High Copy Suppressor Of Mutant In Hub1 And Regulation Of AAA-ATPase Sap1

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A dissertation submitted for the partial fulfillment of BS-MS dual degree in Science

Department of Biological Sciences
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Mohali

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Certificate of Examination

This is to certify that the dissertation titled "**High Copy Suppressor Of Mutant In Hub1 And Regulation Of AAA-ATPase Sap1**" submitted by Arundhathi Dev J R (Reg. No. MS14117) for the partial fulfillment 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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(Supervisor)

Dated: November 25, 2019

Declaration

The work presented in this dissertation has been carried out by me under guidance of Dr. Shravan Kumar Mishra at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made 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 detailed in the bibliography.

Arundhathi Dev J R
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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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Dedicated to Prof. Somdatta Sinha.

Abstract

Hub1 is an unconventional UBL involved in alternative splicing and splice site selection which is essential in *Schizosaccharomyces pombe*. Two functional surfaces of Hub1 have been previously reported. The D22, essential for its interaction with Snu66 and the H63, necessary for its interaction with Prp5. In addition to this, a third surface has been identified in our lab whose mutation shows lethality at 37° C. The *S. pombe* uncharacterized protein SPCPB16A4.06c, along with Zwf1, a glucose-6-phosphate dehydrogenase and Rpl101, a 60S ribosomal protein, are identified to be multi copy suppressors of this growth defect exhibited by Hub1 surface III mutant.

The second experiment done was to study the role played by sumoylation in Tup1 mediated glucose repression by monitoring its relevance in the expression of Sap1_C. The SIN1 Associating Protein, Sap1 is an ATPase of the AAA family whose biological relevance is being studied. The shorter isoform of Sap1, the Sap1_C is expressed only after the Diauxic shift where the glucose repression of Sap1_C is mediated by Tup1. This study shows that Sumoylation of Tup1, which is known to regulate its activity as a repressor, dampens the efficiency of Tup1 facilitated repression of Sap1_C.

Chapter 1

High Copy Suppressors Of Hub1 Surface III Mutation

1.1 Introduction

1.1.1 Ubiquitin like proteins

The conjugation of ubiquitin is one among the many post translational modifications that proteins undergo. Ubiquitin is a 76 amino acid protein which is covalently attached to a target Lysine in the substrate protein either in a monomeric or a Lys-linked polymeric manner. This addition of ubiquitin is called ubiquitination and it involves activation (by ubiquitin activating enzyme E1) conjugation (by ubiquitin conjugating enzyme E2) and ligation (by ubiquiting ligase E3). Ubiquitination is implicated with an array of functions like proteasomal degradation, cellular signaling, cell cycle regulation endocytosis etc.

Ubiquitin like proteins are proteins that are similar in structure to Ubiquitin and share the β grasp fold of ubiquitin. Their mechanistic pathways are similar to those employed by ubiquitin but are distinct none the less. Modification by UBLs have been found to have novel and unique functions. Members of the UBL family includes SUMO, Nedd8, Hub1, ISG15, UCRP and so on. Like ubiquitin, most of the UBLs require DUB like enzymes to remove their C-terminal extensions to facilitate their

conjugation by generating a functional form which contains a C-terminal di-glycine motif.

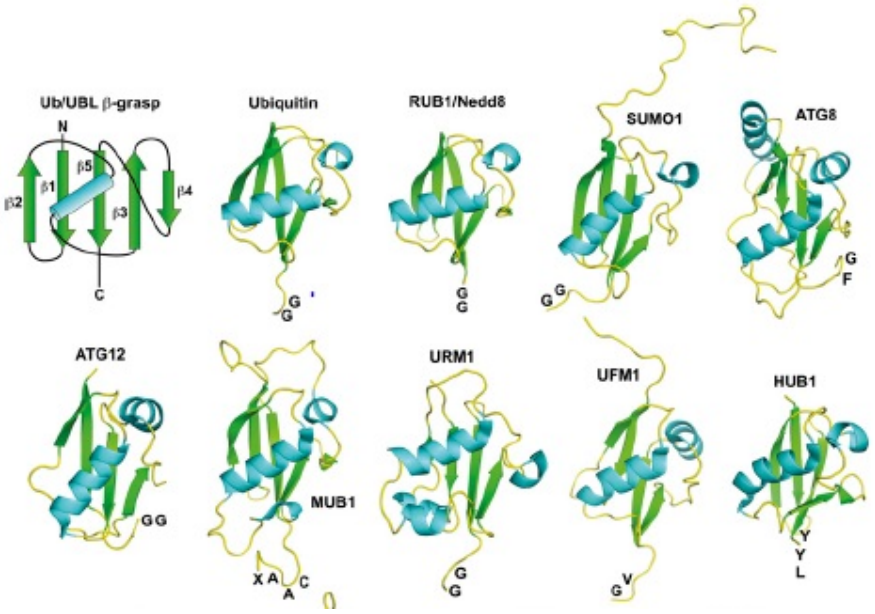


FIGURE 1.1: UBL family of proteins: The three dimensional ribbon diagrams of a few members of the UBL family are shown in its processed form with exposed C-Terminal. The beta-grasp fold is prominent in all proteins. Adapted from Richard D. Vierstra [1]

1.1.2 Hub1 an Unconventional UBL

Homologous to Ubiquitin, Hub1 (also known as UBL5), is a highly conserved 73 amino acid protein, belonging to the UBL family. Despite its low sequence identity with ubiquitin (∼23%) Hub1 has a tertiary structure very similar to ubiquitin. Hub1 is unconventional when compared to other UBLs in that it has a C terminal di-tyrosine followed by a non-conserved residue unlike the characteristic di-glycine. Also, this di-tyrosine appears to be dispensable for its essential function. Another peculiarity of Hub1 is that there has been no reported processing, conjugating or deconjugating enzymes for Hub1 which is typical of other UBLs. It is synthesized in the cell in its native form[2].

The most significant function of Hub1 known to date is its involvement in pre mRNA

splicing. Hub1 is shown to interact non-covalently to Snu66, a component of the tri-snRNP complex. It is also shown to be involved in alternative splicing, non-canonical splice site usage along with other functions like its association in cell cycle progression, polarized growth and mitochondrial unfolded protein response[3].

