute.

Final Elongation: This step is performed at 70-74 °C for 5-15 minutes following the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Final Hold: It is performed for infinite time at 4 °C for storing reaction short-term.

Components

Template DNA: Generally 1ng template DNA is used for 25µl reaction. All reactions performed for this study were 50µl reactions and 0.3µl template was used.

Primers: Primers that are complementary to the 3' ends of each of the sense and anti-sense strand of template DNA. Primers length are usually between 18-28 nucleotides and the GC content between 40-55% because it determines its melting temperature (Tm)

$T_m = 4(G+C) + 2(A+T)$ {for primer length < 20 nucleotides}

$T_m = (\%GC)\ 0.41 + 62.3 - [(500/\text{length of primer}) - 5]$

{for primer length > 20 nucleotides}

dNTPs: They are the building-blocks from which DNA polymerase synthesizes new DNA strand.

Buffer: It provides suitable chemical environment for activity and stability of DNA polymerase.

DNA polymerase: It synthesis new DNA strand. DNA polymerase used for this study was high fidelity Q5 Polymerase *(New England Biolabs).*

Method:

- Thaw all the required solutions (10x buffer, dNTPs, primers) before using them
- Add all the solutions accordingly for 25µl or 50µl reaction in a pcr tube
- Vortex briefly in a micro-centrifuge, so all the solutions settle down
- Then place the pcr tube in pcr machine, giving appropriate temperatures and time for different steps.

2.2 Restriction Digestion and Ligation

Restriction digestion is a process by which DNA is specifically cleaved at one or multiple sites by one or multiple enzymes. Digestion produces either blunt or staggered cuts in the DNA. For digestion of vector generally 1µg of vector is required and for insert digestion, it is first amplified by pcr and then digested.

Method:

- For Insert:
  - Amplify the insert with appropriate primers with pcr (50µl reaction)
  - Run 5µl of pcr product on agarose gel for conformation of amplification
  - Purify pcr products from remaining 45µl of pcr sample (for this study Thermo scientific Gel Extraction kit was used)
  - Digest the purified DNA by adding desired enzymes, buffer complementary to enzymes, 100x BSA
  - Incubation overnight at 37°C
  - Run on agarose gel for confirmation of digestion and Gel filtrate it (for this study Thermo scientific Gel Extraction kit was used).

- For Vector:
  - Add 1 µg of DNA vector along with desired enzymes, buffer and 10X BSA
  - Incubation for 1.5 hr
  - Add 0.5 µl CIP (Calf-Intestinal alkaline Phosphatase) and further incubation for 30 minutes
  - Heat inactivation for 10 minutes at 65°C
  - Run on agarose gel for confirmation of digestion and Gel filtrate it (for this study Thermo scientific Gel Extraction kit was used).

Ligation is a process of insertion of foreign DNA into a vector. The ends of DNA fragments are joined together by the formation of phosphodiester bonds between

3’-hydroxyl of one DNA termini with the 5’-phosphoryl of another. Then the modified vector can be transformed into a host organism and its expression can be checked.

Components: For a typical ligation reaction digested insert, digested vector, T4 ligase buffer and T4 ligase is required. 50-75ng of digested vector was used and the amount of digested insert added was calculated using following formula:
Amount of insert = Amount of vector X (size of insert/size of vector) X MR
{MR = Molar ratio of vector & insert (1:3 to 1:8)}

Method:

- Add in appropriate amount digested insert, digested vector, water,T4 ligase buffer, T4 ligase in an autoclaved micro-centrifuge tube
- Incubation at room temperature for 1-2 hrs.

2.3 Gel Purification/Extraction of DNA

For purification and extraction of DNA from agarose gels, Thermo Scientific Gene JET Gel Extraction Kit was used and the procedure is as follows:

- Add 450 µl of binding buffer (provided in the kit) to the gel slice and melt it at 55°C
- Transfer the sample to spin purification column (provided in the kit) and centrifuge at 13000 rpm for 1 minute and discard the supernatant
- Add 700 µl of wash buffer (provided in the kit) to the column and centrifuge for 1 minute at 13000 rpm and discard the supernatant
- Dry spin for additional 1 minute to remove any ethanol present in the column
- Place the column in a micro - centrifuge tube and add 15 µl of prewarmed water (at 55°C) to the column and spin it for 1 minute at 13000 rpm to elute the DNA.

***Note:** All centrifugation must be done at room temperature.

2.4 PCR Product Purification

For purification of pcr products also same Thermo Scientific's Gene JET Gel Extraction Kit was used according to the following procedure:

- Add 100 µl of binding buffer(provided in the kit) to the pcr products and transfer the products in spin purification centrifuge it for 1 minute at 13000 rpm
- Discard the supernatant and repeat above step again and discard supernatant again
- Add 500 µl of wash buffer(provided in the kit) to the column and discard the supernatant
- Repeat the above step again
- Dry spin the column to remove any ethanol left in the column
- Place the column in micro-centrifuge tube and add 15 µl prewarmed water to the column
- Centrifuge at 13000 rpm for 1 minute to elute the DNA from column.

*__Note:__ All centrifugation must be done at room temperature.

2.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a method which is used to separate a mixed population of DNA fragments based on their sizes. The percentage of agarose determines the pore size of the gel. Electric field helps to move the DNA through agarose matrix. Since, DNA is negatively charged it moves towards the positively charged electrode.

Components: Electrophoresis buffer: usually a Tris-acetate-EDTA (TAE), Loading buffer: which allows the sample to fall into the wells and generally a tracking dye which allow visual monitoring of how far the electrophoresis has proceeded, Ethidium Bromide: a

fluorescent dye used for staining nucleic acids, Transilluminator: which is used to visualize ethidium bromide-stained DNA in gels.

Method: Agarose gel was prepared of required percentage (0.8%, 1%) depending upon the size of DNA and 2 µl of EtBr was added for 50 µl solution. 6X loading buffer was added to the samples before loading them onto the gel. 100 bp or 1kb ladders were also loaded accordingly along with the samples for size estimation. Gel was run in 1X TAE buffer and visualized in BioRad Gel Doc.

2.6 Transformation of E.coli

The purpose of this technique is to introduce a foreign plasmid into a bacteria and to use that bacteria to amplify the plasmid in order to make large quantities of it. After ligation or in principle for amplification of any plasmid, the entire ligation mixture is transformed into bacterial competent cells.

Components: For transformation competent cells, plasmid and LB are required.

Method:

- Add entire ligation mixture to 100 µl of competent cells and incubate on ice for 30 minutes
- Give heat shock at 42°C for 85 seconds and incubate for additional 5 minutes on ice
- Add 1 ml LB and incubate for 1.5 hrs. at 37°C
- Centrifuge at 3000 rpm for 3 minutes and keep around 100 µl of solution and discard rest
- Resuspend the pellet in that 100 µl and spread it on appropriate antibiotic marker containing plate for selection.

