STUDY OF TRANSCRIPTION FACTOR IIIC (TFIIIC) IN DNA DAMAGE REPAIR (DDR) PATHWAY IN S. cerevisiae

M.Sc Thesis

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List of abbreviations

DNA	Deoxy-ribonucleic Acid
RNA	Ribonucleic Acid
NER	Nucleotide Excision Repair
BER	Base Excision Repair
tRNA	Transfer RNA
TFIIIC	Transcription Factor III C
RFC	Replication Factor C
HR	Homologous Recombination
NHEJ	Non-Homologous End Joining
MLB	Modified Lysis Buffer
PFGE	Pulse Field Gel Electrophoresis
MMS	Methyl Methane Sulfonate
qPCR	Quantitative PCR
HA	Haemagglutinin
dNTP	Deoxy-ribonucleoside tri-phosphate
EDTA	Ethylene diamine tetracetic acid
IP	Immunoprecipitation
DDR	DNA Damage Response
DSB	Double Strand Break
SSB	Single Strand Break
PI	Propidium Iodide
FACS	Fluorescence Activated Cell Sorter
COIP	Complex Immunoprecipitaion
ChIP	Chromatin Immunoprecipitation
RPA	Replication Protein A

mRNA	messenger RNA
PMSF	Phenyl methylsulfonyl-fluoride
TEMED	N,N,N',N'-tetramethylethelenediamine
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide Gel Electrophoresis
PCI	Phenol:Chloroform:Isoamylalcohol
PCR	Polymerase chain reaction
YEP	Yeast extract and peptone medium
OD	Optical Density
A_{600}	Absorbance at 600 nm
bp	Base pairs
Pol I	RNA Polymerase I
Pol II	RNA Polymerase II
Pol III	RNA Polymerase III
PIC	Pre initiation Complex
WT	Wild-type strain
Mutant	Tfc3 mutant
Rescued/Res	Tfc3 complemented
Δ	Deletion of a DNA sequence
RT	Room temperature
° C	°C Celsius
V	volt
μg	Microgram
μL	Microliter
ng	Nanogram

nM	Nanomolar
kDa	Kilo Daltons
mg	Milligram
min	Minutes
mL	Milliliter
hr.	Hour

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Abstract

Life as we know exists on earth with DNA as a genetic material. DNA is chemically very stable in nature; still it is prone to damage in a due course of time. In order to keep the information in the DNA safe, DNA damage response pathways (DDR) have evolved in the cell. Other than typical DDR proteins, the proteins such as Pol II (TFIIH) are known to be involved in the DDR pathway, known as 'Transcription coupled repair' (TCR). Also, stalling of replication fork at tRNA genes and suppression of tRNA transcription found in yeast upon replicative stress points out to the fact that there must be some mechanism to safeguard these DNA segments as found in most actively transcribed genes.

Interaction study of TFIIIC with DDR protein followed by complex immunoprecipiatation (COIP) confirmation; a mutant of TFIIIC (tsv115) was taken and its response to the DNA damage in comparison with wild type was analyzed along with the levels of DDR proteins in various concentrations of genotoxins was analyzed using western blotting. Pulse field gel electrophoresis (PFGE) & comet assay were used to determine the extent of DNA damage in mutant, cell cycle progression were checked by fluorescence activated cell sorter (FACS) and to further rule out any artifact interaction of Ddc2 with Tfc1 was checked under increasing concentration of genotoxin. Also, check the presence of chromosomal rearrangement and gene copy no. comparison of mutant with wild type qPCR based analysis was carried out.

TFIIIC is a largest and most important transcription factor in having high affinity towards the DNA specially B-box region; which also exhibit extra transcriptional functions. This study suggests TFIIIC could have role in resuming paused replication fork. The Mec1/Ddc2 complex upon activation of replicative stress pathways removes the PIC (pre initiation complex) via TFIIIC and helping in suppression of RNA pol III to exclude the possibility of replication fork and transcription machinery collision. Thus, prevents the deleterious effect of fork collapse. TFIIIC having a role in DDR pathway other than disassembling of PIC and helping in proper DNA replication needs to be validated as there are not enough evidences in this study.

Keywords: TFIIIC, DDR, TFIIH, Extra transcriptional function, Ddc2, Rad9, PIC & Mec1/Ddc2 complex.

CHAPTER 1

Introduction

1.1. Background

DNA being a genetic material of the cell is also prone to the damage by the various factors, caused by both extrinsic and intrinsic factors (Lindahl, 1993; Sugiyama, *et.al*, 1994). The DNA damage repair pathway is well characterized and well known, especially the proteins involved in signal transduction and repair mechanism, but the way cell detects damage is not so well characterized and mostly unknown. Few proteins such as PARP, MRN, MRX, RPA are known to play a role as a sensor proteins that can actually detect damage and mostly have doughnut like shape or is a single strand DNA binding proteins (Zhou & Elledge, 2000). Also, damage detection is observed to be carried out by the proteins other than dedicated DDR proteins such as RNA polymerase also called detection by proxy (Sancar *et al*., 2004) , stalled replication fork at damaged site etc. So far no report has been observed that indicates the involvement of transcription machinery other than RNA pol II in DNA damage response.

RNA polymerase II is responsible for the transcription of mRNAs in the eukaryotic cells and produces all the protein coding mRNA which makes the DNA more prone to the damage as these region remains uncoiled and is accessible for the chemicals and also due to constant unwinding and rewinding damage is very common in such type of genes (Hanawalt & Spivak, 2008). RNA Pol II participates in the DDR response after stalling of RNA pol II at the damaged site followed by dissociation of RNA pol II, which in turn attracts the DDR proteins ultimately leading to the detection of damage. RNA Pol II has been found to be involved in the NER repair mechanism but no direct evidence in DSB or SSB have been observed yet.

In contrast to the RNA Pol II, RNA Pol III is also very active in cell as it is responsible for the transcription of housekeeping genes and is very actively transcribed (Schramm & Hernandez, 2002), though the length of the transcript is shorter than that of genes transcribed by RNA pol II; possibility of damage remains intact. Recent study suggests that, replication of tDNA is difficult and results in a replication fork pausing (Nguyen *et al*., 2010) though it is quickly relieved and restored, the strand breakage and damage in such an event remains a possibility. Regulation of RNA pol III by the replication checkpoint protein also points out to the fact that RNA pol III might be involved in the DDR pathway. As shown by Nguyen *et al*., 2010*,* regulation seems to be Rad53 dependent. Activation of Rad53 is however Mrc1, Mec1 dependent. As mec1 Δ fails to regulate pre-tRNA level during replicative stress, whereas in wild type pre-tRNA level decreases, suggesting Mec1 dependent regulation of RNA pol III in stress condition. Replication forks pausing at tRNA

gene are a quite normal and is kind of essential for proper replication as the replication fork and transcribing tRNA is fatal as it might result in DNA strand breakage. So, suppression of RNA pol III by the replication stress pathway and pausing of replication fork is a way to ensure proper replication in the cell. Though, what triggers regulation of tRNA gene suppression is not known, the mechanism seems to be Mec1 & Rad53 dependent. Although, Maf1 is also involved in the suppression of RNA pol III, it is clear that it does not play a central role in the regulation (Nguyen *et al*., 2010).

All these facts indicate a possible connection between DDR proteins (Mec1 & Rad53) with RNA pol III. A large scale proteomics analysis of RNA pol III and TFIIIC in the laboratory previously (data not published, manuscript in preparation) shows the interaction of TFIIIC with several DDR proteins. Particularly interesting interaction of Tfc1 and Ddc2 was confirmed by COIP experiment as well. The interaction points out to the fact that the TFIIIC might have a role in DDR pathway or in the replication stress pathway (Nguyen *et al*., 2010).