1.1.3 The Functional Surfaces of Hub1

The first functional surface of Hub1 to be identified, D22, was the surface of Hub1 necessary for its interaction with Snu66[4]. Snu66 has a conserved Hub1 Interaction Domain (HIND) where it interacts to Hub1 by a formation of salt bridge. The 22nd aspartic acid residue is found to be necessary for this interaction and a mutation in this residue (D22A) interferes with its interaction with Snu66.[3]

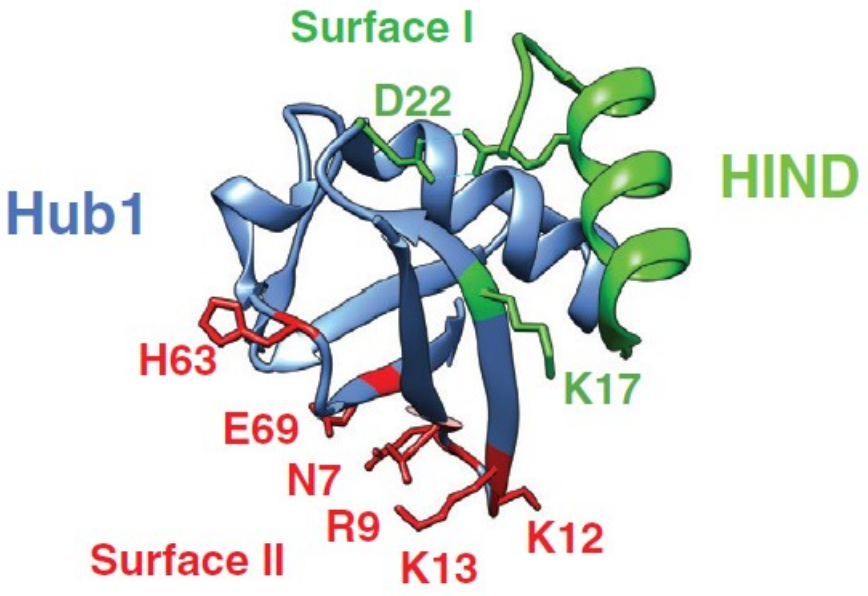


FIGURE 1.2: Three dimensional structure of Hub1 here shown interacting with the HIND domain of Snu66. The surface I of Hub1 (HIND interacting) is marked in green and the surface II (Prp5 interacting) is marked in red. Image from Karaduman et al., 2017[5],

Another surface of Hub1, H63 (surface II) is found to be imperative for its interaction

with the DEAD box Helicase Prp5. This interaction with Prp5 is shown to modulate splice site selection in the early steps of spliceosome assembly. A key mutation which shows interference with this interaction is the Histidine 63 mutation (H63L).

1.1.4 A putative third surface of Hub1

Hub1 is a non essential gene in cerevisiae. But in pombe and higher eukaryotes, the deletion of Hub1 is lethal[6]. But then, if Hub1 is an essential gene and Surface I and II are its functional surfaces, a mutation in these surfaces should prove to be lethal. But this was not the case observed in pombe. The Surface I and II mutations, on their own were not lethal to pombe. Keeping this problem at heart, a PhD fellow from our lab (Kiran K.), in collaboration with Ranabir Das from NCBS worked out the NMR structure of Hub1 and in doing so observed a positively charge surface formed by the 9th and 41st Arginine. It was found that the mutation of these residues to alanine, hub1 R9A,R41A mutant showed growth defect at 37° C.

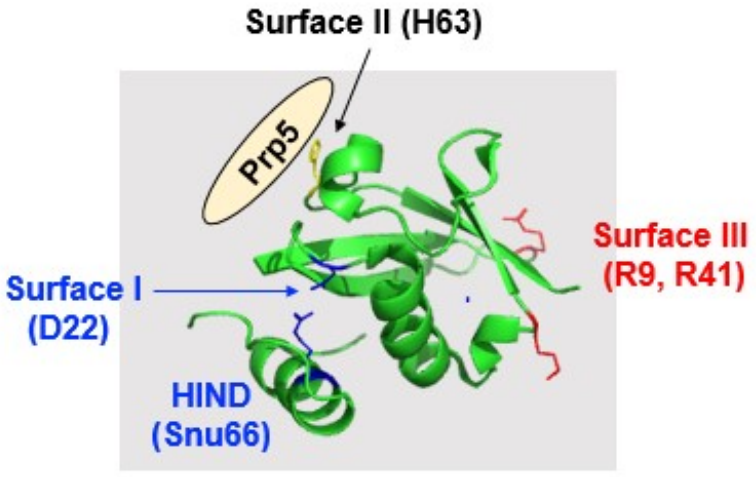
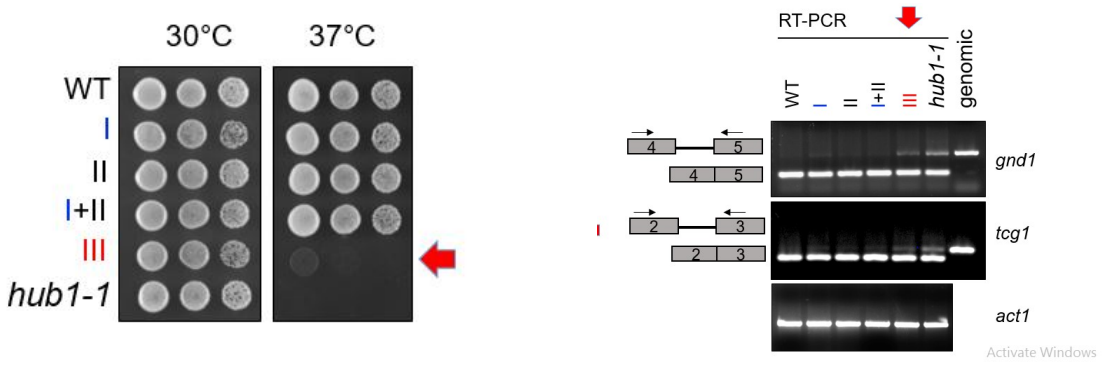


FIGURE 1.3: The 3D ribbon structure of Hub1 showing its interactive surfaces. The newly identified third surface is highlighted in red. Image from Kiran K. Kolathur (NMR in collaboration with Ranabir Das, NCBS).



(A) (B)

FIGURE 1.4: (A)The growth defect of Hub1 third surface mutant in comparison with the surface I, Surface II, a combined Surface I and II mutant and also the *hub1-1*. (B) Splicing assay showing intron retention in Surface III mutant. Red arrow indicates *hub1 R9A, R41A*. Images are adopted from Kiran. K. Kolathur.

The growth defect of this Surface III mutant is comparable to the temperature sensitive mutant of Hub1, the *hub1-1* which is an I42S mutation (the 42nd Ile residue is detrimental for maintaining its tertiary structure and hence this drastic phenotype). To study this growth defect of surface III mutation, splicing assays were performed with *hub1 R9A,R41A* mutant which revealed intron retention in *gnd1* and *tcg1* which are targets of Hub1 and this intron retention detected was similar to *hub1-1*. This putative surface III is therefore an essential surface of Hub1.

1.1.5 Aim

To explore the functional significance of this new identified third surface of Hub1, the study adopted the approach of finding genes that circumvent the growth defect of the Hub1 surface III mutant upon multi copy expression. There are two reasons behind adopting this strategy. Over-expression of a gene product might compensate for the growth compromise caused by the mutated Hub1 if they act in sequence. The over expression of a gene product might help in the functioning of the mutated Hub1 protein if they act in a coordinated manner[7]. A high copy suppressor screen was performed for the *hub1 R9A, R41A* mutant with the *S. pombe* cDNA library cloned under the inducible promoter, *nmt1*.

