Validation of interaction between Fir1 and Rtt103 via Co-Immunoprecipitation

The project is submitted for partial fulfilment of the degree

MASTER OF SCIENCE

to the

Department of Life Science and Biotechnology, Jadavpur University

by

BIJAYA NAG

(Reg no-142826 of 2017-2018)

Under the guidance of

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CERTIFICATE

This is to certify that this thesis entitled “Validation of interaction between Fir1 and Rtt103 via co-immunoprecipitation” submitted in partial fulfilment of the requirement for the award of degree of Master in Life Science and Biotechnology from Jadavpur University, Kolkata, West Bengal is a record of bonafide work carried out by Bijaya Nag in my laboratory.

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Acknowledgement

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Finally I would like to thank all of my family members for their continuous encouragement and care.

Bijaya Nag

CONTENTS

List of Abbreviations

APS	Ammonium per sulphate
DSB	Double strand break
EDTA	Ethylene-diamine-tetra acetic acid
FIR1	Factor Interacting with REF2
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
NHEJ	Non-homologous end joining
HU	Hydroxyurea
mRNA	Messenger RNA
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulfonyl Fluoride
PVDF	Polyvinylidene Fluoride
RTT103	Regulator of Ty1 Transposition
SDS	Sodium dodecyl sulphate
TAP	Tandem affinity purification
TBS	Tris-buffered saline
TBST	Tris Buffered Saline with Tween 20
TCA	Trichloroacetic acid
TEMED	Tetramethylethyldiamine
YKU80	Yeast KU Protein
YPD	Yeast extract peptone dextrose

INTRODUCTION

The whole hereditary information of an organism that is encoded in the DNA is called genome. Genes provide the information for making all proteins that are necessary for the expression of characters.

Genome is found inside every cell,and in those that have nucleus,the genome is situated inside the nucleus.Yeast cell has 16 chromosomes with 5885 potential protein - coding genes.

Different forms of damage constantly occur in the genome and if these are not repaired, can lead to mutations and chromosomal aberrations that result in cell death and diseases like cancer. Hence cells have evolved several mechanisms to detect and repair damage to the genome. Damage to DNA induces a DNA damage response (DDR) that has three outcomes- first the cell cycle checkpoint that arrests the cell cycle in order to prevent damaged DNA from being replicated ; second the DNA repair pathways that repair the break and lastly an apoptotic pathway that leads to death of the cell in case the damage is irreversible. DDR includes functional interactions of DNA repair, transcription and RNA processing machineries. DNA damage is generally induced by UV radiation,genotoxic drugs,ionising radiation,or other metabolites that generate reactive oxygen species.These pathways of repair and checkpoint mediated cell cycle arrest are conserved across the eukaryotes.

A particularly dangerous form of damage is the DNA double – strand break (DSB).These may arise due to exposure of DNA to ionising radiations or genotoxic drugs. Cells repair this form of DNA damage either through non –homologous end joining (NHEJ) or through homology-mediated repair.YKU70/80, a heterodimer plays an important role in repair of DNA breaks by NHEJ. They are also important for many telomeric functions, including loading of telomerase to telomeres protecting them from nucleolytic digestions.In the process of isolating suppresors that contribute to the multiple roles of YKU proteins, RTT103 was identified as a partial suppressor of temperature sensitivity. RTT103 is essential for recovery from DNA double strand breaks in the chromosome.It also associates with the sites of DNA breaks and hence is likely to play a direct role in response to DNA damage.
RTT103(Regulator of Ty1) is an abundant nuclear protein which is also known to function as

transcription terminator is critical for maintaining the genome integrity. RTT103 contains a carboxyl-terminal domain interacting domain (CID) which interacts with C terminal domain of RNA polymerase and proteins are recruited via this interaction to the actively transcribed chromatin. RTT103 is not essential but synthetically lethal with several RNA 3'-ends of genes,like Rat1 and along with them facilitates termination of transcription. It is possible that termination complexes become associated with DNA damage sites in order to prevent synthesis of aberrant transcripts.

Fir1 (Factor Interacting with REF2p) is involved in 3' mRNA processing; it has a small but detectable role in influencing the efficiency of poly (A) site use. It interacts with RNA polyadenylation factors and its deletion results in the shortening of poly (A) tails. Polyadenylation is a part of larger process of gene expression. Poly (A) tails protects mRNA molecule from enzymatic degradation in the cytoplasm and aids in transcription termination, export of the mRNA from the nucleus, and translation. All eukaryotic mRNAs are polyadenylated except replication –dependent histone mRNAs. Processing of eukaryotic mRNA 3'ends is coupled to transcription termination by RNA polymerase II.The same transcribed sequences that signal polyadenylation also contribute to release of polymerase from the template. Defects of mRNA at 3' ends can profoundly affect cell viability, growth and development. This mRNA 3'processing is strongly but transiently inhibited upon DNA-damaging conditions. Fir1 is an APC/C ^{Cdh1} substrate, so it is degraded in the G_1 phase and is abundant in M phase. In our experiment one set of cells were arrested in the S phase by employing Hydroxyurea.