Also, plate assays done on the mutant of Tfc1 & Tfc3 (subunits of TFIIIC) showed sensitivity towards genotoxins supports the fact that there might be connection between these two proteins (previously done work, CCMB, 2014). TFIIIC is a large complex protein which is reported to have role more than just the transcription factor as it has been reported to show other functions as well (Donze, 2012).

1.2 Yeast as a Model Organism

The Yeast, *Saccharomyces cerevisiae,* is unicellular, eukaryotic organism belonging to the Kingdom Fungi & Phyla Ascomycota (Kurtzman & Fell, 2006); with a cell size of about 3-4µm but size may also vary (Walker *et.al*, 2002). Yeast has just over 6000 genes & 16 linear chromosomes, it has a quite compact genome with few introns and short intergenic regions compared to other eukaryotic organisms. Furthermore, the genome of the Yeast is also well characterized and completely sequenced. Though little primitive, Yeast has both sexual and asexual reproduction (Fig. 1).

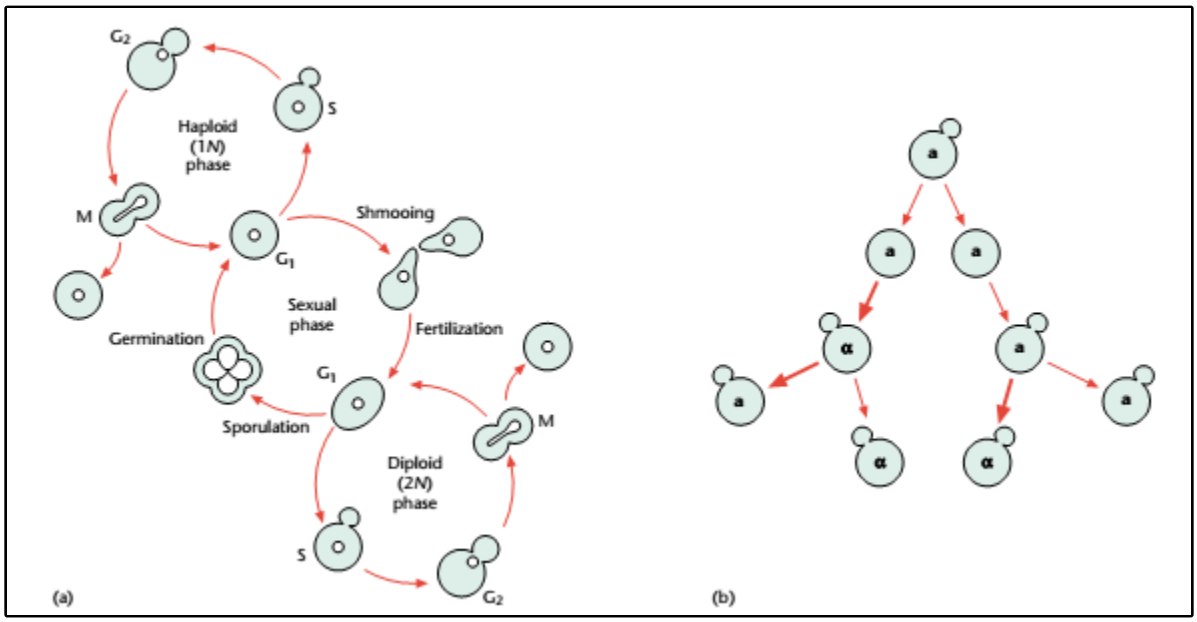


Figure 1: Life cycle of *Saccharomyces cerevisiae*. a) Alternation of generations and vegetative growth. b) Mating type switching in homothallic (Ho+) haploids. Mother cells switch in G1 (Mell & Burgess, 2002).

Being a eukaryotic organism, it can be grown in the laboratory just as bacteria, in YEPD media or simple media as PDA-starch media with doubling time of just above 1hr depending on the strain, making it easier to handle & serving as a good candidate for mutagenesis analysis, cloning & proteomics analysis (Yeast two hybrid system).

With small genome size & high gene density, it also contains most of the homologous counterparts to the higher organism making it perfect system to study the behavior of the proteins, genes and their effect as well as therapeutic effect and application before trying in higher organisms (Mell & Burgess, 2002). In case of DNA damage study yeast does have the orthologous proteins to that of higher organism as Mec1, KU70/KU80 heterodimer, Ddc2, Ddc1, Sml1 etc. (Cherry *et.al*, Saccharomyces genome database) Much of the knowledge regarding DNA damage, cellular check point actually came from the study done on Yeast

(Friedberg, 1991) making the yeast a good candidate as a model organism to study the eukaryotic DNA damage response.

1.3 DNA Damage: Causes and Types of DNA damage

DNA is a genetic material found in every cell of a living organism that stores the information about every protein, enzyme and the hereditary character possessed by an organism and its stability is very much important for a cell to be viable and functional. DNA can be damaged by various reasons such as ROS produced in the cell as a byproduct of an aerobic respiration, ionizing and non-ionizing radiations, replicative errors, collapse of replication fork due to transcription and replication collision and genotoxins etc. (Friedman & Brewer, 1995) (Fig. 2).

Basically, DNA damage can be categorized into two basic categories as endogenous i.e., spontaneous and exogenous i.e., induced. The simplest form of endogenous DNA damage is spontaneous hydrolysis of DNA such as Glycosidic bonds, AP sites hydrolysis, domination of the DNA bases, etc. (Lindahl T, 1993; Sugiyama *et.al,* 1994). Whereas exogenous or environmental DNA damage is brought about by the radiation, such as UV-rays which causes the thymidine dimer formation, ionizing radiations which causes single or double strand breakage (Ravanat JL, *et.al*, 2001). Another type of exogenous DNA damage is caused by the genotoxins i.e., chemicals. There are various types of chemicals that causes DNA damage such as alkylating agents as Methyl Methane Sulfonate and Temozolomide platinum compounds, and the natural product Mitomycin C, that cause DNA damage in the form of intrastrand and interstrand cross-links (Noll *et.al*, 2006) Chemotherapeutic drugs, topoisomerase inhibitors etc.

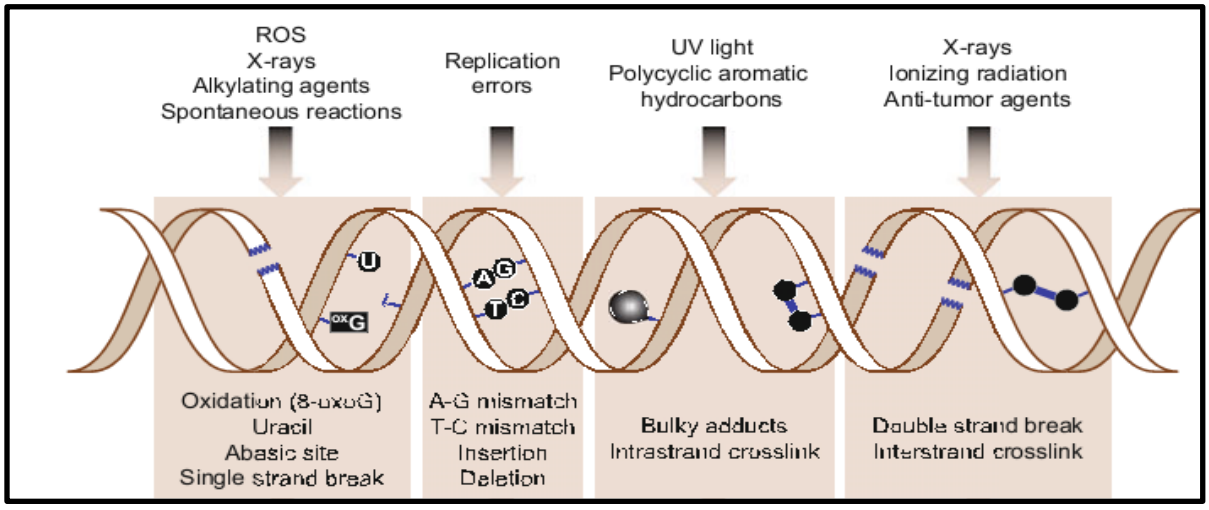


Figure 2: Illustration of Types of DNA damage and its causes. (Dexheimer, 2013)

1.4 DNA Damage response (DDR)

DNA is prone to the damage and its detection and proper repair is very much crucial for the survival & normal functioning of the cell. DNA damage response includes detection & removal of the damage and restoration of duplex, transcription activation of relevant proteins, and cell cycle arrest through activation of the checkpoint proteins & apoptosis which eliminates the highly damaged cells (Sancar *et al*., 2004).