1.2 Experimental Methods

1.2.1 Yeast genomic DNA isolation

An overnight culture of the yeast strain was set up in appropriate growth media at 30°, 250rpm. On reaching saturation, 10 ml cells were harvested at 3000 rpm for 5 minutes at room temperature. The cells were washed in distilled water and resuspended in 200 µl lysis buffer. Lysis was performed by glass bead method where glass beads and 200 µl phenol: chloroform: isoamyl alcohol (PCI) were added. Vigorous vortexing for 1 minute followed by incubation on ice for 1 minute was repeated for 8 cycles. After vortexing, 200 µl TE buffer was added and then centrifuged at 13000 rpm for 10 minutes at RT. The supernatant was transferred to a fresh MCT with an addition of equal volume of chloroform followed by vortexing and centrifuging. The aqueous layer collected after this step was used to precipitate DNA by adding 100% ethanol and incubation at -20° for 20 minutes.

This was followed by centrifugation and the pellet obtained was dissolved in 100 µl TE buffer and treated sequentially with RNAse and Proteinase. A second round of PCI treatment was done followed by Chloroform treatment to remove persisting phenol. The final DNA precipitation was done by the addition of 10 µl ammonium acetate and 1ml 100% ethanol followed by incubation at -20° for an hour. This was then centrifuged, washed with 70% ethanol to remove salts and resuspended in 50 µl NFW for use.

1.2.2 DNA techniques

All the vectors used in this study are *S. pombe* expression vectors which are under the thiamine repressible promoter *nmt3*, *nmt81* or *nmt1*. All constructs have an N-terminal *3MYC* epitope tag. Plasmids with point mutations were created by site directed mutagenesis via the Spicing by Overlap Extension (SOE) PCR method.

1.2.3 Competent cell preparation and Transformation

Competent cells preparation and Transformation was done in accordance to the published protocol for *S. cerevisiae*[8]. Overnight cultures were grown in appropriate media at 30° C with shaking and then re-inoculated in fresh 50 ml YEL media at 0.2 OD_{600} and left to grow until 0.6-0.9 OD_{600}. The cells were then harvested by centrifugation at 3000rpm at room temperature for 5 minutes and then washed in half the volume of sterile water followed by one tenth the initial volume of SORB and pelleted again. After decanting the SORB, the pellet was resuspended in 360 µl SORB followed by an addition of 40 µl of salmon sperm DNA (denatured). 10 µl of these competent cells were mixed with 1 µl of plasmid DNA and then suspended in a six fold volume of sterile PEG (40%). This was followed by mild vortexing and an incubation at 30° C for 30 minutes. A heat shock was given to the cells at 42° C for 5 minutes and they were kept on ice for 5 minutes. The cells were then plated in appropriate selection plates and incubated at 30° C until the colonies appeared.

1.2.4 High copy suppressor screen

The high copy suppressor screen for the Hub1 surface III mutant was performed by transforming 100 µl of competent cells (of P46 strain with the *hub1-R9A,R41A* mutation) with 10 µl of *S. pombe* cDNA library constructed under the inducible *nmt1* promoter and plated in the thiamine limiting EMM-LEU plate and incubated at 37° C for 7-8 days until the colonies appeared. The colonies were then individually streaked on SC- LEU plates and later growth assays were performed for each colony obtained to re-verify their suppression.

1.2.5 Growth assays

The cells were suspended in 1 ml sterile water and their OD_{600} was measured. 2OD cells were taken in a microliter plate and appropriate water was added to make up the volume to 250 µl and fivefold serial dilutions of this was made followed by dilution spotting of the cells in appropriate selection plates with and without thiamine.

Plates were then incubated at 30° C and 37° C for 3-4 days until the growth appeared.

1.2.6 Western Blot

A 24 hours primary culture was set up in SC-LEU media and was left to reach saturation until the culture turned pink. From this culture, a secondary culture was set up at 0.1 OD_{600} in EMM-LEU media to induce protein expression and left to grow till 0.7-1 OD_{600}. To perform western blot, 1 OD_{600} cells were harvested and total proteins were precipitated using TCA[8]. 20 µl of the protein was loaded on SDS- PAGE for electrophoresis and later transferred to PVDF membrane (110mA, 2.5 hours)and blocked with skimmed milk (5%) for 1 hour. The PVDF membrane was incubated with primary antibody (in TBST buffer) for 3 hours followed by 3 TBST buffer washes and then incubated with secondary antibody (HRP conjugated) in skimmed milk for 1 hour. The blot was washed in TBST buffer 3 times and peroxide and luminol were used to initiate chemiluminescence in order to visualize.

1.3 Result

1.3.1 An Unconventional Clone

In a Multi copy Suppressor screen for the Hub1 R9AR41A mutant with the *S. pombe* cDNA library (under *nmt1* promoter) (performed by Kiran, a PhD fellow in Lab) a clone (HCS) which was clearly rescuing the growth defect of the *hub1-R9A,R41A* mutant at 37° C was isolated and upon C-term sequencing was found to be a partial clone of *tcg1*. Tcg1 is a single stranded TG1-3 DNA binding protein. Aside from telomere maintenance, it has a role in meiosis. From mass spectrometric analysis of the spliceosome, Tcg1 is also found to be part of the spliceosome.

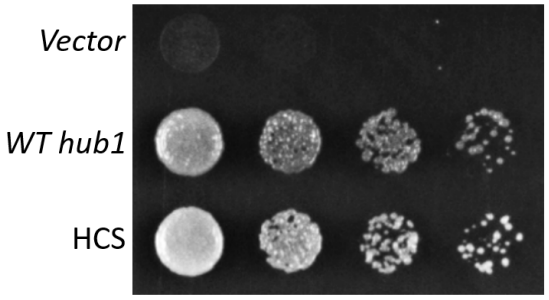


FIGURE 1.5: The suppressor clone HCS which was identified(by Kiran, a PhD fellow in lab) in a high copy suppressor screen for the *hub1 R9A, R41A* mutant at 37° C.

To verify the suppression of Hub1 R9A, R41A mutant by Tcg1, *tcg1* ORF was independently cloned in a multi copy expression vector under an inducible promoter *nmt3*. But on performing the growth assays, it was observed that as opposed to conferring growth advantage at 37° C, Tcg1 overexpression was growth inhibitory and was found to be cytotoxic even at optimum growth conditions.