The role of 3'-end processing in DDR supports the idea that the steady state levels of different mRNAs change upon DNA damaging conditions due to regulation of not only their biosynthetic but also their turnover. Yeast strains containing a disrupted FIR1 gene are slightly less efficient.

In our experiment we are trying to validate whether RTT103and FIR1 interacts with each since the interaction is already observed in yeast two hybrid assay. There is also growing evidence that the connections between mRNA processing and DNA damage responses may be disrupted in human diseases, potentially contributing to genome instability and carcinogenesis.

TAP-tag purification

Tandem affinity purification (**TAP**) is a purification technique for studying protein–protein interactions. It involves creating a fusion protein with a designed piece, the TAP tag, on the end.

In the original version of the technique, the protein of interest with the TAP tag first binds to beads coated with IgG, the TAP tag is then broken apart by an enzyme, and finally a different part of the TAP tag binds reversibly to beads of a different type. After the protein of interest has been washed through two affinity columns, it can be examined for binding partners.

The original TAP method involves the fusion of the TAP tag to the C-terminus of the protein under study. The TAP tag consists of calmodulin binding peptide (CBP) from the N-terminal, followed by tobacco etch virus protease (TEV protease) cleavage site and Protein A, which binds tightly to IgG. The relative order of the modules of the tag is important because Protein A needs to be at the extreme end of the fusion protein so that the entire complex can be retrieved using an IgG matrix.

History

TAP tagging was invented by a research team working in the European Molecular Biology Laboratory at late 1990s and proposed as a new tool for proteome exploration. It was used by the team to characterize several protein complexes.

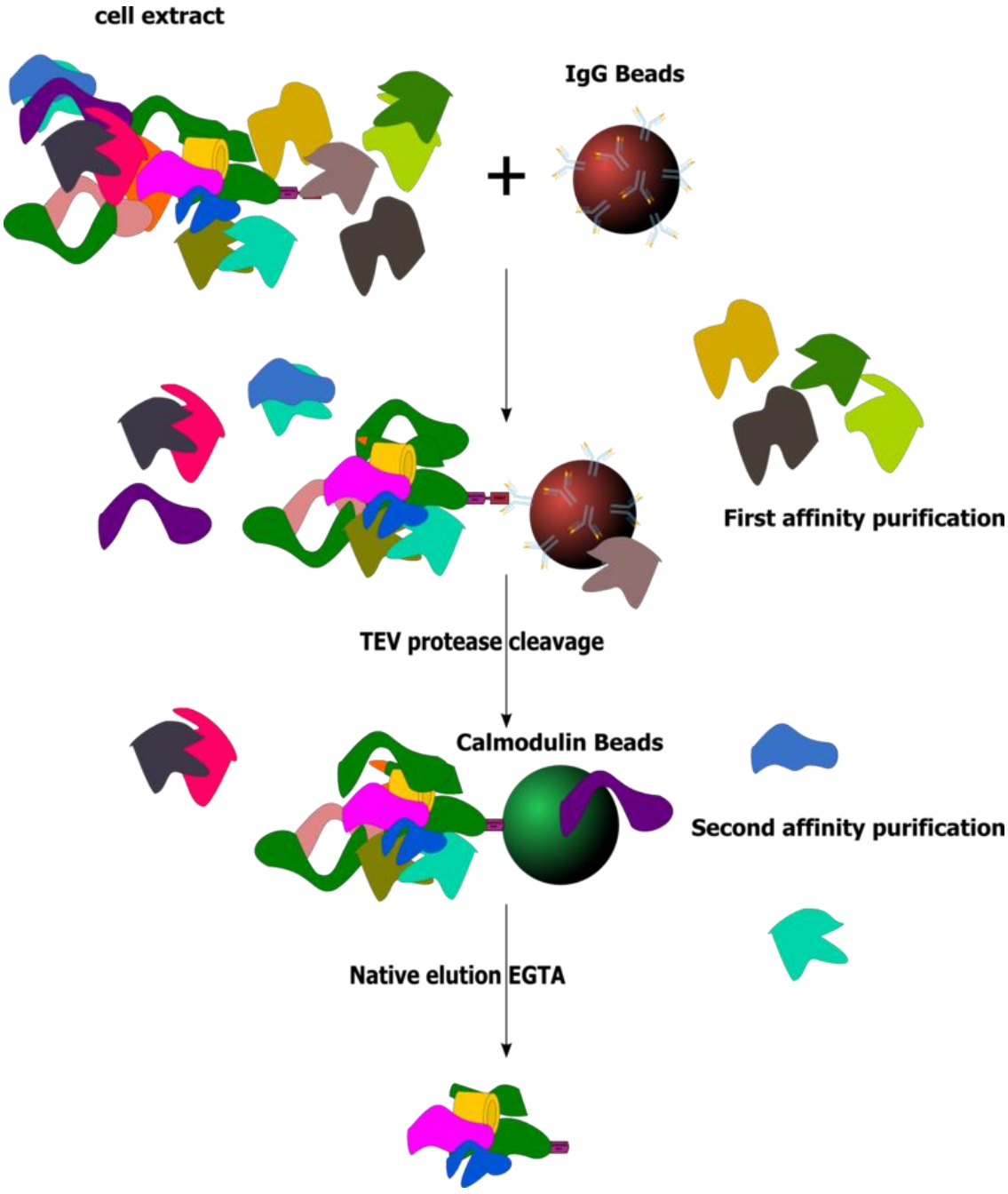
Once the fusion protein is translated within the host, the new protein at one end of the fusion protein would be able to interact with other proteins. Subsequently, the fusion protein is retrieved from the host by breaking the cells and retrieving the fusion protein through affinity selection, together with the other constituents attached to the new protein, by means of an IgG matrix.