In general DDR is a complex signal transduction pathway that leads to damage repair & proper cycling of the cells insuring that correct information encoded in the DNA is passed down the generation and maintain the proper cell functioning. As all other signal transduction pathway functioning in the cell the DDR (DNA damage response) pathway also consists of various proteins, which can be broadly classified into three categories; sensors, mediators and effectors (Zhou & Elledge, 2000) (Fig 3).

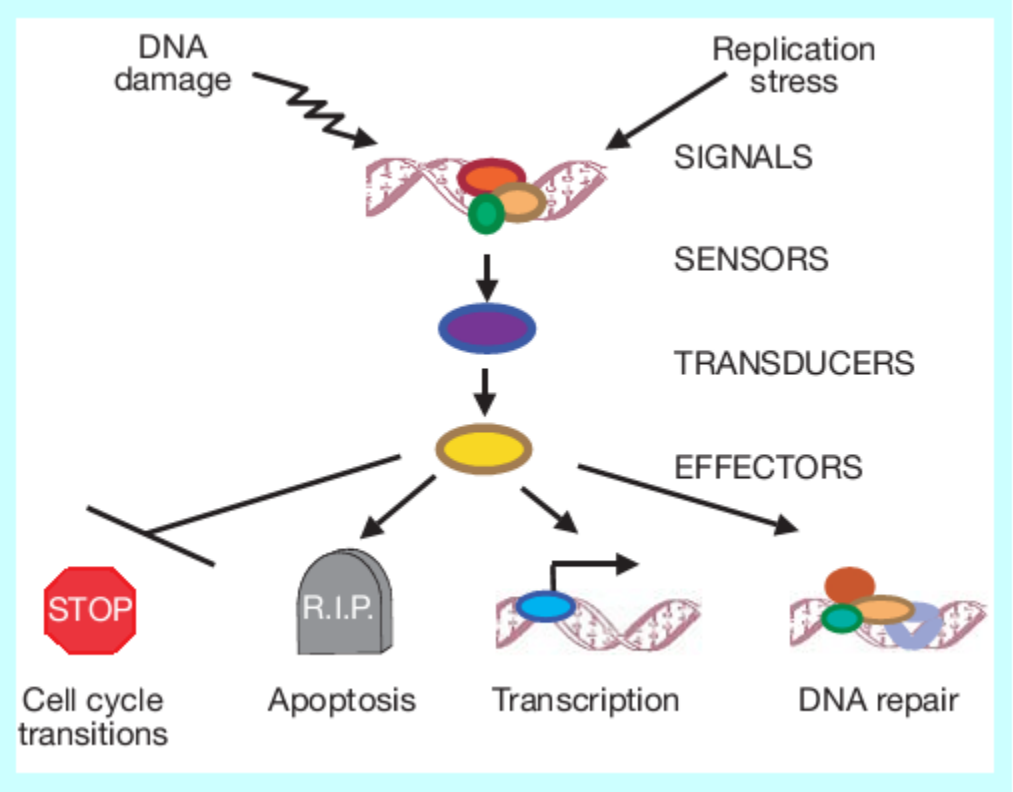


Figure 3: A contemporary view of the general outline of the DNA damage response signal-transduction pathway. Arrowheads represent activating events and perpendicular ends represent inhibitory events. Cell-cycle arrest is depicted with a stop sign, apoptosis with a tombstone. The DNA helix with an arrow represents damage-induced transcription, while the DNA helix with several oval-shaped subunits represents damage-induced repair. For the

purpose of simplicity, the network of interacting pathways is depicted as a linear pathway consisting of signals, sensor, transducers, and effectors (Zhou & Elledge, 2000).

1.4.1 Sensors

These are the proteins that primary sense the damage in the DNA itself and activate the downstream proteins in the signal transduction pathway. Unlike enzymes, sensors bind to the damaged DNA and also bind to the undamaged DNA while searching for damage. They have non-negligible affinity to the DNA and spend time bound to the DNA, which is not damaged than the damaged one. So, the specificity of sensors for the damaged DNA is very less, instead of binding DNA only upon damage and activating the signal transduction pathway, DNA damage sensors are thus function in quite a different manner scanning the genome all the time and working rather than a switch which is only activated upon the damage like other systems that exists in the cell (Sancar *et al*., 2004). Proteins that have DNA binding property, such as poly ADP Ribose polymerase (PARP), DNA-pk has long been considered as a sensor protein, other proteins include the proteins having similar property and bind to the DNA as a doughnut shape warping DNA in between such as PCNA, which has also been considered to have role as a sensors. Many proteins have been suspected to be a sensor as not much is known about the sensors compared to the mediators and effectors. Such proteins include Rad17, Ddc2, and Mec3 of budding yeast whose homologous in mammals are Rad1, Rad8, and Hus1 as illustrated in table no.1. Similarly, other proteins include BRCA1 where it is a part of BASC (BRCA1-associated genome surveillance complex) that contains ATM, the Nbs1/MRE11/RAD50 complex, MutS/MutH in mismatch repair, etc. still the knowledge about the sensors remains limited (Zhou & Elledge, 2000).

The mechanisms by which sensors recognize damage is also varied with the type of proteins; it can be though explained by dividing them into four types of mechanism.

i) Direct damage recognition:
   Direct damage recognition includes the type of damage detecting mechanism, where the damage is detected and repaired by the same enzyme using simple induced fit mechanism. Example of such repair is the damage repair by the phytolyase DNA glycosylase (Sancar *et al*., 2004).

ii) Multistep damage recognition:
   Multistep damage recognition involves multiple proteins and steps of damage recognition through action of multiple proteins; it can be further divided into two categories:
   a) Molecular Matchmaker: Molecular match maker is the protein that brings two proteins together which otherwise will remain solitary using ATP, such as

RFC (Replication Factor C) in eukaryotic, UvrA in bacterial system and XPC in Bacterial and Mammalian NER system. Where RFC loads PCNA on the DNA, UvrA recognizes DNA damage and loads UvrB (Sancar *et al*., 2004).

b) Combinatorial Recognition: Mechanism common for a transcription regulation and rarely used for the DNA damage recognition as well. Genes contain coding region and multiple regulatory elements, depending on which of the sites are occupied transcription is switched on and off, in DNA repair however there is only one recognition DNA element to which the repair factor might bind. In this way combinatorial chemistry here is used in a broad sense for several DNA repair functions (Sancar *et al*., 2004).

iii) Recognition by Proxy:

The mechanism of damage detection, where the detection is indirect, and the protein is not a DDR protein. For example, DNA damage in bacteria at actively transcribed region of the genome is detected due to the arrest of RNA polymerase at the damage site where stalled RNA polymerase attracts proteins having affinity with RNA polymerase in elongation mode, one such protein is TRCF (transcription-repair coupling factor) which activates UvrA on the damaged site also, transcribed region is marked by the RNA polymerase so is repaired quickly (Sancar *et al*., 2004).

iv) Recognition of DNA repair intermediates:

During DNA repair, many intermediate structures are created such as nicked DNA, excised DNA segment, Holiday junction, which might also be detected by the repair system and may initiate whole new repair sequences.