1.3.2 Addressing the cytotoxicity of Tcg1 overexpression

Keeping in mind the role of Tcg1 in telomere maintenance and also its inclusion in the spliceosome, we suspected that the cytotoxicity caused by Tcg1 overexpression

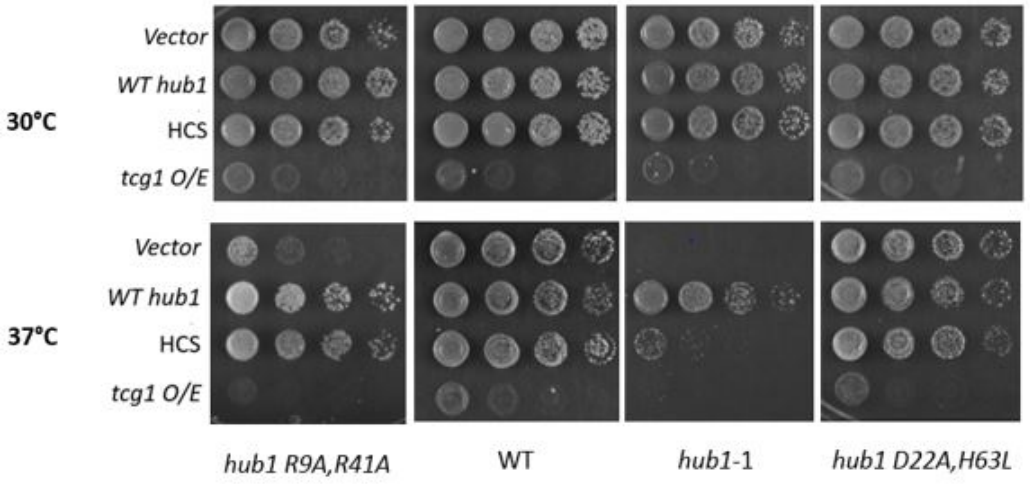


FIGURE 1.6: Growth assay showing cytotoxicity of Tcg1 over expression in Hub1 surface III mutant followed by similar phenotype observed in wild type cells, *hub1-1* cells and also Hub1 surface I and II mutant.

might be because of either splicing defects or problems involving chromatin stability. To further understand this, Tcg1 was over expressed in wild type cells and the levels of Rap1, Hif2, Rxt2 and Dsh1 were monitored. We know from our previous studies[9] that these proteins have functions in chromatin stability, telomere maintenance, transcriptional regulation and the like. It is also seen that levels of these protein drop in the event of inefficient splicing.

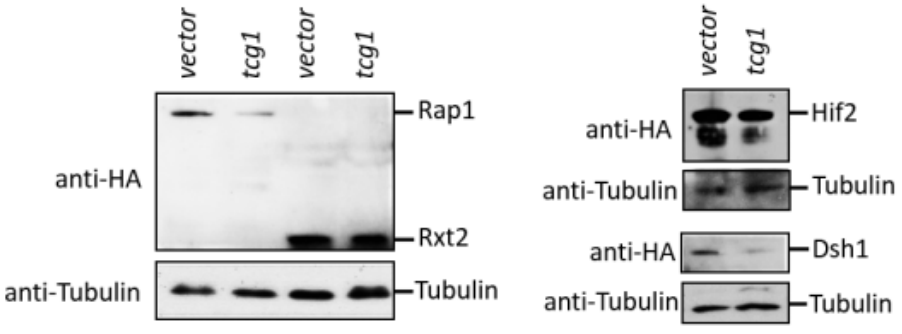


FIGURE 1.7: Western blots showing the levels of proteins Rap1, Rxt2, Dsh1 and Hif2 (all tagged with HA) upon Tcg1 overexpression. A significant reduction in the levels of these proteins is observed as compared to the WT levels (transformed with empty vector).

As seen in figure(1.7) Tcg1 over expression led to a reduction in the levels of all the

four proteins leading us to believe that this might be a consequence of splicing defects. However, further experiments like splicing assays must be performed in order to substantiate this observation and to make conclusive deductions.

1.3.3 A second insert in the plasmid

The clone HCS obtained from the High copy suppressor screen was then sequenced from N-terminus and in doing so, it was revealed that the HCS housed two inserts instead of one. The vector was found to contain a 3000 base pair insert, of which the first 1600 base pairs contained the 5' UTR, ORF and the 3'UTR of *spcpb16a4.06c* and the latter 1400 base pair part contained a partial clone of tcg1 starting from the 86th base pair with a Sal1 restriction site separating the two clones. *SPCPB16A4.06c* is a *S. pombe* specific uncharacterized protein that is 126 amino acids long. Very little is known about the protein other than the observation in Marguerat S. et al. (2012)[10] where the RNA levels of this protein is detected in increased levels following amino acid starvation of the cell.

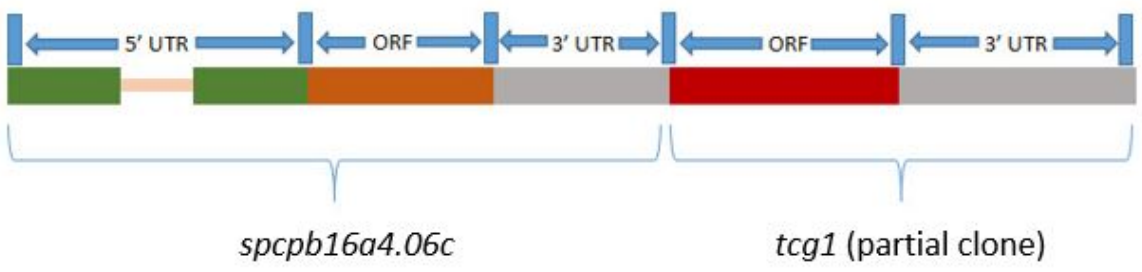


FIGURE 1.8: Schematic of HCS

1.3.4 *SPCPB16A4.06c* is the real suppressor

To understand this suppressor better, the two parts of the insert were individually cloned and the growth assay was repeated. Here it was observed that the 1600 base

pair long segment that contained the uncharacterized protein was in fact responsible for the growth rescue at 37° C. Subsequently, the ORF of *spcpb16a4.06c* was independently cloned and the growth assay was performed by transforming the *hub1* mutants with this clone. Rescue of growth defect at 37° C was observed. However, this rescue was not as significant as the rescue by the clone containing the entire 1600 fragment which included both the 5' and the 3' UTR.

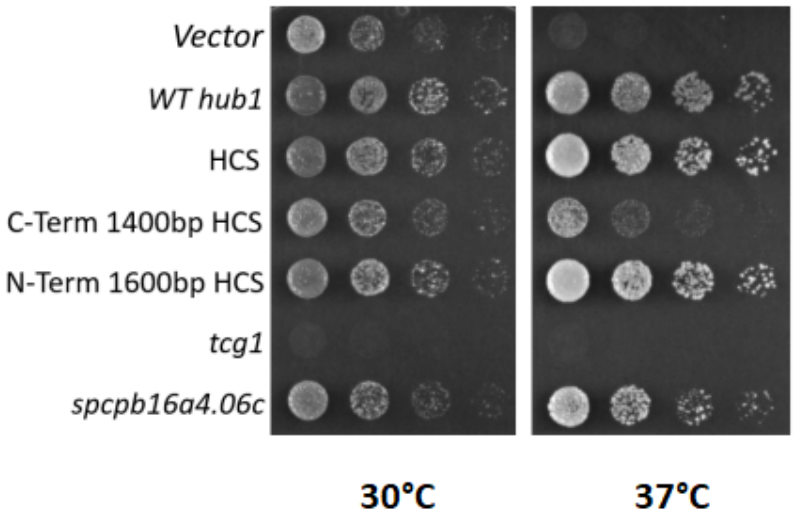


FIGURE 1.9: Growth assay of the *hub1 R9,R41A* mutant by transforming HCS, its two inserts and the ORFs contained in the two inserts respectively. It can be observed that the rescue is by *spcpbl6a4.06c*

1.3.5 Start codon mutated *spcpb16a4.06c* does not rescue the growth defect at 37°C

The observation that a *S. pombe* specific uncharacterized protein would be a potential interactor of a highly conserved protein like Hub1 was surprising. For this reason, we speculated that the rescue might be because of the RNA itself having a regulatory role and might not be because of the protein product. To substantiate this speculation, a site directed mutagenesis was performed where the start codon of *spcpb16a4.06c* was mutated to isoleucine in the 1600bp fragment so that the protein translation was inhibited. It was observed that on mutating the start codon (Met to Ile), the 1600bp

fragment lost its ability to rescue the growth defect of the *hub1* mutant thus leading us to conclude that it is the protein itself being responsible for the rescued phenotype.