Process

After washing, TEV protease is introduced to elute the bound material at the TEV protease cleavage site. This elute is then incubated with calmodulin-coated beads in the presence of calcium. This second affinity step is required to remove the TEV protease as well as traces of

contaminants remaining after the first affinity step. After washing, the elute is then released with ethylene glycol tetra acetic acid (EGTA).

The native elution, consisting of the new protein and its interacting protein partners as well as CBP, can now be analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) or be identified by mass spectrometry.



Advantages

An advantage of this method is that there can be real determination of protein partners quantitatively in vivo without prior knowledge of complex composition. It is also simple to execute and often provides high yield. One of the obstacles of studying protein interaction is the contamination of the target protein especially when we don't have any prior knowledge of it. TAP offers an effective and highly specific means to purify target protein. After 2 successive affinity purifications, the chance for contaminants to be retained in the elute reduces significantly.

Disadvantages

However, there is also the possibility that a tag added to a protein might obscure binding of the new protein to its interacting partners. In addition, the tag may also affect protein expression levels. On the other hand, the tag may also not be sufficiently exposed to the affinity beads, hence skewing the results.

There may also be a possibility of a cleavage of the proteins by the TEV protease, although this is unlikely to be frequent given the high specificity of the TEV protease.

Suitability

As this method involves at least 2 rounds of washing, it may not be suitable for screening transient protein interactions, unlike the yeast two-hybrid method or *in vivo* crosslinking with photo-reactive amino acid analogs. However, it is a good method for testing stable protein interactions and allows various degrees of investigation by controlling the number of times the protein complex is purified.

Applications

In 2002, the TAP tag was first used with mass spectrometry in a large-scale approach to systematically analyse the proteomics of yeast by characterizing multiprotein complexes .Study revealed 491 complexes, 257 of them wholly new. The rest were familiar from other

research, but now virtually all of them were found to have new components. They drew up a map relating all the protein in components functionally in a complex network.

Co-immunoprecipitation

It is a technique of precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein. The process can be used to isolate and concentrate a particular protein from a sample containing many thousands of different proteins. It requires that antibody coupled to a solid substrate at some in the experiment.

AIM

To validate the interaction of Fir1 with Rtt103 using both wild type and hydroxyurea treated cells by co-immunoprecipitation method.

WORK PLAN

- Standardization of buffer for TAP-tag purification.
- Comparing the protein bands in the cell extracts.
- To observe the interaction of Rtt103 and Fir1 by Tandem Affinity Purification and Western Blotting.
- To check the synchronization of yeast cells post hydroxyurea release.

MATERIALS

1xTransfer Buffer(1 litre)

- Tris methylamine - 3.3 gm
- Glycine-14.5 gm
- Methanol-100 ml
- Volume make up till 1000 ml

10X TBS(200 ml)

- NaCl-16 gm
- Tris-4.8 gm
- Adjust pH to 7.6
- Tween 20- 200 µl

5X SDS Running Buffer(500 ml)

- Tris-7.5 gm
- Glycine-47 gm
- SDS-2.5 gm

YPD(Yeast Peptone Dextrose)(100 ml)

- Yeast Extract-1 gm
- Peptone-2 gm
- Dextrose-2 gm
- Agar-2 gm

Buffer A

- 50 mM HEPES-KOH
- 140 mM EDTA
- 1% Triton X-100

- 0.1% Na deoxycholate
- 1mM PMSF
- Protease Inhibitor Cocktail

Buffer B

- 200 Mm Tris-Cl
- 320 mM Ammonium sulphate
- 50 mM $MgCl_2$
- 10 mM EGTA
- 20 mM EDTA
- 1 mM DTT
- 20% glycerol
- 1 mM PMSF
- Protein inhibitor complex