(Sancar *et al*., 2004)

1.4.2 Mediators

In comparison with the sensors, knowledge of mediators is more advanced in the DNA damage response. Regarding the mediators of DDR, mainly comes the protein kinases, in contrast to the protein kinases ATM & ATR kinases (PI (3) K family member) have the central role. Among the two ATM and ATR function of ATM is better understood due to availability of mutation in the human causing a disease AT. ATM has role in phosphorylating several key proteins such as P53, Mdm2, BRCA1, Chk2, and Nbs1 in response to DNA damage shown in the figure 4. Mutation in the AT patient makes them sensitive towards IR- radiation. Whereas not much is known regarding the ATR protein kinase; as no ATR mutation is found in human and also in other organism. Although, the evidences from the yeast (*S. cerevisiae)* of ATM and ATR homologue (table 1) supports the role of ATR in the DNA damage response & the fact that ATM and ATR both shares many phosphorylation substrates also points out to the same fact. One such substrate is p53 which is phosphorylated at Ser15 by both the

kinases (Zhou & Elledge, 2000). Also, BRCA1 phosphorylation due to DNA damage by IR is ATM dependent whereas Hydroxyurea (HU) is ATR dependent (Tibbetts, *et.al*). The ATR homologues in yeast (Mec1) have shown sensitivity towards replication stress and have been found to be essential for the recovery (Desany *et.al*, 1998). The experiments performed on the knockout mice have shown that ATR might have role in the replication monitoring as null mutant die whereas null mutant of ATM survives with retarded growth. This observation supports the fact that ATM and ATR is the major player in case of DNA damage response (Zhou & Elledge, 2000).

How the ATM/ATR kinases are controlled against various stimuli is unknown but some studies suggest that these kinases are activated in *vivo*, and small amount of ATM is activated in *vitro* also by the DSB (double strand break). However ATM alone binds to the DNA or other proteins are required for it to bind to DNA remains a question. For instance, ATM might also resemble DNA-pk which itself has an affinity for DSB but is enhanced upon ku70/ku80 addition, indicating that ATM might be activated and enhanced by some other proteins. The activity of ATM and ATR could be regulated in a similar manner (Zhou & Elledge, 2000).Apart from just transducing the signal, these kinases are known to arrest the cell cycle progression upon activation due to DNA damage, at the cell cycle checkpoint, these proteins play a crucial role telling the cell whether to go ahead or not depending on the condition of the cell if there is damage in the cell these kinases along with activation of the downstream proteins in the pathway also inhibits the cell cycle progression and arrests the cell till the damage is not repaired (Sancar *et al*., 2004)

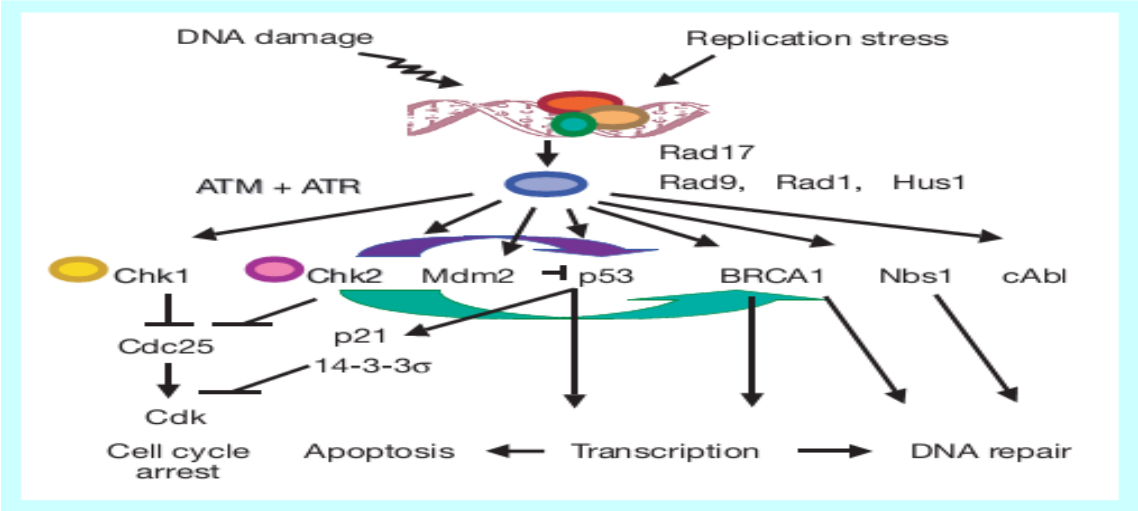


Figure 4: Organization of the mammalian DNA damage response pathway. Arrowheads represent positively acting steps while perpendicular ends represent inhibitory steps. Gene names are shown at the approximate positions where their encoded proteins function in the pathway. Although the general organization of the pathway is correct, some details are

omitted, especially concerning the relationship between the ATR/ATM and Hus1/Rad17/Rad9/Rad1 proteins, which may participate in mutual regulation (Zhou & Elledge, 2000).

1.4.3 Effectors

Effectors are the proteins that actually do repair of the damaged DNA, ATM/ATR kinases activates i.e., phosphorylate the Chk1 and Chk2 kinases (Two serine/threonine kinases that are structurally unrelated but that share some overlapping substrate specificity). In yeast homologue of the Chk2 is Rad53 and Cds1 (table 1). In mammals Chk2 has been found to prevent p53 phosphorylation. Also, Chk1 & Chk2 have been found to have role in arresting the cells in the G2 phase by inactivation and transport of cdc25 into the cytoplasm. However, progress on Chk1 has been slow due to non-viability of Chk1 null mutant as they undergo p53 dependent apoptosis. The observation of chk1 being essential points out towards its possible role in the S phase as facilitating DNA replication though the evidence is not so clear (Zhou & Elledge, 2000).

Table 1: Orthologous checkpoint proteins; in fission yeast, budding yeast and mammals (Zhou & Elledge, 2000).

Protein function	*S. pombe*	*S. cerevisiae*	Mammals
Sensors			
RFC1-like	Rad17	Rad24	RAD17
PCNA-like	Rad9	Ddc1	RAD9
	Hus1	Mec3	HUS1
	Rad1	Rad17	RAD1
BRCT-containing	Rhp9/Crb2	Rad9	BRCA1
DSB recognition/repair			
	Rad32	Mre11	MRE11
		Rad50	RAD50
	1	1	NBS1
Transducers			
PI3-kinases	Tel1	Tel1	ATM
	Rad3	Mec1	ATR
Rad3 regulatory subunit	Rad26	Ddc2	1
Effector kinases	Chk1	Chk1	CHK1
	Cds1	Rad53	CHK2
Downstream effectors	—	—	p53

1. 5 DNA damage repair Mechanism

DNA damage response leads to the detection of the damaged site followed by the cell cycle arrest, various protein kinases activation, and phosphorylation of the substrates. The most important event in the DNA damage response is the activation of the DNA repair mechanism to repair the damage and restore the DNA in its original form.

Depending on the types of damage (Fig 5), repair mechanism can be of following types:

1.5.1 Direct repair

Direct repair, type of DNA repair mechanism; where DNA is repaired by direct reversal of the damage. There are two types of such direct repair mechanism: photo reversal of UV-induced pyrimidine dimer formation by phytolyase and the removal of O^6-Methyl group from O^6-MethylGuanine (O^6MeGua) in DNA by methyl guanine DNA Methyltransferase. Photolyase is not present in many species, including humans, whereas methylguanine DNA methyltransferase has nearly universal distribution in nature(Sancar *et al*., 2004).