2.7 His-tag Protein Purification using Ni-NTA beads

Polyhistidine-tag is at least six histidine long amino acid motif in proteins, often at the N- or C-terminus of the protein. Polyhistidine-tags are often used for affinity purification of proteins. Polyhistidine binds strongly to divalent metal ions like nickel. The protein binds to the nickel ions, which are immobilised in a column. The protein can be eluted by imidazole which competes with the polyhistidine tag for binding to the column.

Components:

- B-Per Reagent: It is a lysis reagent designed to extract soluble protein from bacterial cells. (From Thermo Scientific)
- Ni/NTA Agarose: They are magnetic particles coated with Ni-NTA Agarose affinity purification matrix and are used for immobilizing and purifying recombinant proteins carrying a His-tag. (From QIAGEN)
- PMSF (phenylmethylsulfonyl fluoride): It is a serine protease inhibitor that reacts with serine residues to inhibit trypsin, chymotrypsin, thrombin and papain, used for preparation of cell lysates.

Method:

- Expression of cells in bacterial strain BL-21(DE3)
- Inoculation of primary culture in 5ml LB overnight with necessary antibiotic marker at 37°C
- Addition of primary culture to 500ml LB for secondary culture and inoculation at 37°C until O.D reaches 0.6-0.8
- Addition of 100 µm IPTG for induction of protein synthesis and overnight inoculation at 18 °C
- Centrifugation at 4000 rpm at 4°C for 10 minutes and through the supernatant
- Washing of the pellet with 50ml 1X PBS and transfer it in 50ml falcon tubes
- Centrifugation again at 8000 rpm at 4°C for 10 minutes and discard of supernatant
- Addition of 40ml B-Per reagent along with 1mM PMSF + 100 µl protease inhibitor + 0.1 µl/ml Nuclease to the pellet with intense vortexing
- Incubation for 10 minutes at room temperature

- Centrifugation at 10000 rpm for 20 minutes at 4°C
- Transfer of supernatant in 1ml prewashed {3 times with lysis buffer(1X PBS + 10mM Imidazole)} Ni-NTA beads
- Incubation in ice on shaker for 1 hr.
- Transfer of supernatant-Ni-NTA mixture to the column and collection of flow through in 50ml falcon tube and pass it through the column again
- Washing of column twice with 10ml wash buffer (1X PBS + 20mM Imidazole)
- Elution of protein with elution buffer (1X PBS + 250mM Imidazole) and collection of 6-8 fractions in 1.5ml micro-centrifuge tubes

Table 2.1: 10X PBS solution components

Components	Amount
KH_2PO_4	0.26 g
Na_2HPO_4-$7H_20$	2.17 g
NaCl	8.71 g
KCl	0.201 g
H_20	800 mL
	Adjust pH to 7.4 and bring volume to 1 L with H_20

2.8 SDS - PAGE

It is a very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. Binding of SDS to the polypeptide chain in most proteins, imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis. The pore size of gel depends on the percentage of Acrylamide/Bisacrylamide which in turn depends on the size of protein. Two classes of

gels namely Stacking & Resolving gel make up a SDS PAGE gel. Stacking gel (usually large pore size) imparts equal negative charge to every protein and Resolving gel separates protein based on their charge to mass ratio.

Components:

- Buffer: Choice of buffer affects the mobility of buffer counterions and thereby the resolution of gel. It should not react or modify the protein running on the gel.
- Acrylamide: When mixed with water it shows slow, spontaneous autopolymerisation, joining molecules together by head on tail fashion to form long single-chain polymers.
- Bisacrylamide: It is used as a cross linking agent for polyacrylamide gels. It can crosslink two polyacrylamide chains to one another, resulting in a gel.
- Sodium Dodecyl Sulfate (SDS): It is a strong denaturing agent used to denature the proteins from their native state.
- Ammonium Persulfate (APS): It is the source of free radicals and is the initiator of gel formation.
- TEMED: It stabilizes free radicals and improves polymerisation.
- Coomassie Brilliant Blue (CBB): It is an anionic dye which non-specifically binds to proteins. Excess of dye is removed by destaining solutions which contains acetic acid and ethanol. The proteins are fixed by acetic acid and are detected as blue bands on a clear background.

Method:

- Prepare the required percentage of gel according to the size of the proteins
- Add 50 µl of the HU + 15%DTT (9:1) buffer to the sample proteins and heat them at 65°C for 10 minutes
- Load the sample and relevant protein ladder onto the gel and let it run
- Stain the gel overnight with CBB
- Destain the gel with a solution of 10% acetic acid + 25% ethanol
- Visualisation of gel under BioRad Gel Doc.

2.9 Yeast Two - Hybrid Assay (Y2H)

Yeast two-hybrid is based on the reconstitution of a functional transcription factor (TF) when two proteins or polypeptides of interest interact. The downstream reporter gene gets activated by the binding of a transcription factor onto an upstream activating sequence (UAS). Transcription factor is split into two separate fragments binding domain (BD) and activating domain (AD). The two proteins are fused to these two domains, protein fused to BD is referred to as 'bait', while protein fused to AD is 'prey'. Upon interaction between the bait and prey, the BD and AD are brought in close proximity and a functional TF is reconstructed upstream of the reporter gene.

Components: Usually the target proteins namely 'prey' and 'bait' along with their positive and negative controls.

Method:

- Transformation Protocol:
  - Take 10 µl of competent cells and add 2 µl of plasmid
  - Add 6X 40% Polyethylene glycol (PEG) solution and incubate at 30°C for 30 minutes
  - Give heat shock ay 42°C for 15-30 minutes for *S. cerevisiae* (5-7 minutes for *S. pombe*)
  - Put on ice (only for *S. cerevisiae*)
  - Add 100 µl of water and spread on appropriate media plates (For this study SC-Ura-Leu plates were used)
  - Incubate at 30°C for 2-3 days

- Spotting Protocol:
  - Take around 10 colonies with a sterile toothpick
  - Add 500 µl of sterile water and measure O.D

- Bring the O.D at same value for all the samples by either adding colonies or water
- Make 5 serial dilutions in a micro titre
- Stamp all the serial dilutions on respective media (For this study stamping was done on SC-Ura-Leu-His and SC-Ura-Leu-Ade plates)
- Incubate the plates at 30°C for 2-3 days and check for interaction.