Buffer C

- 50 mM Hepes pH 7.4
- 0.25% NP40
- 250 mM NaOAc
- 50 mM EDTA
- 5% GLYCEROL
- 1 mM DTT

Laemmali/Magic Buffer(for 10 ml)

- 2.4 ml 1M Tris-Cl pH 6.8
- 4 ml 100% Glycerol
- 0.8 g SDS
- 4 mg Bromophenol Blue
- 0.5 ml β-mercaptoethanol
- 3.1 ml H_2O

SDS GEL COMPOSITION

Constituents	**10% 15ml Resolving gel**	**30% 5ml Stacking gel**
H_2O	5.9	3.4
Acrylamide mix	5.0	0.83
Tris(pH 8.8)	3.8	**_**
Tris(pH 6.8)	**_**	0.63
SDS(10%)	0.15	0.05
Ammonium persulfate	0.15	0.05
TEMED	0.006	0.005

METHODS

1. Extraction of whole cell protein from yeast cells by trichloroacetic acid (TCA) method

- The yeast cells are inoculated in YPD and grown overnight at 37 C. The cells are collected by centrifugation and resuspended in 200 µl of 20% of TCA; glass beads are added up to the meniscus and lysed by vortexing for 3 minutes.
- Suspension is recovered and transferred to a new eppendorf tube. The glass beads are washed twice with 200 µl of 5% TCA and washes are added to the previous suspension.
- The eppendorf is centrifuged at 13000 rpm for 10 minutes. At this stage a protein pellet is obtained. The supernatant is discarded well and the pellet is resuspended in 200 µl of 1X Laemmli buffer.
- The Laemmli buffer turns red because of low pH, so 1M Tris base is added until it turns blue (usually 50 µl).
- The sample is boiled for 3 minutes (in dry water bath) and centrifuged again at 3000 rpm for 10 minutes. The protein sample is transferred to a new tube and the pellet is discarded (unlysed cells mainly).

SDS-PAGE and western blotting

The protein extract obtained for TAP-tagging is used for Western Blot to compare the protein bands expressed in Fir1, Rtt103 and Yku80.10% Resolving Gel and 30% Stacking Gel is used. After the gel is polymerized, it is placed in the electrophoresis tank and run. The samples are allowed to stack at 60V and resolved at 120V.Once the protein has resolved ,they are stained using Coomassie brilliant blue.

For detecting a specific protein in the extract using antibody, the separated proteins are transferred to a PDVF membrane, pre-soaked 100% methanol after separating the proteins by SDS-PAGE. The membrane was equilibrated with transfer buffer before onto the dry blotter. The order of assembly was “Three filter papers-gel-membrane-three filter papers”. All the above mentioned materials were equilibrated in transfer buffer; the gel was kept towards the cathode. Transfer was performed by applying 400mA for 1-2 hours at room temperature. The

membrane is washed with 1X TBST after transfer. Skimmed milk powder (2 gm in 40ml 1X TBST) is used for blocking. It is kept on dancing shaker for 1 hour. Myc antibody is used to tag Fir1.The incubation with primary antibody (anti-myc) is done at room temperature for 2 hours with shaking. The membrane is washed with 1X TBST three times, 5 minutes for each wash. The incubation with secondary antibody (HRP-tagged IgG) is done at RT for 2 hours. The membrane is washed with 1X TBST three times, 5 minutes each. The presence of protein is detected by chemiluminesce (H_2O_2-Luminol).

Extract Preparation:

50 ml of *S.cerevisiae* strains are grown to 0.8-1.0 O.D.The cells are collected by centrifugation.Pellets are resuspended in 1 ml Buffer A. These are transferred to 2 ml eppendorfs containing glass beads up to the meniscus and lysed by vortexing in a Bead beater for 8 cycles (1st four cycles for 40 seconds each rest for 30 seconds with 2 minutes incubation in ice.).The eppendorfs are sealed with paraffin strips prior to vortexing. The vortexed tubes are pierced and each one is fitted to the mouth of another tube and inserted into a 50ml falcon. The falcons are given a short spin at 4000 rpm. The eppendorfs are spinned at 4ºC for 10 minutes at 1500 rpm. Tubes are placed in ice. The supernatant is transferred to another 2 ml eppendorf. An aliquot is kept for analysis.

Pre-clear:

To the remaining supernatant 100µl-200µl of pre-washed sepharose beads(in lysis buffer) is added and incubated at 4ºC on an end-over-end rotar.The beads are spinned down at 15000 rpm for 4 minutes and the supernatant is discarded.