1.5.2 Base excision repair (BER)

Repair of a single nucleotide lesion by the removal of a single base pair is the base excision repair, where only the affected base is removed by one of several DNA glycosylases which can also be spontaneously producing abasic sites. DNA repair by the BER happen in the case of mismatch, oxidation of the base etc. The APE1 endonuclease which is responsible for the incision of the DNA, results in a nick, the nick is then repaired by the DNA pol-β which performs a one-nucleotide gap filling reaction and removes the 5' deoxy-ribose via its lyase activity. Then the sealing of the gap is performed by the XRCC1-LigaseIII complex.

Another, pathway for the BER is the long patch repair, where DNA polδ/ε is involved and proliferating cell nuclear antigen (PCNA) complex for synthesizing (2-10bp)and FEN1 to remove the displaced DNA flap and Ligase I for sealing (Mostoslavsky, 2008).

1.5.3 Nucleotide Excision repair (NER)

Nucleotide excision repair is the main mechanism to remove bulky lesion formed by the exposure to the radiations, chemical or protein added to the DNA. In this mechanism the damaged base is removed by the excision nuclease, it can remove multiple and a single nucleotide from the DNA duplex. The basic steps of NER are; (a) damage recognition, (b) dual incisions bracketing the lesion to form a 12–13-nt oligomer in prokaryotes or a24–32nt oligomer in eukaryotes, (c) release of the excised oligomer, (d) repair synthesis to fill in the resulting gap, and (e) ligation (Sancar *et al*., 2004).

But depending on the type of detection system and the proteins involved in the repair NER can be of two types i.e., GG-NER (global genomic-NER) and TC-NER (transcription coupled-NER).

In GG-NER; the XPC-hHR23B complex first screen for the disrupted base-pairing & in TC-NER; the stalled RNA polymerase is recognized and displaced by the CSA and CSB proteins. The subsequent steps of GG-NER and TC-NER are identical. The XPB/XPD helicases subunit of the TFIIH transcription factor opens ~30 bp of DNA surrounding the damage, while XPA screens for the abnormal structures. RPA stabilizes the “bubble" intermediate. The XPG and ERCC1/XPF endonuclease subsequently cleave the 3' and 5' ends in the damaged strand, respectively. This generates a 24-32 bp gap that is filled by the DNA replication machinery (Mostoslavsky, 2008).

1.5.4 Recombinational repair

Recombinational repair, deals with the whole different types of DNA damage. It is dedicated repair mechanism for the DNA double strand break (DSB) in the cell. There are in general two types of the Recombinational repair mechanism known.

i) Homologous recombination (HR): When there is a DSB in the cell it is followed by ATM phosphorylation and binding of RAD50/MRE11/NBS1 (MRN) complex, whose 5'-3' exonuclease activity exposes both 3'ends. The replication-protein-A (RPA) protein binds the single stranded DNA and facilitates assembly of the RAD51 nucleoprotein (including associated factors XRCC2,XRCC3,RAD51B, C and D). RAD51 exchanges the single strand with the same sequence from the homologous double-stranded DNA molecule. Here, the nuclear translocation of the RAD51 and synapsis of the sequences depends on the BRCA2 and the transport is probably facilitated by the Cohesins. RAD54 another protein in the pathway is a member of the SWI/SNF family of ATP-dependent remodeling complexes, likely involved in chromatin accessibility. DNA is synthesized using the information on the intact DNA sequence of the homologous template and the Holliday-junction is resolved by the resolution (Mostoslavsky, 2008).

ii) Non-homologous end joining (NHEJ): Usually in G1, when second copy is not available and in haploid cells, cells simply links the ends of a DSB without any template, using the end-binding proteins Ku70-Ku80, which facilitate DNA end-alignment and end-protection together with DNA-PKcs. Ligation is facilitated by the XRCC4-LIgaseIV complex. End-joining is sometimes associated with a gain or loss of a few nucleotides if processing of the ends occurs, a step that requires Artemis (Mostoslavsky, 2008).

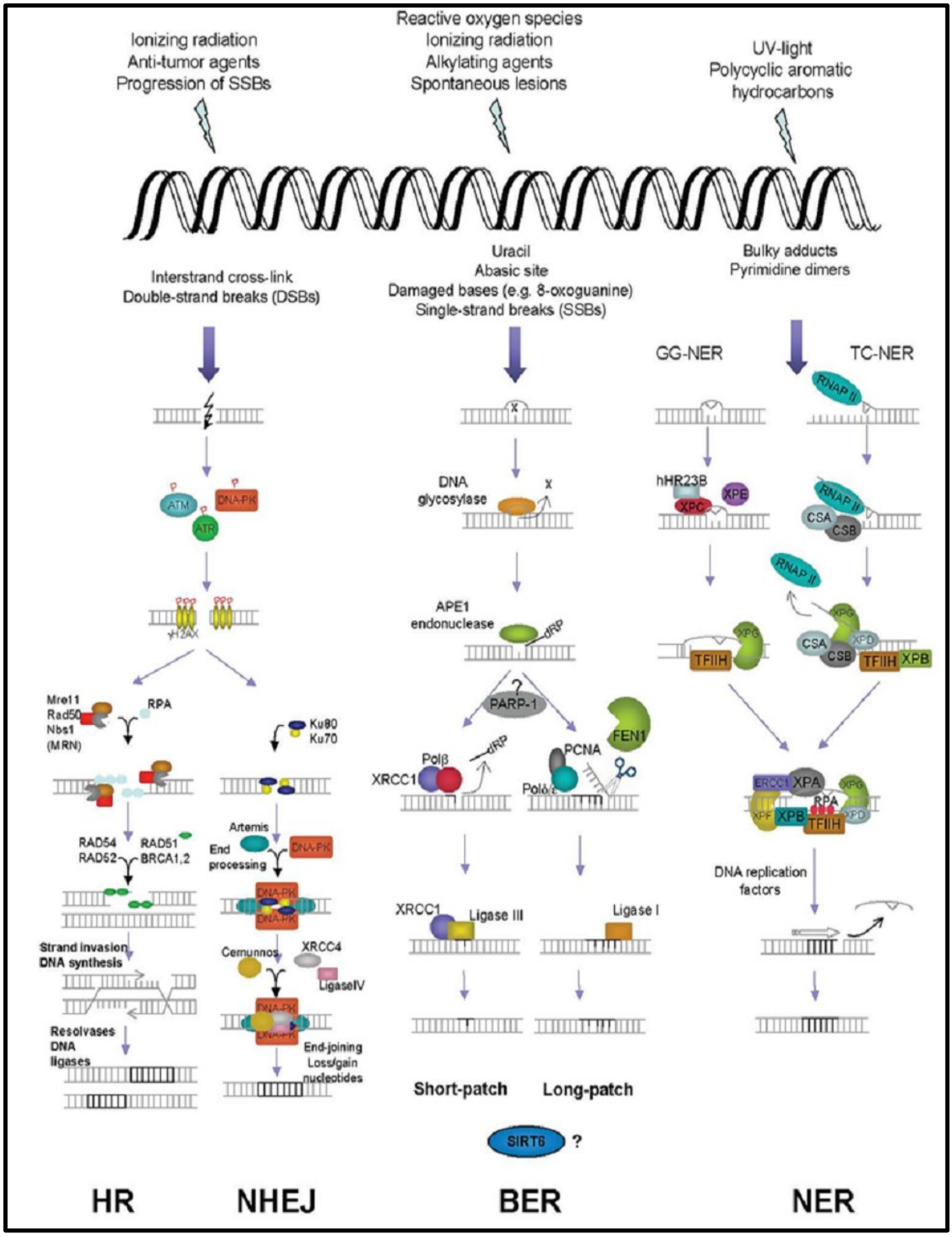


Figure 5: Diagrammatic representation of the DNA damage repair mechanism as described above (Mostoslavsky, 2008)