2.10 Preparation of Competent Cells

Natural competence is the ability of a cell to uptake a foreign DNA from its environment. Artificial competence can be induced in laboratory that involve making the cell passively permeable to DNA by exposing it to conditions that do not normally occur in nature. Cells are incubated with divalent cations typically $CaCl_2$ that surrounds the cell and help in uptake of foreign DNA into the cell because DNA is negatively charged.

* **Note**: Work under sterile conditions and use everything autoclaved.

- PJ – 69 7a Competent Cells for Yeast:
  - Inoculate overnight at 30°C single colony of *S. cerevisiae* PJ – 69 7a strain in 5ml of YPAD medium
  - Transfer it to either 50 ml or 100 ml YPAD medium and inoculate at 30°C till the $O.D_{600}$ reaches 0.6 – 1.0
  - Centrifuge at 3000 rpm for 5 minutes at RT and discard supernatant
  - Wash the pellet with sterile 25ml water and centrifuge again
  - Wash the pellet with sterile SORB with $1\backslash10^{th}$ of culture volume
  - Add 360 µl of SORB for 50 ml culture
  - Add 40-50 µl denatured single stranded Salmon sperm DNA to the culture
  - Aliquot 100 µl of competent cells in 1.5 ml micro centrifuge tubes and freeze them in -80°C.

❖ YHY23P Competent Cells for Yeast:

- Repeat the above procedure with YHY23P strain of *S. cerevisiae*, instead of PJ – 69 7a strain.

***Note:** YHY23P strain has already an Ura plasmid, that is why this strain is used for complementation studies.

▪ YPAD medium (1L):

Table 2.2: Solution A (2X)

Components	Amount
Yeast Extract	10 g
Bacto Peptone	20 g
Distilled Water	450ml

Table 2.3: Solution B (10X)

Components	Amount
Glucose	200 g
Adenine Sulphate	1 g
Uridine	1 g
L – Histidine	1 g
L – Leucine	1 g
Distilled Water	1 L

***Note**: Autoclave Solutions A and B separately and add 450ml of solution A + 100 ml of solution B + 450 ml of distilled water for YPAD medium.

❖ E.coli DH5α Chemical Competent Cells:

- Streak the cells from DH5α strain on a fresh agar plate without any antibiotic and incubate it overnight at 37°C
- Pick a single colony and inoculate it in 5ml of LB medium overnight at 37°C

- Transfer 500 µl of the overnight culture into 50ml of Super Optimal Broth (SOB) medium and inoculate it at 37°C until the O.D reaches 0.5
- Incubate the culture on ice for 10 minutes
- Transfer the culture in 50ml falcon tubes and centrifuge for 10 minutes at 3500 rpm at 4°C
- Discard the supernatant and resuspend the pellet in 25 ml of Transformation buffer # 1
- Incubate it on ice for 10 minutes
- Centrifuge the solution for 10 minutes at 3500 rpm at 4°C
- Discard the supernatant and resuspend the pellet in 4ml of Transformation buffer # 2 and add 140 µl of DMSO
- Incubate the suspension on ice for 15 minutes and add another 140 µl of DMSO
- Distribute the suspension in aliquots of 200 µl and store immediately at -80°C
- During transformation add equivalent amount of Transformation buffer # 3.

Table 2.4: Transformation Buffer # 1 (100ml)

Stock	Final Concentration
1M MOPS (pH = 6.5)	10mM
1M KCl	100mM
1M $MnCl_2$	45mM
1M $CaCl_2$	10mM
1M KAc (pH = 7.5)	10mM
Adjust volume to 100 ml with sterile water	

- Transformation Buffer # 2 (100 ml): All components of Transformation buffer # 1 and additionally add 12.5 ml of 80% glycerol.

Table 2.5: Transformation Buffer # 3 (100ml)

Stock	Final Concentration
1M $CaCl_2$	100mM
1M $MgCl_2$	50mM

2.11 Complementation Assay

Complementation is a way of determining whether two mutants that cause the same or similar phenotype (e.g. his-) are due to mutations in the same or different genes. Complementation is due to the interaction of gene products produced by different genes and is not due to interaction of the genes themselves.

Components: For performing complementation, target mutants along with their necessary positive and negative controls are required.

Method:

- Transformation: Refer to Transformation part of section 2.9

- Complementation Experiment: Refer to Spotting part of section 2.9

*Note: For this study spotting was done on EMM-Leu-Ura and selection spotting was done on EMM-Leu+5FOA (5-Fluoroorotic Acid) plates.

FOA Selection: URA 3 is a gene on chromosome V in yeast and it encodes for orotidine 5-phosphate decarboxylase (ODCase), an enzyme that catalyses one reaction involved in the synthesis of pyrimidine ribonucleotides in yeast RNA. Loss of ODCase leads to growth defect unless uracil is provided in the media. However, if 5-FOA is added to the media, ODCase converts 5-FOA into the toxic substance 5-fluorouracil causing cell death.

2.12 Circular Dichroism

Circular Dichroism (CD) relies on the differential absorption of left and right circularly polarised radiation by chromophores which either possess intrinsic chirality or are placed in chiral environments. Proteins have a number of chromophores which can give rise to CD signals. Secondary structure can be determined in the far UV spectral region (190-250nm); at 218 nm β-sheet absorption occurs, at 208 & 222nm α-helix absorption occurs and at 205-208nm random coil absorption occurs. The near UV spectral region (250-350nm) is sensitive to certain aspects of tertiary structure. In this region the chromophores are the aromatic amino acids and disulfide bonds, and the CD signals they produce infers about the overall tertiary structure of the protein.

Components: For performing a CD protein sample (0.2mg/ml) and a buffer (usually the same buffer in which the protein is eluted), CD cuvette (5mm for this study).

Method:

- Add 200 µl of buffer for blank spectra
- Add 200 µl of protein for its CD spectra

Spectrometer and Software: Chirascan™-plus CD Spectrometer and software = Pro Data Viewer and Chirascan.

- Parameters and analysis:

Mean Residue Ellipticity (MRE) = $\frac{\text{Raw Ellipticity (mdeg) X MRW}}{\text{10 X conc. (mg/ml) X pathlength (cm)}}$

MRW = Molecular Weight (Da)

Total # of amino acids

*Note: For this study pathlength was 0.5cm, total no. of amino acids was 91 and the wavelength was between 200-280nm and the CD spectra of the proteins was recorded along with the 6X His tag, since, removing His tag from the Hub1, it precipitates on its own.

- *S. pombe* Hub1 including His tag (First 20aa with 6xHis are from vector) (pI = 8.50)

MGSSHHHHHHSSGLVPRGSHMIEVLCNDRLGKKVRVKCMPDDTVGDFKKLVAA
QTGTDPRRIVLKKWHSVFKDNITLADYEIHDGMSLEMYYS

2.13 Fluorescence Spectroscopy:

Fluorescence spectroscopy is a type of electromagnetic spectroscopy which tells about the fluorescence emitted by the sample. Usually UV light is used to excite the electrons to higher energy levels and causes them to emit light; typically, but not necessarily visible light. Fluorescence is observed when the electrons drops down to one of the various vibrational levels of the ground electronic state.