Process downstream:

The beads are again washed in Briggs lysis buffer and spinned down at 1500 rpm for 4 minutes and the supernatant is discarded.1X 80µl Laemmli Buffer was added and boiled them at 100 ºC for 5 minutes then vortexed. The sample is then spinned at 15000 rpm for 5 minutes and supernatant is collected.40µl of this pull down sample is used for Western Blot.

RESULTS

In this study the interaction between Fir1 and Rtt103 was determined by performing co-immunoprecipitation. Earlier in the lab, a physical interaction between these two proteins of interest was established by yeast two hybrid method. For Co-IP, the Buffers were selected depending upon their ionic strength. The ionic strength of Buffer A (140 mM NaCl)is lower to that of Buffer B (250mM NaOAc) and Buffer C (320 mM).Buffer A was finally selected for Co-IP as higher concentration of salt in lysis buffer is more disruptive of protein-protein interaction and can also perturb SDS-Polyacrylamide gel electrophoresis. In the strain expressing Fir1 tagged with a 13myc epitope was grown in YPD broth, overnight and extracts are prepared in different lysis buffers-Buffer A, Buffer B, Buffer C. Proteins were also precipitated by TCA method. Duplicate set of sample extract was used for each buffer, except TCA.For Buffer A, duplicate set of 10µl and 20µl of sample extract was loaded onto gel, for the rest of the buffers and TCA extract,20µl was loaded .The extracts were run in SDS Gel and protein bands obtained were compared. Marker was loaded in one of the wells to observe the molecular weight of the proteins. The higher molecular protein bands in buffer A were more resolved compared to the other buffers and TCA extract. The bands in 10µl of Buffer A are much fainter compared to 20µl extract protein bands.

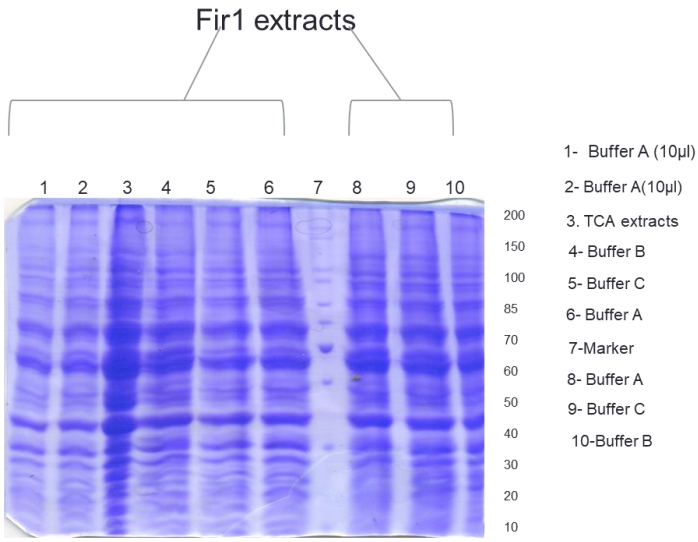


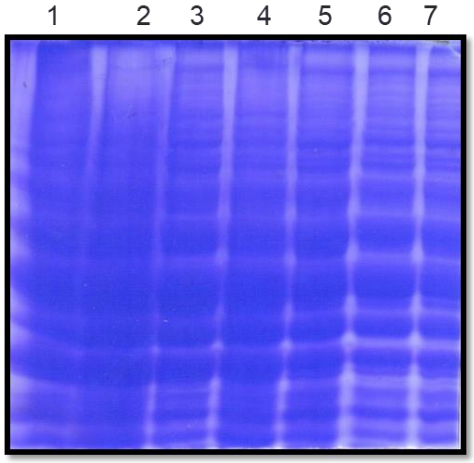
Fig 1: Standardization of Buffer for TAP-tag purification

The protein bands in the Buffer A are much more resolved compared to Buffer B and Buffer C due to its appropriate ionic strength (140 Mm NaCl) ,which is the natural ionic

strength of a cell and stabilizes the protein aptly. Hence it is chosen for TAP-tag purification technique.

Protein band comparision of Normal and HU treated extracts

All the three strains-Rtt103-TAP, Fir1-myc and Yku80-TAP are treated with 200mM hydroxyurea and both the wild type and S-phase arrested cell extracts were run on SDS-polyacrylamide gel to confirm for Co-IP.



1-Fir1-myc TCA extract
2-Fir1 –myc HU extract
3-Yku80-TAP HU extract
4-Rtt103-TAP HU extract
5-Fir1-myc normal extract
6-Yku80-TAP Normal extract
7-Rtt103-TAP normal extract

FIG 2: Confirmation of extracts for co-immunoprecipitation.Rtt103-TAP M.W 67.475 kDa and Fir1-13 myc M.W. 113.72 kDa

Protein content was found satisfactory and the extracts were confirmed for co-immunoprecipitation.