1.6 Research Plan

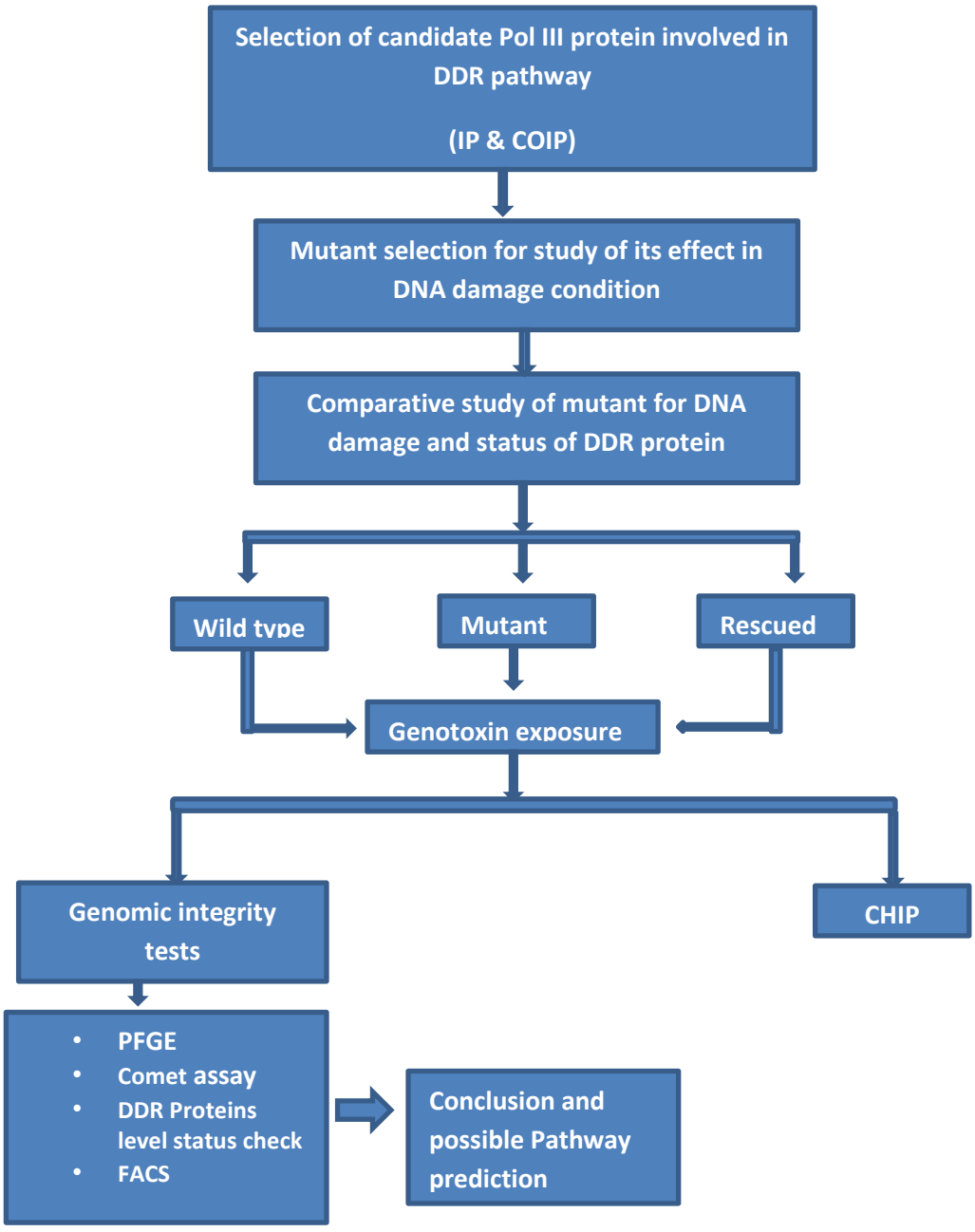


Figure 6: Schematic of research work plan

1.7 Hypothesis

TFIIIC is a DNA binding protein that has A & B-box binding domains and exhibits Extratranscriptional function. It interacts with DNA Damage Response (DDR) protein, which was observed in the large scale proteomics analysis of Yeast cell extract (unpublished data, CCMB, 2014) and confirmed by CoIP experiment. Furthermore, plate assay of TFIIIC mutant showed sensitivity towards genotoxins. Thus, the sensitivity of the mutant to DNA damage and interaction of TFIIIC with DDR proteins could be due to TFIIIC having a role in DDR pathway.

1.8 Objectives

1.8.1 General Objectives

- To Study the role of RNA Pol III transcription factor TFIIIC in TCR (transcription Coupled Repair) pathway in *S. cerevisiae*

1.8.2 Specific Objectives

1. To analyze the growth curve of wild type (YPH500), Tfc3 mutant & Tfc3 complemented.
2. To construct RAD53-HA tagged strain using the genomic tagging method as described by Janke *et.al*, 2004.
3. To analyze Interaction of TFIIIC with key DDR proteins (Ddc2 and Rad 53).
4. To analyze the Genome integrity and status of DNA Damage Response (DDR) proteins in TFIIIC mutant upon exposure to genotoxins.
5. To analyze the cell cycle progression of all strains, and validation by quantitative PCR based method and comet assay.
6. Identification of specific place & role of TFIIIC in existing DDR pathway, i.e., Extratranscriptional (ETC) function.

1.9 Rationale

Maintaining genomic integrity in the cell is very important for the viability of cells. In order to do that cells have evolved very complex mechanism of the constant surveillance system to detect & repair any DNA damage that can occur in due course of time. So far proteins involved in the DNA repair mechanism & signal transduction pathway are well

characterized. Still, much remains uncharacterized regarding sensor proteins and proteins that are involved in signal transduction above that of Protein kinases are still not well understood.

Interesting possible role of TFIIIC in the pathway detected during various experiment in the laboratory (CCMB) propelled this study. Owing to already known role of RNA pol II in the DDR also signifies that RNA pol III might also be involved in the pathway, which has never been reported before. In order to distinguish between possible roles of RNA pol III where TFIIIC was found to be the most promising candidate (large scale proteomics analysis-CCMB), this study was carried out.

Hence, this study tends to uncover the role of TFIIIC in the DNA damage response pathway and where in the picture it might fit with contrast to the whole story.

CHAPTER 2

Literature review

2.1 Transcription machinery

A cell, a basic unit of life contains a DNA as a genetic material which is expressed through protein via RNA. In order for it to do so, there is a basic process going on called transcription that forms RNA uses the DNA as a template, which is then translated to the protein by the cellular machinery ribosomes. Central to the whole process of expression of the information coded in the DNA is a molecule called RNA polymerase that is actually responsible for forming RNA uses a DNA strand as a template which then ultimately leads to the expression as a protein which might or might not be visible as a phenotype. The process, Transcription is conserved throughout all the life form on earth. The major classes of life forms i.e., Prokaryotes & Eukaryotes, also shares same basic process, involving the same type of the enzyme RNA polymerase. Transcription begins with a binding of RNA polymerase (holoenzyme) to the special DNA sequence called promoter region consisting of consensus sequences such as a TATA - box. Activation of RNA polymerase then initiates the transcription process called initiation, which is followed by the termination. Initiation also leads to the clearing of the promoter region so that transcription can begin again (Clancy, 2008). In general, transcription involves RNA polymerase, DNA and some other proteins such as inducer etc. But the number and type of RNA polymerase involves differing in different life form, in prokaryotes and in eukaryotes though in archea and prokaryotes difference is not much (Clancy, 2008).