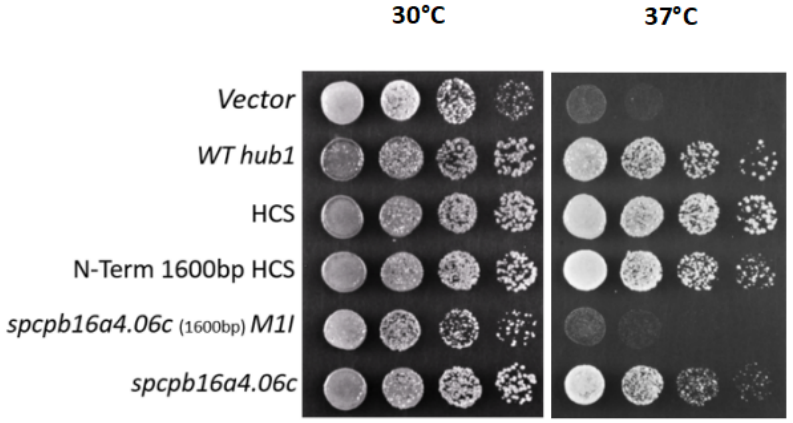


FIGURE 1.10: Growth assay performed with the start codon mutated *spcpb16a4.06c* in the T600 bp insert of HCS. No growth is observed at 37° C showing that the rescue of growth defect caused by the Hub1 surface III (R9A, R41A) mutation is by the protein product and not the RNA.

1.3.6 Other multi copy suppressors of the surface III mutant

A high copy suppressor screen was performed once again for the Hub1 surface III mutant with the *S. pombe* cDNA library in an effort to identify other suppressors. In this screen, a total of 5 colonies were obtained of which 4 were found to rescue the mutant's growth defect on performing growth assay at 37° C, here identified as Clones 1,2,3 and 4 respectively. Plasmids were isolated from these cells via yeast shuttle prep protocol.

DNA could be isolated from clone 1. The other three clones were sequenced to identify the genes. Clone 4 was identified as *rpl101*, a 60S ribosomal protein having RNA binding functions. Clones 2 and 3 were both found to contain the same gene,

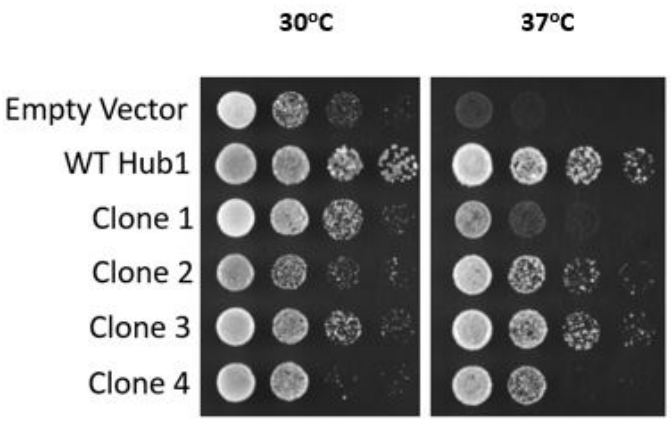


FIGURE 1.11: The growth assays performed with the colonies obtained from the high copy suppressor screen performed with the Hub1 surface III mutant. They are identified here as clones 1, 2, 3 and 4 respectively.

zwf1, which is a glucose-6-phosphate dehydrogenase whose activity is rate limiting for the pentose phosphate pathway which is responsible for the oxidative conversion of glucose-6-phosphate to ribulose-5-phosphate.

Clone 1	no DNA isolated
Clone 2	*zwf1*
Clone 3	*zwf1*
Clone 4	*rpl101*

TABLE 1.1: The list of clones isolated from the high copy suppressor screen and the genes contained in them

1.4 Discussion

The high copy suppressor screen was performed for the *hub1 R9A,R41A* mutant in order to find out the interactors of this Hub1 surface. So far, in this set of experiments, 3 different suppressors were identified. It was unanticipated that a *S. pombe* specific uncharacterized protein would be an interactor of a highly conserved protein like Hub1. However, very little is known about the protein and a credible structure prediction could not be made using bioinformatics tools. Hence, not much could be inferred about this protein. Another interactor found through this screen is *rpl101* which codes for the 60S ribosomal protein L1-B. The protein is a structural constituent of the ribosome and is involved in RNA binding. Lastly, *zwf1* was also identified in the screen, which is part of the pentose pathway shunt. Their significance in relation to Hub1 surface III is a question that yet needs to be answered. Further studies connecting these proteins with Hub1 might help us to shine light on the function of this new surface identified.

Tcg1 which was initially expected to rescue the lethality of Hub1 surface III mutant at 37° C had turned out to be cytotoxic instead. The cytotoxicity of Tcg1 over expression might, in part, be attributed to the inefficient splicing as indicated by a decrease in the levels of Rap1, Dsh1, Rxt2 and Hif2. From mass spectrometric analysis, Tcg1 is seen to be part of the spliceosome. Considering this, the observed decrement in levels of the aforementioned proteins leads us to suspect that Tcg1 might be a negative regulator of the spliceosome. This observation needs to be substantiated by splicing assays.

Chapter 2

Role Of Sumoylation In Tup1 Mediated Repression Of Sap1_C

2.1 Introduction

2.1.1 Tup1: A global transcriptional co-repressor in yeast

The 713 amino acid protein Tup1 is implicated in regulation of transcriptional activity of the *Saccharomyces cerevisiae* genome. Analogous to the *Drosophila* Groucho, Tup1 is a member of an evolutionarily conserved family of co-repressors that share a similar domain organization and mode of action. Four subunits of Tup1 associate with a single Cyc8/Ssn6 subunit to form the Tup1-Cyc8 co-repressor complex, which primarily functions by either evoking a repressed chromosomal state, consequently switching off transcription or by direct inhibition of transcription activation[11].

The functional domains of Tup1 can be largely classified into three regions. The N-terminal 72 amino acids which are required for its complex formation with Cyc8, the middle region, capable of histone binding, which is responsible for most of the repression activity and the C-terminal domain which has its characteristic 7 WD repeats which structurally forms a seven bladed β propeller and is involved in $MAT\alpha 2$ binding[12].