Co-immunoprecipitation analysis of Rtt103 and Fir1

The interaction of Fir1 with Rtt103 is already been found by Yeast Two Hybrid Assay, in order to validate this result we are performing Co-IP of Fir1 with Rtt103.Immunoprecipitation was done with TAP, which has affinity for IgG and Fir1 was tagged with 13 Myc and immunoprobed with Anti-myc antibody.

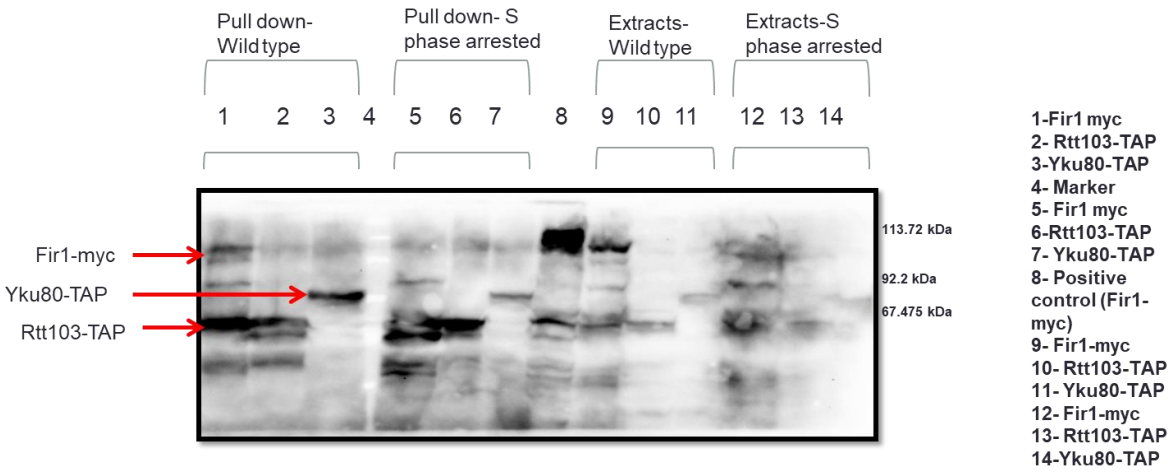


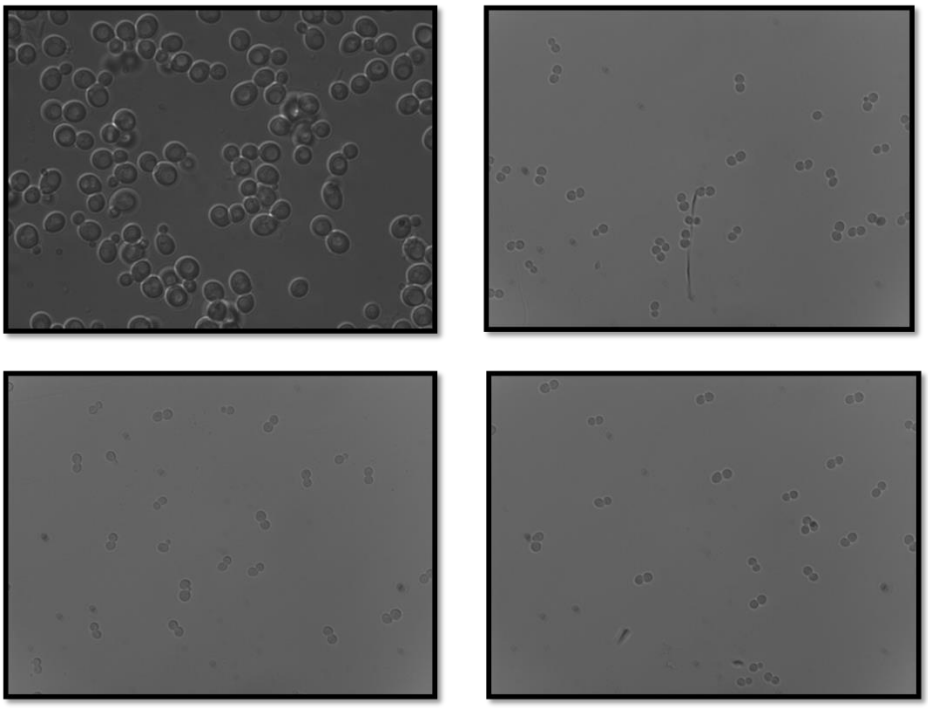
Fig 3: **Pulling down Fir1-Myc in both Wild type and S phase arrested condition with Rtt103-TAP.40µL of extract was taken as input and loaded on a 10% gel. The Western Blot was developed with anti-myc antibody.**

Co-IP results reveal that there is an interaction between Fir1 and Rtt103 in Fig 3 .The Yku80-TAP-tagged extract is taken as a negative control as it does not interact with Rtt103 or Fir1. Fir1-Myc extract are taken as positive control.