The fluorescence of a folded protein is a mixture of the fluorescence from individual aromatic residues. Most of the intrinsic fluorescence is due to the excitation of tryptophan residues, with some due to tyrosine and phenylalanine. Tryptophan absorption takes place at 280 nm and emission in the range of 300 – 350 nm depending in the polarity of local environment. Tryptophan can be used to estimate the nature of its microenvironment. For example, the addition of a surfactant to a protein which contains a tryptophan which is exposed to the aqueous solvent will cause a blue-shifted emission spectrum if the tryptophan is embedded in the surfactant vesicle or micelle.

Components: Typically for performing a fluorescence spectroscopy experiment target proteins are required.

Spectrometer and Software: Varian Cary 50 Spectrometer and software = carywin.

- Parameters: slit width = 2.5nm, excitation = 280 nm, emission = 300-350nm.

*__Note__: His tag from both the proteins (Hub1 & Fum1) was not removed. {pI Hub1 = 8.50, pI Fum1 = 8.84}

2.14 Thioflavin T (ThT) Assay

Thioflavin T is a benzothiazole dye that exhibits enhanced fluorescence upon binding to misfolded protein aggregates called amyloid fibrils both ex vivo and *in vitro*. When it binds to beta sheet-rich structures, such as those in amyloid aggregates, the dye displays enhanced fluorescence and a characteristic red shift of its emission spectrum [Khurana R. et al, 2005].

Components: For performing ThT assay target proteins and ThT stock is required.

Method:

- Prepare the stock of ThT (For this study 500 µM stock was prepared)
- Add 5-10 µM of ThT to the sample (500 µl) and mix gently (For this study 5 µM ThT was added)
- Measure the spectra using fluorimeter.

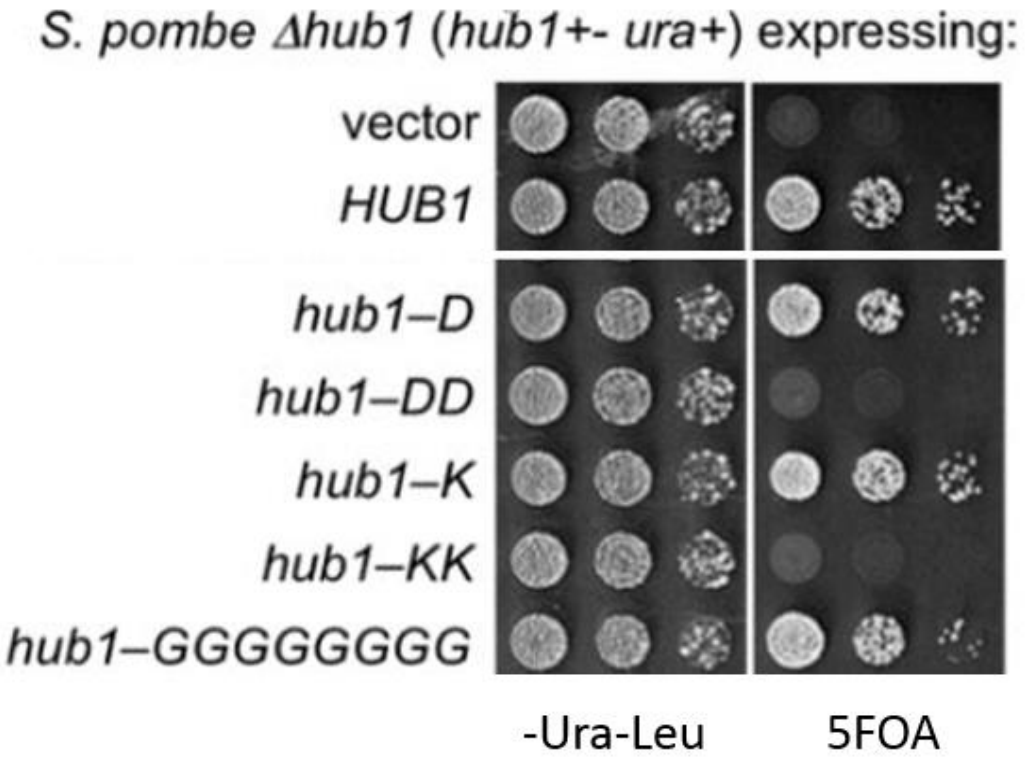
  - Parameters: slit width = 5nm, cuvette = 3mm, excitation = 450nm, emission = 482nm

CHAPTER 3

RESULTS & DISCUSSIONS

3.1 Complementation Assay in *Δhub1 S. pombe*

Various C-terminal extensions of Hub1 are not able to rescue the growth defect in Δ*hub1* strain, for instance, *hub1-DD* and *hub1-KK*. Whereas glycine mutation *hub1-GG* behaves like WT Hub1 and can rescue the growth defect in Δ*hub1* strain (figure 3.1). [Mishra SK. *et al*, 2011]



*__Note:__ Image added by the permission of S.K Mishra [Mishra SK. *et al*, 2011]

Figure 3.1: Complementation assay in *S. pombe Δhub1*. hub1-D, hub1-DD, hub1-K, hub1-KK, hub1-GGGGGGGG are C-terminal extensions of Hub1. Note that the addition of more than one charged residue to the C-terminal of Hub1, leads to the loss of function of Hub1. However, addition of any number of uncharged residues to the C-terminus does not affect the function of Hub1.

Since, aspartic acid is a negatively charged amino acid and lysine is a positively charged amino acid and it might be because of the charging at C-terminal, they are not

complementing. So, the idea was to create such a mutant in which charge is neutralised, therefore DKG was added to the Hub1 C-terminal to make a neutralised C-terminal mutant *hub1-DKG*. Also, C-terminal might be essential in splicing, so by adding some amino acids and increasing its length might have affected splicing and thus Hub1 becomes non-functional. Since, glycine is a relatively small amino acid as compared to aspartic acid and lysine, C-terminal in case of *hub1-GG* might have been more flexible, thus might have behaved like WT Hub1. So, various Hub1 mutants like *hub1-GDD*, *hub1-GGG* and *hub1-DDG* (refer section 2.2) were cloned in pREP81x vector and glycine was added to provide more flexibility to C-terminal. Complementation assay using these C-terminal extensions was performed and the results are depicted in figure 3.2.