As , many as 3% of the *Saccharomyces cerevisiae* genes are repressed by the Tup1-Cyc8 complex[13] which includes genes specifying mating type, glucose repressed genes, sporulation related and DNA damage response genes. Though not an essential gene in cerevisiae, the deletion of *TUP1* confers distinct phenotypes like slow growth, flocculation[14], negligible sporulation, mating type defects in *MAT*α cells, derepression of a number of genes that are glucose repressed and also confers the cells an ability to take up dTMP from growth media.

2.1.2 Sumoylation of Tup1

Post translational modification by SUMO, or the Small Ubiquitin like Modifier is a very versatile protein tag owing to its role in regulation of transcription, chromatin structure, protein localization, DNA repair, signal transduction and so on. It is a member of the UBL family of proteins and its mechanistic pathway is similar to ubiquitin. A study by Ng et al, (2015) showed that Tup1 is sumoylated at two specific lysine residues, K229 and K270, at specific conditions[15].

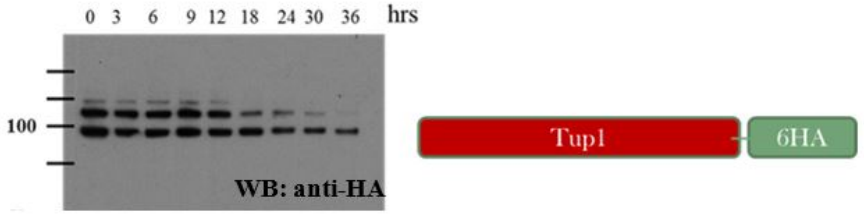


FIGURE 2.1: Western blot showing Tup1- HA levels in cells harvested at different time points of a culture. The higher molecular weight protein bands were found to be its multi-sumoylated forms. The level of sumoylation is seen to decrease progressively after the diauxic shift. Image from Poulami Choudhuri.

In our lab, it was shown (by Poulami, a PhD fellow) that the level of sumoylation of Tup1 reduced progressively after the diauxic shift. Given, the role of sumoylation in mediating its transcriptional repression, this decrease in the sumoylation after the

diauxic shift might be play a role in the inhibition of Tup1 mediated glucose repression of genes post the diauxic shift.

2.1.3 The AAA ATPase Sap1

The ATPases Associated with diverse cellular Activities are abbreviated as AAA proteins. These are a large family of proteins who share a structurally conserved module and employ ATP binding and hydrolysis to act on its target substrates. Their functions, however, are vastly diverse in that they are involved in cell cycle regulation, cellular transport, organelle biogenesis, protein homeostasis and so on.

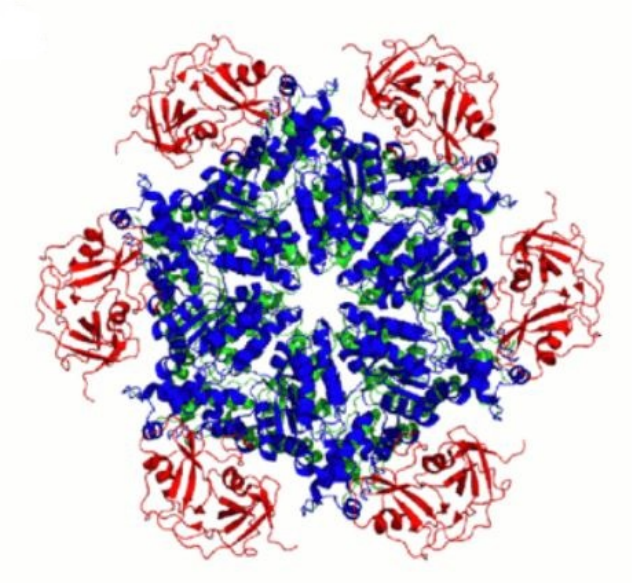


FIGURE 2.2: The 3D ribbon structure of Cdc48 showing its AAA-ATPase domains (shown here in green and blue)

The protein Sap1 is named so for its interaction with SIN1 (Sin1 Associated Protein)[16]. It is an ATPase and the sequence and structure predictions of Sap1 shows significant homology to the conserved family of AAA proteins making it a AAA ATPase. The SIN1 protein primarily functions as a transcriptional repressor and antagonizes the function of the SWI/SNF ATP dependent chromatin remodeling complex[17]. Sap1 is shown to interact with SIN1 both in vitro and in vivo but the function of Sap1 remains unclear and is being studied upon. Sap1 is also found to have a SUMO Interaction

Motif (SIM) where it is seen to bind to a non-conjugatable form of SUMO[18]. In our lab, Sap1 is also shown to interact with Snu66 (Experiment performed by a PhD fellow, Poulami)˙

2.1.4 Sap1, the shorter isoform and Tup1

Protein isoforms are proteins which are formed from the same gene locus that are splice variants or result from PTMs or various promoters. They can be functionally similar or have unique functions. On studying Sap1, it was found in our lab that a shorter isoform of Sap1, the Sap1_C is also being expressed. The promoter for this shorter isoform is found to be present within the ORF of SAP1. The expression of Sap1_C is not constitutive.

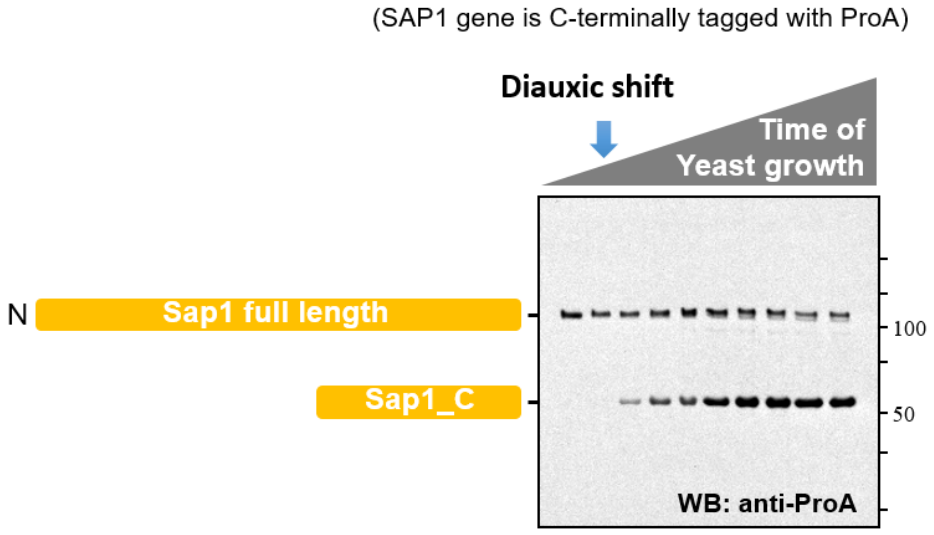


FIGURE 2.3: The expression of Sap1_C, the shorter isoform of Sap1. This expression is seen to start and gradually increase after the diauxic shift. Image from Dr. S.K. Mishra.