2.1.1 Transcription machinery of Prokaryotes

In prokaryotes there is only one type of RNA polymerase is present also called RNA polymerase holoenzyme. Comprising of four catalytic subunits two α, β, β'and ω, that lacks promoter selectivity and a sigma (σ) subunit which is responsible for the transcription initiation in a promoter dependent fashion (Sadhale *et.al,* 2007). This sigma factor recognizes the promoter in its group which is not seen or recognized by other sigma factor, in this way bacterium despite has only one type of RNA polymerase managed to accomplish transcription of all genes by using different sigma subunits that are specific for different promoter (Clancy, 2008).

2.1.2 Transcription machinery of Eukaryotes

Eukaryotic transcriptional machinery is a lot more complex than that of the prokaryotic transcriptional machinery, consisting of more than one type of RNA polymerases and more complex regulatory mechanisms involving many transcription factors, as it has to function in

a much larger genome packed into a higher chromatin structure. The transcription in eukaryotic organism is carried out by three different RNA polymerases I, II & III involving many transcription factors and different component of the core protein (Sadhale *et.al*, 2007).

i) RNA polymerase I (RNA Pol I)

RNA polymerase I, also known as RNA Pol I transcribes only ribosomal RNA except 5s rRNA. Pol I consists of 14 subunits, of which 12 of its subunits have identical or similar counterparts in RNA polymerase II and RNA polymerase III. The other two subunits relate to initiation factors of RNA Pol II and have structural homologous counterparts in Pol III (Engel *et.al,* 2013). Transcription by RNA Pol I is confined to the nucleolus region as copies of rRNA DNA is present in the region as a tandem repeat. Each copy contains 18s, 5.8s and the 28s RNA molecules. These molecules are interlaced with spacers, ITS1 and ITS2 also it is flanked by 5' external transcribed spacer and a downstream 3' external transcribed spacer (Zentner *et.al,* 2011; Edger *et.al*, 2014).

ii) RNA Polymerase II (RNA Pol II)

RNA Polymerase II catalyzes the synthesis of most of the proteins in the cell, as all the mRNA coding for most of the proteins are synthesized by the RNA Polymerase II. From the previous studies on Yeast as well as humans, the RNA pol II is composed of 12 subunits, Rpb1 to Rpb12, among which Rpb5, Rpb6, Rpb8, Rpb10 and Rpb12 are shared by other two polymerases, and, Rpb1, Rpb2, Rpb3/Rpb11 and Rpb6 are homologous to bacterial core RNA polymerase subunits β´, β, αand ω respectively (Hampsey, 1998).

iii) RNA polymerase III(RNA Pol III)

RNA polymerase III catalyzes the synthesis of mostly tRNA genes, 5s rRNA and U6 snRNA. Like other RNA polymerases it also shares some subunits (Rpb5, Rpb6, Rpb8, Rpb10, and Rpb12) with pol I and Pol II, but apart from these shared subunit it is composed of 17 subunits. Seven subunits show a high ˚C of structural conservation and two, AC40 and AC19 (Orthologs of Rpb3 andRpb11, respectively) is shared with Pol I. Additionally; RNA Pol III comprises the sub complexes C37/C53 and C82/C34/C31 (Vannini, 2012; Sentenac & Riva, 2013).

2.2 RNA Pol III Transcription

RNA pol III transcription system is responsible for the transcription of most housekeeping genes, has been of interest also due to its complex structure in terms of its architecture (Sentenac & Riva, 2013). Pol III transcription is somewhat different from that of Pol II. Genes transcribed by the Pol III encode the catalytic RNAs and they are usually less than 400bp long in nature. Due to short length of the pol III genes its termination is also quite simple than that of Pol II as it recognizes simple T residue repeat as a termination signal. RNA molecules produced by the pol III transcription is involved mostly in the metabolic processes such as tRNA, 5sRNA, U6 RNA (part of splicesosome) etc. In a way it is responsible for the transcription of mostly the housekeeping genes (Schramm & Hernandez, 2002).

The complexity of RNA pol III promoter structure is more than a simple Pol I promoter, but is not much more complex than that of Pol II, comprising of less regulatory region as found in the case of Pol II which is regulated through a complex interaction of promoter and enhancers. Three types of promoter structures are found in the pol III transcription system. Two of which are intragenic and TATA less and one is gene external and hence TATA dependent. Transcription by pol III is well understood and explained, which is regulated by three major transcription factors namely; TFIIIA, TFIIIB & TFIIIC (Schramm & Hernandez, 2002; Sentenac & Riva, 2013).

Different types of promoters found in Pol III transcription system operate on different categories of genes transcribed by the pol III (Fig 7).

i) Type I promoter: Promoter is specific to the 5s RNA genes comprising of ICR (internal control region) where ICR itself is composed of A-box, IE (intermediate element) and C-box followed by usual termination signals and starts with TSS (transcription start site) (Schramm & Hernandez, 2002; Vannini, 2012).

ii) Type II promoter: promoter is specific to the tRNA genes containing A-box consensus sequence followed by B-box consensus sequence and termination signal (Schramm & Hernandez, 2002; Vannini, 2012).

iii) Type III promoter: Promoter is specific to the U6 RNA gene, which is little different from the rest two types of promoter in having a TATA box and in human it also contains a distal sequence element (DSE) and proximal sequence element (PSE) followed by gene body and then the termination signal. In case of yeast it also contains A-box and B-box downstream of the termination signal as shown in the figure 7 (Schramm & Hernandez, 2002; Vannini, 2012).

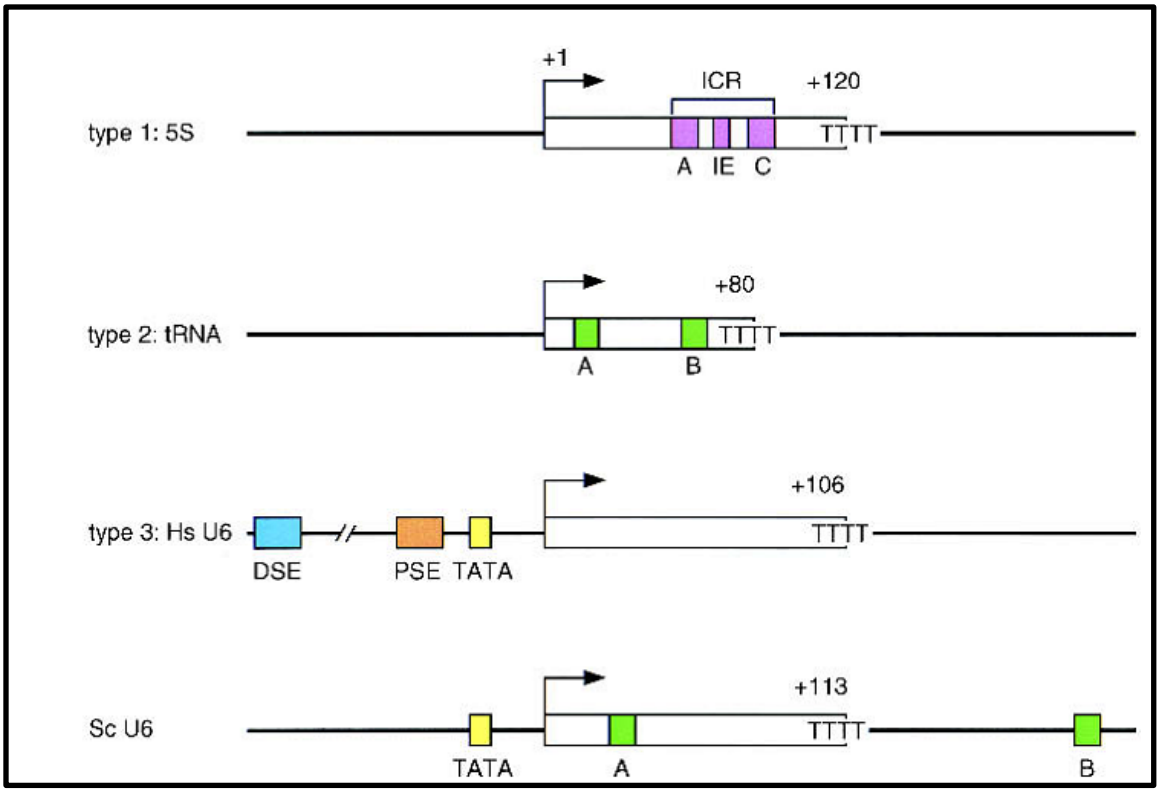


Figure 7: Three types of promoters found in the eukaryotic Pol III transcription system (Schramm L. and Hernandez N., 2002)**.**