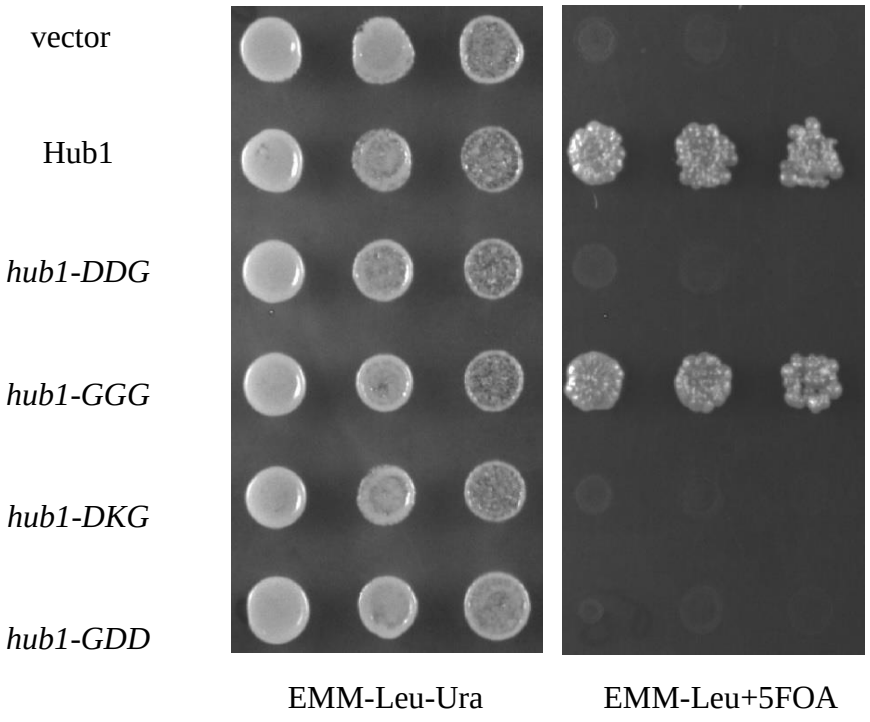


Figure 3.2: Complementation assay of *S. pombe Δhub1*. Complementation assay using *S. pombe Δhub1* (lethal) by expression of Hub1 variants. *hub1-DDG, hub1-GGG, hub1-DKG, hub1-GDD* are the C-terminal extensions of Hub1. Note that only WT Hub1 and hub1 having *GGG* extension are able to rescue the growth defect as indicated by this complementation assay. By contrast, addition of amino acids other than Gly to the C-terminal of Hub1 leads to a non-functional Hub1.

These C-terminal extensions of Hub1 were cloned in pREP81x plasmid (Ura plasmid) and transformed in YHY23P (Leu plasmid) competent cells. Serial dilutions (1:4) of the mutants of Hub1 was done and they were spotted on plates (EMM-Ura-Leu and EMM-Leu+5FOA). EMM-Ura-Leu plate is a control plate, on which every Hub1 extension grows. EMM-Leu+5FOA is the selection plate, on which only that Hub1 extension will grow, which Leu plasmid will complement, since cells will exclude the Ura plasmid in the presence of FOA. As seen in figure 3.1, only Hub1 WT and *hub1-GGG* complements the growth of Hub1. Also when the charge is neutralised as in case of *hub1-DKG*, there is no complementation, which indicates that a particular charge might not be responsible for the loss of function of Hub1. Flexibility of the C-terminal might be necessary for the function of Hub1, since the mutants *hub1-DDG* and *hub1-GDD* also did not complement the growth but *hub1-GGG* does.

3.2 Characterisation of secondary structure of Hub1 WT and *hub1-DD*

Hub1 consists of a half-open β barrel along with two flanking α helices. Since *hub1-DD* does not complement the growth of *S. pombe* (figure 3.1), the motivation was to find out as to what is changed in the Hub1 protein by addition of just two or more than two Aspartic Acid residues, where adding only one aspartic acid is fine. To understand this, first two aspartic acid residues were added to the C-terminus of Hub1 via cloning (refer section 2.2) in pET28a vector and the clones were confirmed by sequencing. The proteins Hub1 and *hub1-DD* were purified via Ni-NTA protein purification (refer section 2.7). Circular Dichroism (CD) experiment (refer section 2.12) was performed with these two proteins and the result of the experiment is shown in figure 3.3.

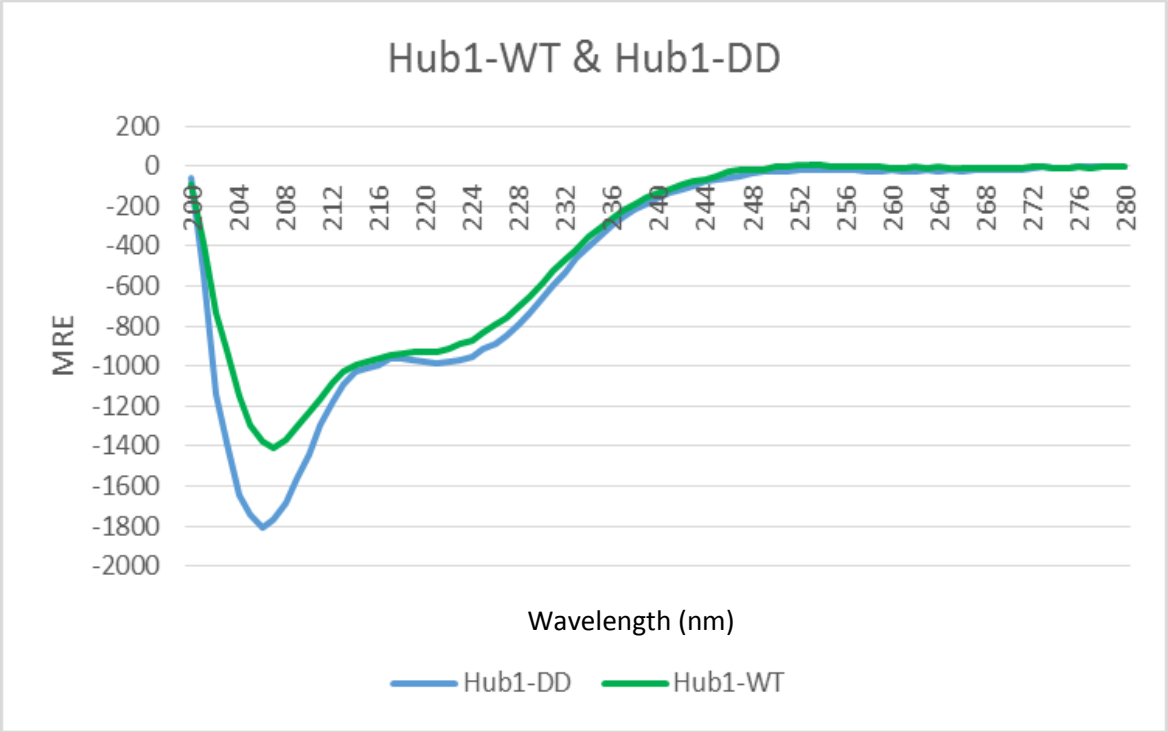


Figure 3.3: Circular Dichroism of proteins Hub1 and *hub1-DD*. Previously it was shown that when two aspartic acids are added to the C-terminal of Hub1, it leads to a loss of function of Hub1. Here the aspartic acid extension of Hub1 (*hub1-DD*) secondary structure is compared with WT Hub1.The CD spectra for both the proteins were taken at equivalent protein concentration (0.18mg/ml).