It was found to be under glucose repression, whereby genes primarily involved in catabolizing other carbon sources are inhibited from expression in favour of utilizing only

glucose. When glucose levels are substantial in the growth medium, yeast cells prefer glycolysis and usually undergo logarithmic growth. This repression is dependent on the cell's ability to detect extracellular glucose concentrations[19]. When the growth media becomes exhausted of glucose, the cells undergo a diauxic shift whereby the cells rapidly switch from fermentative growth to respiration and starts using ethanol as its primary carbon source. Once this switch happens, the glucose repressed genes starts getting expressed in the cells. Sap1_C expression happens in this mode. It is expressed only post diauxic shift. A genetic screen performed by Dr. Mishra between LacZ under the Sap1_C promoter and deletion strains revealed that the expression of the shorter isoform of Sap1 is regulated by Tup1.

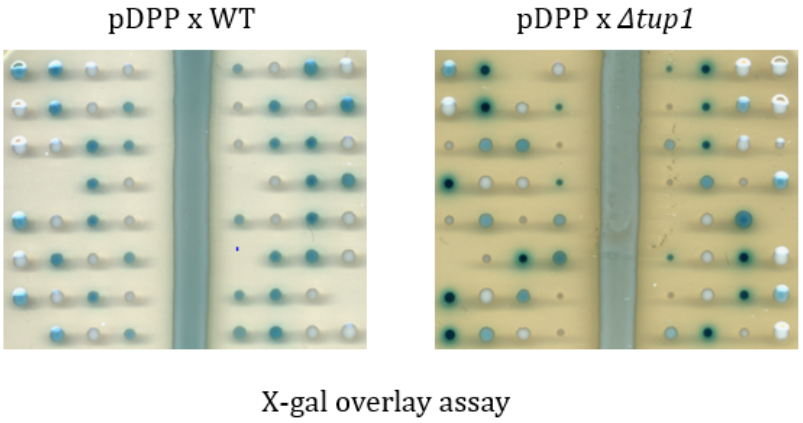


FIGURE 2.4: A genetic screen between LacZ under Sap1_C internal promoter (pDPP) and deletion strains, showed that Tup1 regulates the internal promoter of Sap1. Image from Dr. S.K. Mishra.

Tup1 is already known for its role in mediating glucose repression in cerevisiae along with. It is observed that in *TUP1* deletion mutants, Sap1_C is no longer regulated or under glucose repression and its expression is seen to be constitutive, independent of the diauxic shift.

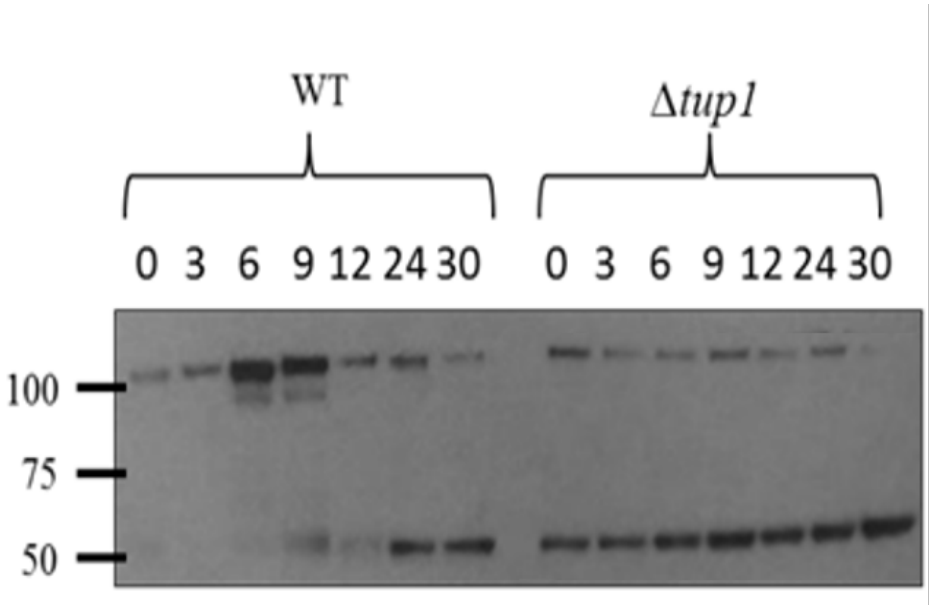


FIGURE 2.5: Sap1_C expresses after diauxic shift in WT cells whereas in the tup1 deletion strain it expresses from the beginning of growth. Image from Poulami Choudhuri.

2.1.5 Aim

From figures 2.3 and 2.4 we see that Sap1_C expression is being regulated by Tup1. Considering that Tup1 is sumoylated at two specific lysine residues – K229 and K270. The findings in Ng et al,(2015) shows that this sumoylation controls the timing of Tup1 regulated repression of transcription. In this context, my experiment was aimed at studying the impact of sumoylation of Tup1 on its regulation of the Sap1_C expression. We hypothesized that the binding of Tup1 at Sap1_C promoter might be dependent on its sumoylation. As the cell grows and undergoes diauxic shift the sumoylation of Tup1 is inhibited and it falls off from the promoter region, thus no longer repressing the transcription of Sap1_C.

2.2 Experimental Methods

2.2.1 ONPG ASSAY FOR β GALACTOSIDASE

2.2.1.1 Cell Harvesting

A primary culture was left to grow overnight at 30° from which a secondary culture was inoculated at 0.1 OD_{600} and left to grow till 0.8 OD_{600}. From the 2° culture a tertiary culture was started at 0.1 OD. Cells were harvested at roughly 7 hours (approx. 1 OD600) and around 25 hours where the OD_{600} was around 7. 3 OD_{600} cells were harvested for each sample, frozen using liquid nitrogen and stored at -80° C.

2.2.1.2 The ONPG assay

The harvested cells were resuspended in 200 µl of Z buffer and then lysed by two freeze thaw cycles (using liquid nitrogen). To measure the β galactosidase activity, the reaction was initiated by an addition of 500 µl of Z buffer with 1.35 µl of ONPG solution. The samples were observed for appearance of yellow colouration while incubating at 30°. On appearance of fairly intense colour, the reaction was quenched by adding 500 µl of Na2CO3 (1M) while also noting the incubation time. This was followed by centrifugation and then the absorbance value of the samples at 405 nm was measured using Bio Photometer against a blank containing Z buffer, ONPG and Na_2CO_3 solutions.

The β Galactosidase activity was calculated using the following formula:

$$\beta = \frac{1000 * OD_{405}}{t * V * OD_{600}}, \tag{2.1}$$

Where t is the incubation time in minutes and V is the volume of cells harvested in ml.

Z BUFFER Composition:

$Na_2HPO_4 \cdot 7\,H_2O$(0.06M), $NaH_2PO_4 \cdot H_2O$(0.04M), KCl(0.01M), $MgSO_4$(0.001M), β-mercaptoethanol(0.05M) (to be added freshly before use).

pH adjusted to 7.0.
ONPG solution: ONPG 4mg/mL in 0.1M phosphate buffer pH 7.0 to be freshly prepared.

Phosphate Buffer
$Na_2HPO_4 \cdot 7\,H_2O$(0.06M), $NaH_2PO_4 \cdot H_2O$(0.04M)
pH adjusted to 7.0.