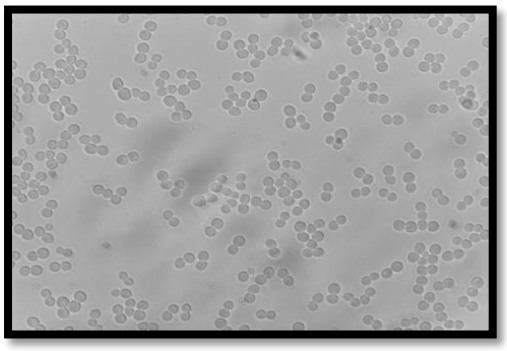
Cell cycle arrest by Hydroxyurea In order to determine whether the interaction of Rtt103 and Fir1 is cell cycle dependent, we arrested the cells in S phase.Fir1 strain was inoculated in 300ml YPD broth overnight at 30°C. O.D was measured to be 0.965. 200mM of hydroxyurea was added to it. Incubated for half an hour. A slide is prepared and checked

under fluorescence microscope for cell arrest. Cells are washed with fresh YPD broth and then with dH_2O.Cells are again checked under fluorescence microscope post release at 40 minutes interval. Cells are counted for percentage of synchronization.

Time post HU treatment=0 minutes



Time post HU RELEASE=40 MINUTES



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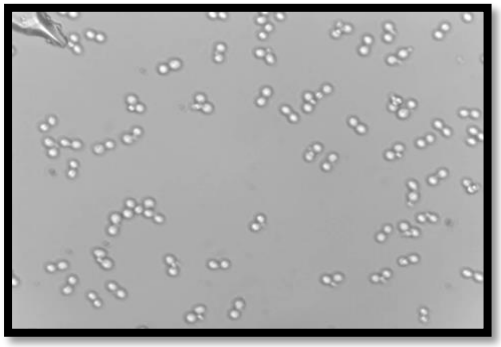
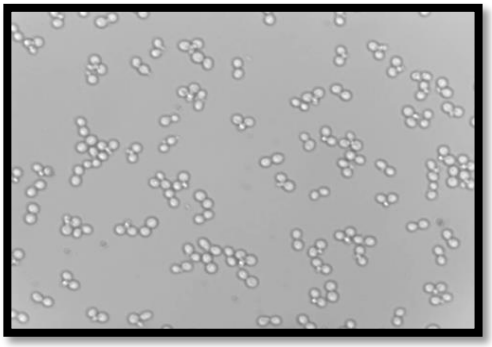

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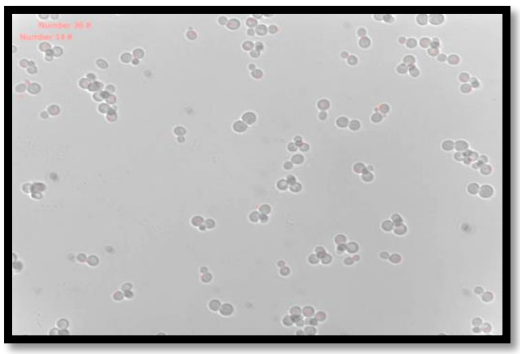
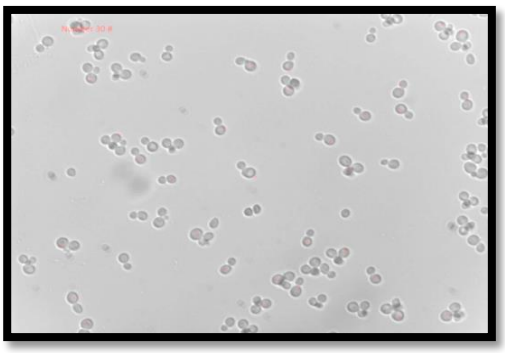

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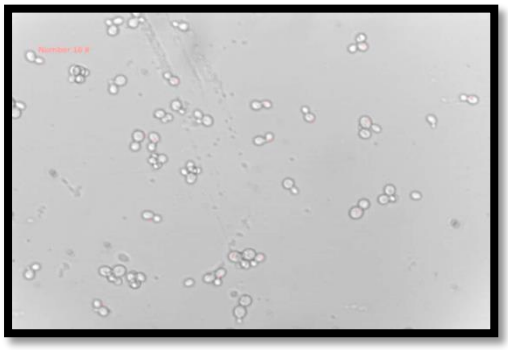
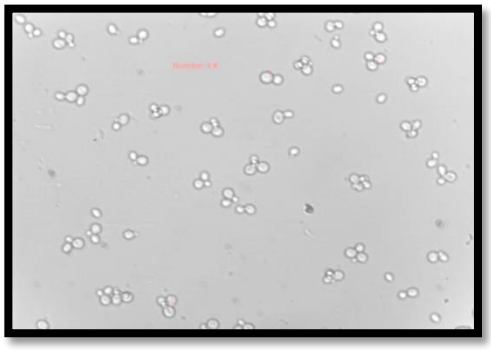