*Note: Concentration of the protein was measured using Bradford assay. However, Bradford is not very reliable, so, concentration of proteins needs to be estimated using more powerful techniques.

As shown in the above figure there seems to be a clear but minor difference in the secondary structure composition of two proteins. It is clear that there is an increase in the percentage of either α helices or random coil content and this difference in the secondary structure of *hub1-DD* leads to the loss of function of Hub1. But this difference in the secondary structure of protein does not affect its binding with Snu66 [Mishra SK. *et al*, 2011]. So, it can be inferred that C-terminal of Hub1 does not have a role in binding with Snu66, however, it must be involved in splicing since adding two aspartic acid to Hub1 does not complement growth in Δ*hub1* strain.

3.3 Interaction study of Hub1 and Fum1

Hub1 localisation is both nuclear and cytosolic and Fum1 is present in mitochondria and cytosol. Inside the nucleus, Hub1 is involved in splicing, whereas function for its cytosolic presence is unclear. It was found that in cytosol *S. pombe* Hub1 interacts with Fum1, whereas *S. cerevisiae* does not interact with Fum1 (unpublished S.K Mishra). *S. pombe* and *S. cerevisiae* has a high sequence homology and there are only few amino acid differences from *S. cerevisiae* to *S. pombe*, one is tryptophan (W) which is present at 47th position in *S. pombe* as opposed to glycine (G) in *S. cerevisiae* (figure 3.4).

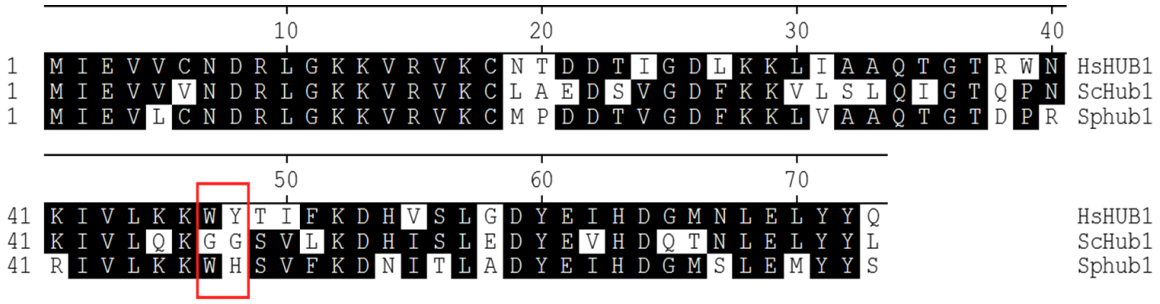


Figure 3.4: Comparison of Hub1 sequences between *Homo sapiens*, *S. cerevisiae* and *S. pombe*. Hub1 is highly conserved. Trp residue is present at the 47th position of *S. pombe* and *Homo sapiens,* however, Gly is present in *S. cerevisiae* (red box). This Trp residue of S. pombe might be involved in the Hub1-Fum1 interaction.

The two-hybrid assay (refer section 2.9) was performed with *S. pombe* Hub1 and Fum1, to understand if *S. pombe* Hub1 interacts with Fum1 or not (figure 3.5). The tryptophan residue at the 47th position of *S. pombe* was changed to glycine (*hub1W47G*) to know if tryptophan has a role in the Hub1-Fum1 interaction or not.