2.3 Result

2.3.1 Construction of Strains

Plasmid pSL9 consisting of 300 base pairs of the Sap1_C internal promoter (pDPP) fused with LacZ were used as reporters of Sap1_C activity.
Strains Sc71(*tup1Δ::KANMX[pTUP1-HA/CEN URA3*])and
Sc72(*tup1Δ::KANMX[pTUP1-HA-K229,270R/CEN URA3*]) with *TUP1* cloned in *pRS316* plasmid were obtained from James L. Manley[15]. The reporters could not be used in the strains owing to both *pSL9* and *pRS316* having *URA3* marker. As an alternative the experiment was pursued in the manner elucidated in the schematic given below:

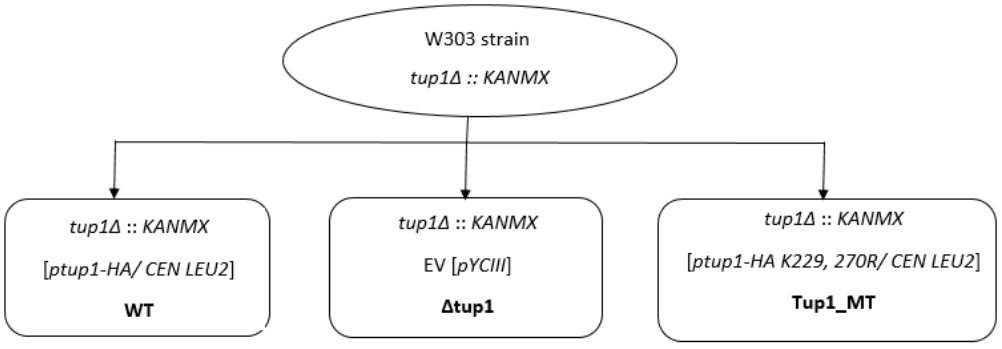


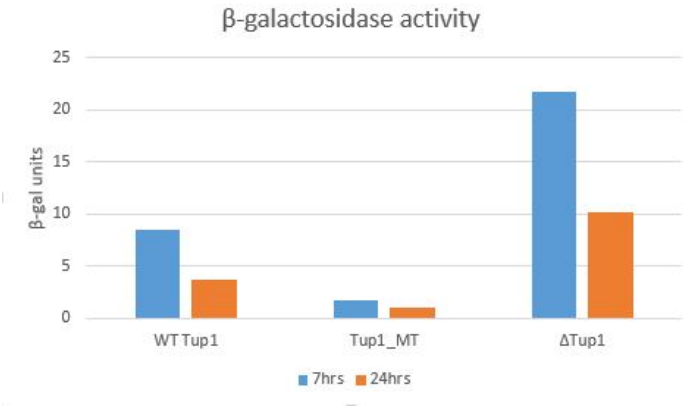
FIGURE 2.6: A schematic of the strain construction for experimental progression[20].

The β galactosidase activity reporters were then transformed in these strains WT, Δtup1 and the Tup1_MT which has K229, 270R mutated to perform the ONPG assay.

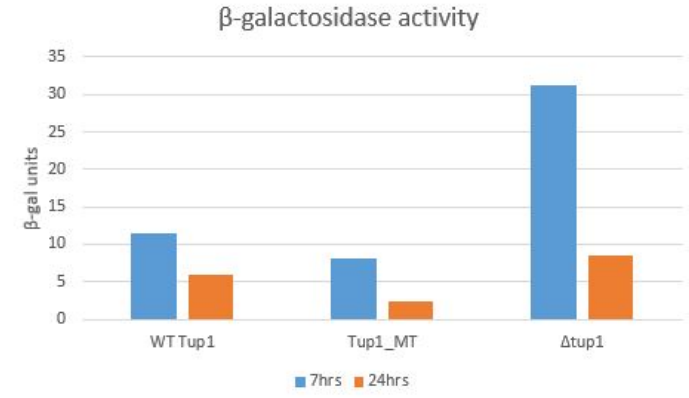
2.3.2 Sap1_C is over expressed in Δ*tup1* mutant

Compared to the WT, the β galactosidase activity is increased 3 fold in Δ*tup1* mutant suggesting a significant over expression of Sap1_C in Δ*tup1* mutant. However

the increment in the level of Sap1_C post the diauxic shift could not be captured by performing the assay. The β galactosidase activity units are observed to be decreasing as opposed to increasing after the diauxic shift. This is in contradiction to the previous observations.



(A)



(B)

FIGURE 2.7: The β galactosidase activity units of WT, Tup1_MT and Δtup1 cells where cells are harvested around 1 OD_{600} (7 hours) and 7 OD_{600} (24 hours). The graphs A and B are for two different sets of samples

2.3.3 Sumoylation deficient Tup1 is a better suppressor of Sap1_C

From the data obtained from the β galactosidase assay, it appears that sumoylation decreases the efficiency of Tup1 in repressing Sap1_C. We had expected that the activity shown by Tup1_MT to be comparable to Δtup1 but this is not the case.

The β galactosidase activity shown by sumoylation deficient Tup1 mutant Tup1_MT (K229R, K270R) appears to be lesser in comparison to the activity shown by WT cells suggesting that sumoylation of Tup1 reduces its efficient repression of the Sap1_C.

2.4 Discussion

From the ONPG assay, it is seen that Tup1 is indeed the regulator of Sap1_C. The increased β galactosidase activity shown by the Δtup1 mutants suggests a constitutive expression of the shorter isoform. The glucose dependent repression however, could not be captured by ONPG assay. The increase in β galactosidase activity after the diauxic shift is not visible in this assay. The sumoylation deficient Tup1_MT showed a decreased β galactosidase activity. This suggests that sumoylation of Tup1 plays a negative regulatory role in its repression of Sap1_C. The lower β galactosidase activity of Tup1_MT compared to the wild type cells shows that sumoylation deficient Tup1 is a better repressor of Sap1_C than WT Tup1. These results are not in consensus with Ng et al,2015, which establishes that the depletion of sumoylation of Tup1 leads to increased transcription. Further conclusions can be drawn if we get corroborating results on monitoring protein levels of Sap1_C in Tup1_MT cells through western blot.

Appendix A

Strains used

A.1 *S. pombe*

SP1	h- WT
SP2	h+ WT
SP140	h-JY41 rap1-6HA::NAT-NT2
SP143	h-JY41 hif2-6HA::NAT-NT2
SP144	h-JY41 rxt2-6HA::NAT-NT2
SP145	h-JY41 dsh1-6HA::NAT-NT2
hub1-1	h-PEM2 hub1-142S::NAT-NT2

Table A.1: *S. pombe* strains

A.2 *S.cerevisiae*

Sc 71	tup1Δ::kanMX[ptup1-6HA/CEN URA3]
Sc 72	tup1Δ::kanMX[ptup1-6HA-K229,270R/CEN URA3]
Sc 104	tup1Δ::kanMX[YCplacIII]
Sc 105	tup1Δ::kanMX[ptup1-HA/CEN LEU3]
Sc 106	tup1Δ::kanMX[ptup1-HA/K229,270R/CEN URA3]

Table A.2: *S. cerevisiae* strains

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