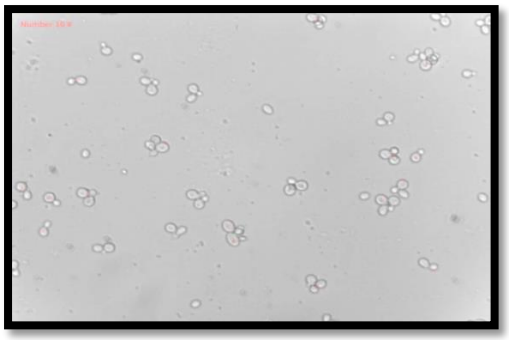

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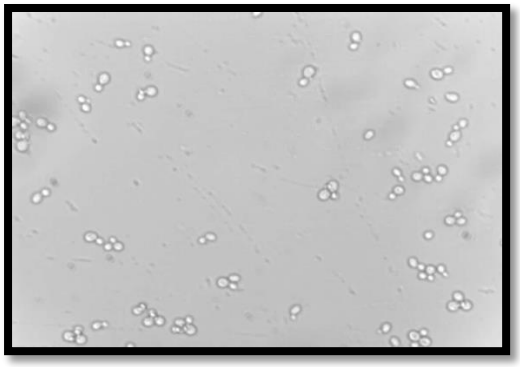

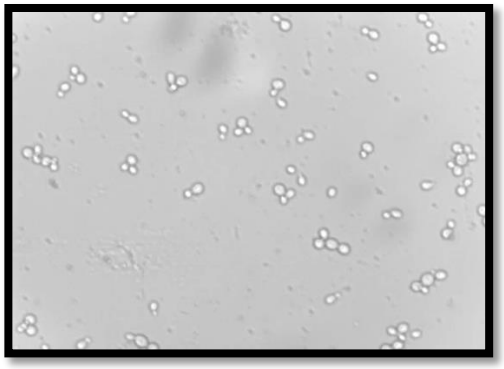

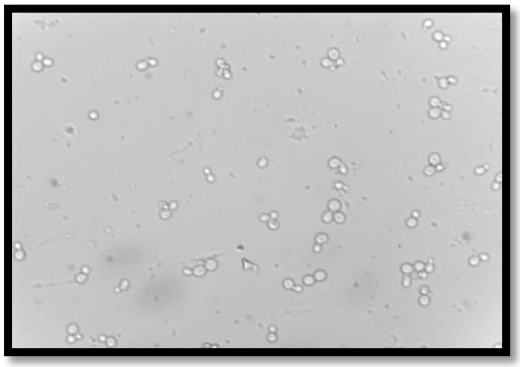

Observed percentage of cell cycle synchronization post HU treatment

Time post Hydroxyurea treatment(in minutes)	**No. of cells observed**	**Small buds(G1)**	**Medium buds(S phase)**	**Large buds(late phase)**	**% Synchronization**
0	**100**	**17**	**83**	**-**	**83**

Observed percentage of cell cycle synchronization post HU release

Time post Hydroxyurea Release(in minutes)	**No. of cells observed**	**Small buds (G1)**	**Medium buds (S phase)**	**Large buds(late s)**	**% Synchronization**
40	100	3	4	93	93
80	100	42	1	57	57
120	100	27	65	8	65
160	100	52	43	5	52
200	100	50	44	6	50

The cell cycle synchronization percentage was characterized till 200 minutes. The level of Fir1 13 myc at each stage has to be characterized via TCA extraction by subjecting it to Western Blot.

DISCUSSION

In this work, we have validated the interaction of Rtt103, a protein involved in transcription termination and Fir1, involved in transcription termination and Fir1, protein involved in 3'mRNA processing .Rtt103 is essential for survival when genome integrity is compromised.Rtt103 also has a role in responding to DNA breaks. It is possible that termination complexes may be associated with DNA damage sites to disengage RNA polymerase from the damaged sites. Rtt103 contains a carboxyl –terminal domain of RNA polymerase Ⅱ and proteins are recruited via this interaction to the actively transcribed chromatin.

Rtt103 is shown to be synthetically lethal with several RNA 3'-end processing factors, and its interaction with Fir1 was found in yeast two hybrid assay, which can be further studied.Fir1 is absent in G1 phase being a APC/C substrate ,whether this affects the function of Rtt103 can also further investigated. It may show mechanistic basis of prevention of aberrant transcripts and promote genome stability.

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