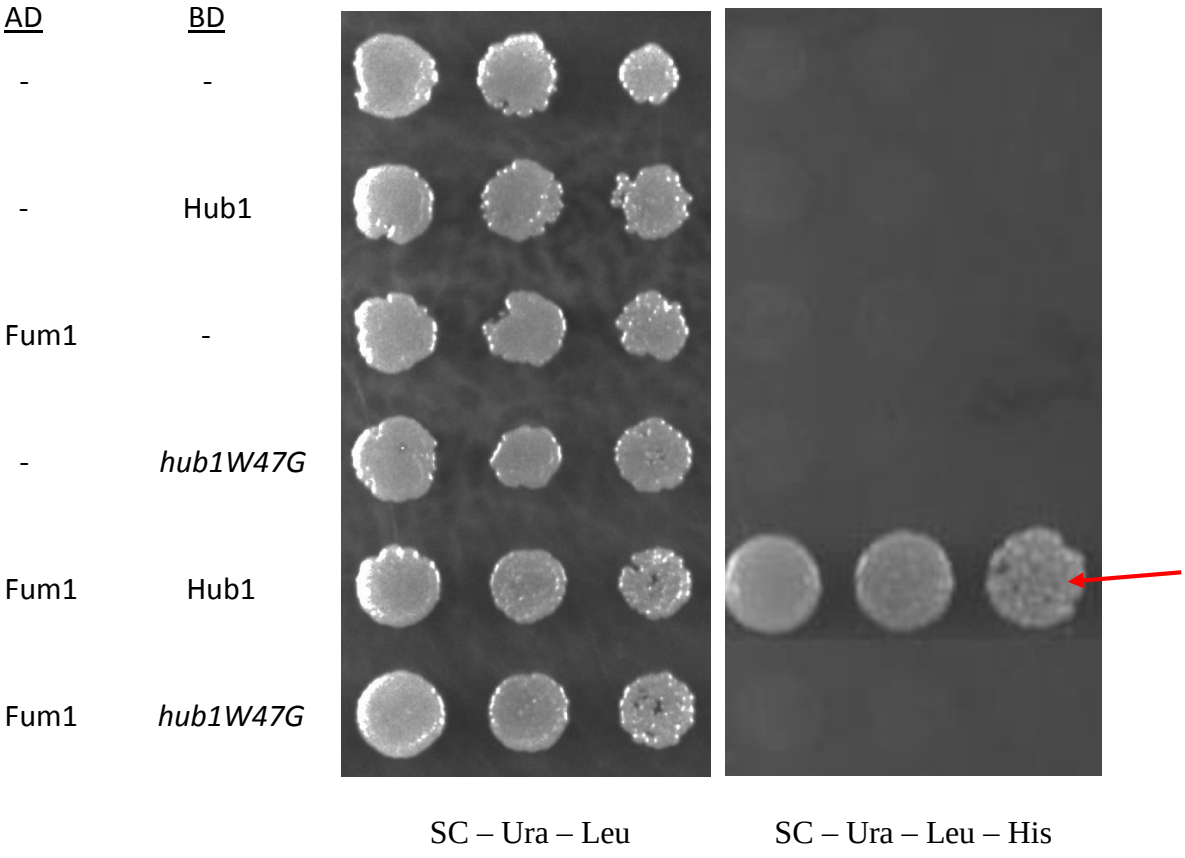


Figure 3.5: Yeast Two-Hybrid assay of Hub1 and Fum1. "-" represents the empty vector with no Hub1 or no Fum1 gene and *hub1W47G* is the point mutation in *S. pombe* where Trp residue present on the 47th position of S. pombe has been mutated to Gly residue, since Gly is present at the 47th position of *S. cerevisiae* and *S. cerevisiae* Hub1 does not interact with Fum1. Only when *S. pombe* WT Hub1 and Fum1 interact, His reporter gets activated and there is growth on –His media. However, there is no interaction between *hub1W47G* and Fum1.

Fum1 was fused with activation domain (pGAD vector), whereas Hub1 and its mutant *hub1W47G* was fused with binding domain (pGBDU vector). It is clear from the above figure that there is interaction between Hub1 and Fum1 (red arrow) indicated by the growth on –His media, as due to their interaction upstream activating sequence gets activated which is reported by His reporter. When tryptophan is changed to glycine there is no interaction, indicating either tryptophan could be the site of interaction directly or it is altering the protein in such a way that the protein then interacts with Fum1. But it is certain that tryptophan is playing an important role in Hub1-Fum1 interaction.

3.4 Documentation of Hub1-Fum1 complex

Hub1 interacts with Fum1 and the complex precipitates when the proteins are mixed together *in vitro*. When these proteins are mixed together in equimolar ratio, white precipitate can be seen clearly, when they are incubated at 4°C. However, when incubated at room temperature (25°C) no such precipitate was visible. *S. pombe* Hub1 has a Trp residue and by two-hybrid study it was seen that this Trp residue either directly or indirectly plays a role in Hub1-Fum1 interaction. So, in order to understand if Hub1 and Fum1 are making a complex fluorescence spectroscopy experiment (refer section 2.13) was performed. Tryptophan excitation peak is at 280nm and emission peak ranges from 300 – 350nm, depending in the polarity of local environment. The peak of the tryptophan shows either red shift or blue shift depending upon the local environment of tryptophan, when two proteins are mixed together.

(figure 3.6)

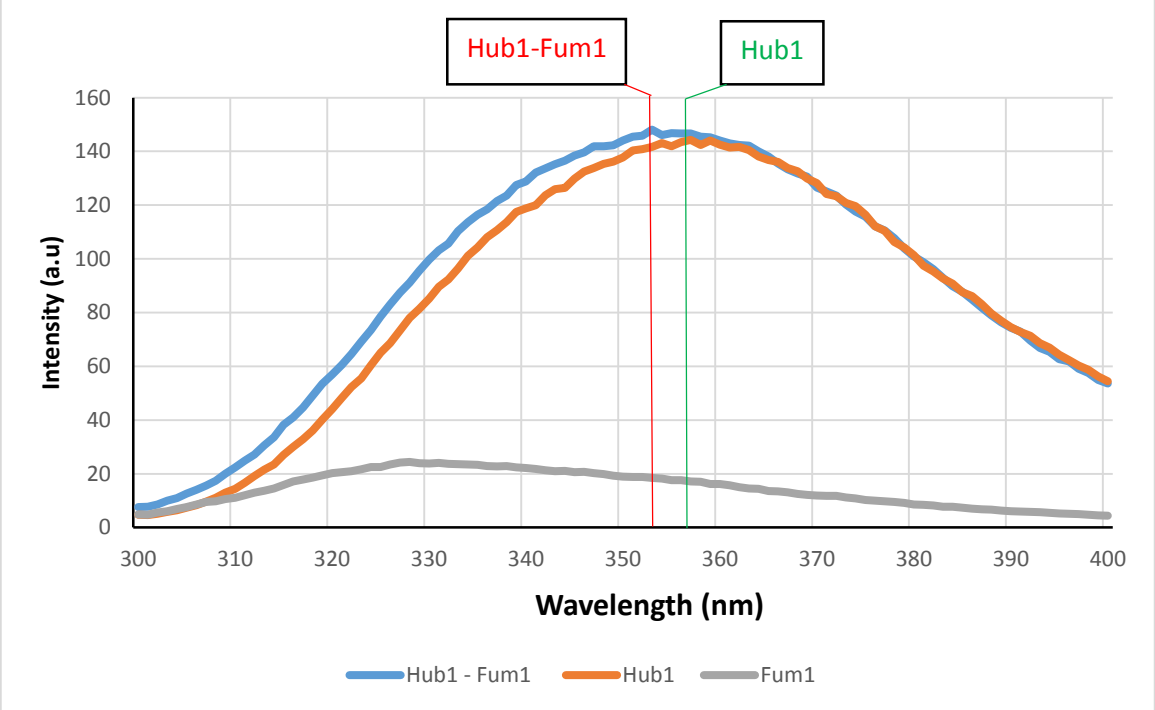


Figure 3.6: Fluorescence Spectroscopy of Hub1, Fum1 and Hub1-Fum1 mixed together. Yeast two-hybrid assay indicated that *S. pombe* Hub1 interacts Fum1. When Hub1 and Fum1 are mixed together in equimolar ratio a clear white precipitate in visible implying Hub1 and

Fum1 together are making a complex. *S. pombe* Hub1 has a Trp residue which is fluorophore and has the maximum intensity of fluorescence at 357nm (indicated by green line). With the addition of Fum1 there is a red shift in the fluorescence spectra and then the maxima shifts to 353nm (indicated by red line), indicating the Trp residue of *S. pombe* Hub1 is getting buried in the presence of Hub1 and together they are making a complex.

Hub1 has a Trp residue at its 47th position and the fluorescence intensity by that residue is depicted in figure 3.6. The fluorescence intensity of Hub1 is maximum at wavelength 357 nm. When the two proteins are mixed in equimolar ratio and their spectra is recorded, the maximum intensity of Hub1+Fum1 decreases to wavelength 353 nm. The wavelength of maximum intensity is getting reduced causing a red shift in the spectra. It signifies that the Trp residue of *S. pombe* Hub1 might be getting buried upon binding with Fum1.

3.5 Characterisation of the Hub1-Fum1 complex

Mutations in fumarase is associated with a number of diseases [Bayley JP. *et al*, 2008]. Furthermore, porcine fumarase I reported to form fractal aggregates *in vitro* [Barteri M. *et al*, 2007]. Formation of these aggregates is induced by oxidizing conditions. As seen in the fluorescence spectroscopy experiment that Hub1 and Fum1, when mixed together forms a complex. Since, Hub1 is interacting with Fum1 as reported by yeast-two hybrid assay and together they form a complex as indicated by fluorescence experiment, there is be a possibility that the Hub1-Fum1 complex might be making amyloids and Hub1 might have role in the diseases caused by Fum1. To understand this Thioflavin T (ThT) binding assay of Hub1, Fum1 and Hub1-Fum1 complex at 4°C and 25°C was performed for the amyloids formation (figure 3.7). When ThT binds to amyloids, enhanced fluorescence spectrum is observed (refer section 2.14).

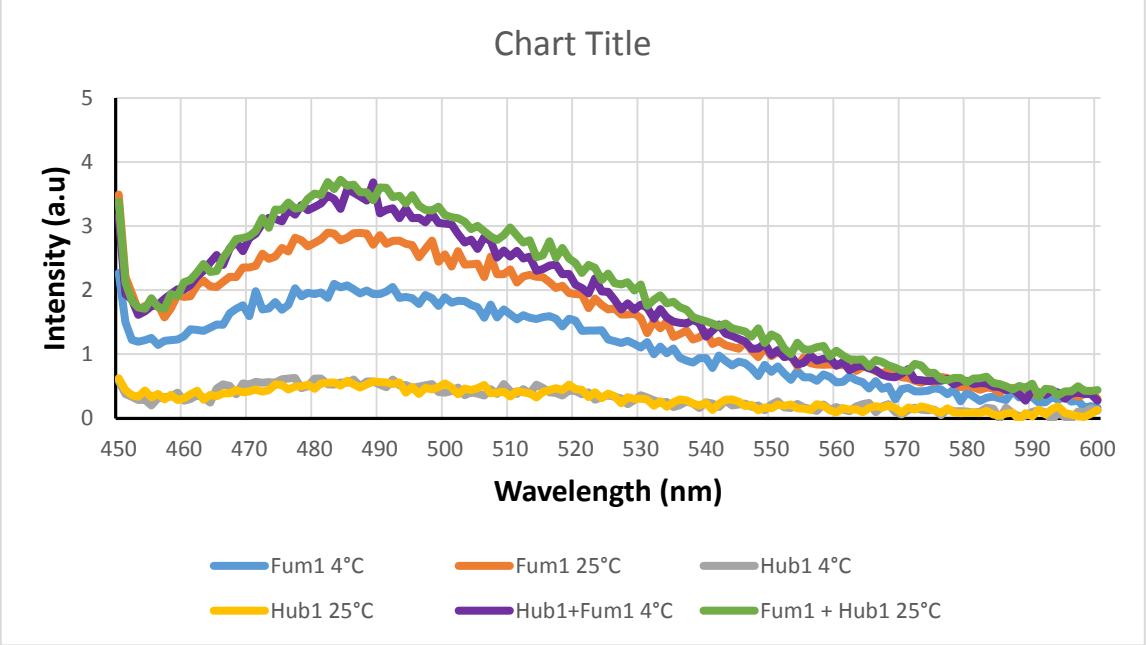


Figure 3.7: Thioflavin T assay of Hub1, Fum1 and Hub1-Fum1 complex at 4°C and 25°C. *S. pombe* Hub1 forms a complex with Fum1 when they are mixed together in equimolar ratio, as indicated by fluorescence studies. The Hub1-Fum1 complex might be leading to the formation of the amyloids. ThT dye binding to amyloids gives an enhanced fluorescence spectra. Hub1, Fum1 and Hub1-Fum1 complex was incubated at 4°C and 25°C. There is no significant enhancement in the fluorescence spectra upon adding of ThT, however, the emission by the proteins lies the region of amyloids i.e. around 480nm. Hence, there might be an amyloids formation by Hub1-Fum1 complex but might not be that significant.

As indicated by figure 3.6, no significant enhanced fluorescence is observed when Hub1 and Fum1 are mixed together but emission peak of Hub1-Fum1 complex lies is around 480nm, which is equivalent to that of amyloids. There was a clear white precipitate observed when Hub1 and Fum1 were mixed together and kept at 4°C, while no precipitate was observed at 25°C. Therefore, it needs to be investigated further whether Hub1-Fum1 complex is actually forming amyloids or is it something else. Hub1 might still have a role in the diseases caused by Fum1 and it might be leading to the formation of fractals aggregates in Fum1 that are already reported in porcine [Barteri M. *et al*, 2007].

3.6: Conclusions and Future Prospects

In the first part of my project, I have shown by complementation assay of *S. pombe* using various C-terminal extensions of Hub1 that the non – functional extensions of Hub1 are not because of any particular charge. But presence of any charge on the C-terminal of Hub1 abolishes its function whereas glycine does not. Secondary structure prediction of Hub1 and *hub1-DD* shows a clear but minor difference between the two proteins. However, aspartic acid or lysine extension of Hub1 leads to the loss of function of Hub1, but does not affect Hub1 binding with HIND domain, suggesting Hub1 folds properly even in the presence of charged amino acids on its C-terminal. It needs to be further investigated why some of the extensions of Hub1 C-terminal, by charge residues leads to the loss of function of Hub1. Furthermore, C-terminal of Hub1 should also be extended by hydrophobic amino acids like leucine or valine to understand if they are able to rescue the growth defect in *Δhub1 S. pombe* strain.

In the second part of the project I have shown that *S. pombe* Hub1 interacts with Fum1 (fumarase) and Trp residue present on the 47th position of *S. pombe* Hub1 is either directly or indirectly involved in this interaction. Hub1 and Fum1 forms a complex and they precipitate together *in vitro* in solution. The Hub1-Fum1 complex shows a clear binding of ThT suggesting towards amyloids like structures. However, further characterization of Hub1-Fum1 complex is required and the exact nature of this complex needs to be explored by advanced techniques like Atomic Force Microscopy (AFM), Fluorescence Anisotropy. Furthermore, fractals aggregates formed by Fum1 that are reported in porcine [Barteri M. *et al*, 2007] might be forming in *S. pombe* also and Hub1 might have a role in the propagation of such fractal aggregates.

Mutagenesis of Fumarase should be done to identify the changes which leads to stronger interaction with Hub1 and these identified mutations might be involved in already reported diseases caused by mutations in Fumarase [Bayley JP. *et al*, 2008].

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