2.3 Transcription factors of RNA Pol III

RNA polymerase III has three types of the transcription factors, TFIIIA, TFIIIB, & TFIIIC depending on the type of the promoter different transcription factor alone or in co-ordination with each other regulates the transcription by Pol III (Schramm & Hernandez, 2002).

2.3.1 TFIIIA

TFIIIA is a transcription factor of Pol III required for the transcription of 5S RNA, which recognizes ICR of the 5s RNA promoter and binds to the C-box element found in the promoter. It is a ~50 KDa protein (Budding Yeast) and is poorly conserved between the organisms except C_2H_2 zinc fingers domain. Out of 9, zinc finger domains; 1 and 7 are essential for the transcription machinery assembly (Engelke *et.al*, 1980; Acker, Conesa, & Lefebvre, 2012).

2.3.2 TFIIIB

TFIIIB is the core initiation factor that recruits pol III on all pol III-transcribed genes. The budding yeast TFIIIB is comprised of three subunits: TATA-binding protein (TBP), TFIIB-

related factor (Brf1), and B double prime (B" or Bdp1) (Geiduschek and Kassavetis, 2001). Yeast *SNR6* and four tRNA genes which contain a TATA box (-30 bp relative to start site) in the upstream region, can be efficiently transcribed, *in vitro*, in a TFIIIC-independent manner (Dieci *et.al*, 2000). However, despite the presence of a TATA box in the upstream region of *SCR1*, TFIIIC is strictly required for the *in vitro* transcription of *SCR1* gene (Dieci *et.al*, 2002). Rest of the pol III genes lack the TATA box and hence require TFIIIC-dependent recruitment of TFIIIB, which otherwise cannot bind to TATA-less promoters. The order of recruitment of transcription apparatus on tRNA gene promoters follows TFIIIC–(Brf1-TBP-Bdp1, three subunits of TFIIIB)–pol III (Acker *et.al*, 2012).

2.3.3 TFIIIC

The requirement of factors IIIB and IIIC, present in the mammalian cell-free soluble extract in the accurate transcription of purified tRNA gene was demonstrated more than three decades ago (Segall *et.al*, 1980). In *S. cerevisae* TFIIIC consists of two large subassemblies called τA and τB (Tau A and B) (Schultz *et al*., 1989; Marzouki *et.al*, 1986). TFIIIC is a complex protein consisting of 6 subunits with an apparent migration rate of 138-, 131-,95-, 91-, 60- and 50-kDa on SDS-PAGE, binds the intragenic promoter region and facilitates the transcription complex assembly process (Acker *et al*., 2012).

2.3.4 TFIIIC is responsible for transcription of ClassIII genes

τB likely comprises the three subunits of 138-,91-and 60-kDa,Tfc3,Tfc6 andTfc8, respectively have strong affinity towards B-box and also binds to the sequence, where as τA comprises of subunits, 131-, 95- and 55-kDa sub-units, Tfc4, Tfc1andTfc7, respectively is thought to bind A-box consensus sequence. Subunits 95- and 138- KDa (Table 2) are in close contact with DNA though binding to the DNA requires whole complex of subunits (Acker *et al*., 2012). Despite differences in internal promoter elements compared to those of tRNA genes, occupancy of the 95-kDa subunits is same in the absence of A box sequence (Braun et.al, 1992) (Fig 8).

Table 2: Yeast TFIIIC subunits description and the functions along with the name and moleclar weight (Acker *et al*., 2012).

Domain	Subunit name	Functions/properties	Domain	Subunit name	Functions/properties
τA	Tfc1 (95 kDa)	A-box binding , Highly conserved protein , Phosphorylated subunit , Essential for start site selection, Scaffold subunit for TFIIIC ,Interactions with Tfc3, Tfc6 and Tfc7	τB	Tfc3 (138 kDa)	B-box binding (in cooperation With Tfc6) Phosphorylated subunit Interaction with Tfc1
	Tfc7 (55 kDa)	Located in the vicinity of box A Phosphorylated subunit Interaction with Tfc1 Chimeric subunit in Hemi ascomycetes		Tfc6 (91 kDa)	B box binding (in cooperation with Tfc3), Identified at the 3' end of tRNA and 5S rRNA genes, Phosphorylated subunit, Interactions with Tfc1 and Tfc8, Structure determination of a large C-terminal fragment (β-propeller domain) bound to Tfc8
	Tfc4 (131 kDa)	TFIIIB assembly , Highly conserved protein, Phosphorylated subunit, Located upstream of the TSS Interactions with Brf1 and Bdp1, 11 tetratricopeptide repeats (TPR)		Tfc8 (60 kDa)	TFIIIB assembly , Interactions with Tfc6 and TBP, Structure determination of Tfc8 Bound to a large C-terminal Fragment of Tfc6, Identifications of a β propeller domain involved in Tfc6 interaction and a novel α/β fold involved in the functional, interaction to TBP

2.3.5 TFIIIC recruits TFIIIB to the transcriptionally active gene body

The first step in the transcription initiation of the tRNA genes, i.e., most of the genes transcribed by the RNA Pol III is the recruitment of the TFIIIC except for type 3 promoter, the experiment was carried out where, various TFIIIB components were added sequentially to a TFIIIC–DNA complex suggest that the TFIIIC–DNA complex interacts initially with the Brf1 component of TFIIIB (Braun *et.al*, 1992). The interacting subunit is most probably Tfc4 subunit which protrudes upstream of the TSS. Like the interaction of TFIIIC with the DNA A & B box interaction with TFIIIB is also very flexible in nature (Schramm & Hernandez, 2002).

2.3.6 TFIIIC in Elongation & Reinitiation

TFIIIC though, is required for the initiation of the transcription start, from the various in-vitro experiments done suggests that elongating RNA pol III replaces the TFIIIC in the absence of TFIIIB this indicates that TFIIIC is not required for the transcription elongation but TFIIIB is needed for RNA Pol III recruits. The result is also supported by the in-vivo

experiments where, TFIIIC binding is only slightly influenced by either the growth phase or the presence of RNA Pol III transcription repressor Maf1, which strongly alter RNA Pol III occupancy (Acker *et al*., 2012).

In short tRNA genes or a genes transcribed by the RNA Pol III, reinitiation is not affected by the absence of TFIIIC and has been found to occur with the help of TFIIIB alone but however in the longer gene TFIIIB is not sufficient alone and TFIIIC is also required (Dieci *et.al*, 1996; Ferrari *et.al*, 2004). The 131-kDa subunit of TFIIIC have been found to interact with the C53 subunit of RNA Pol III which is associated with the transcription termination and reinitiation, points out to the fact that TFIIIC might have very important role in the transcription reinitiation by the RNA pol III (Acker *et al*., 2012).

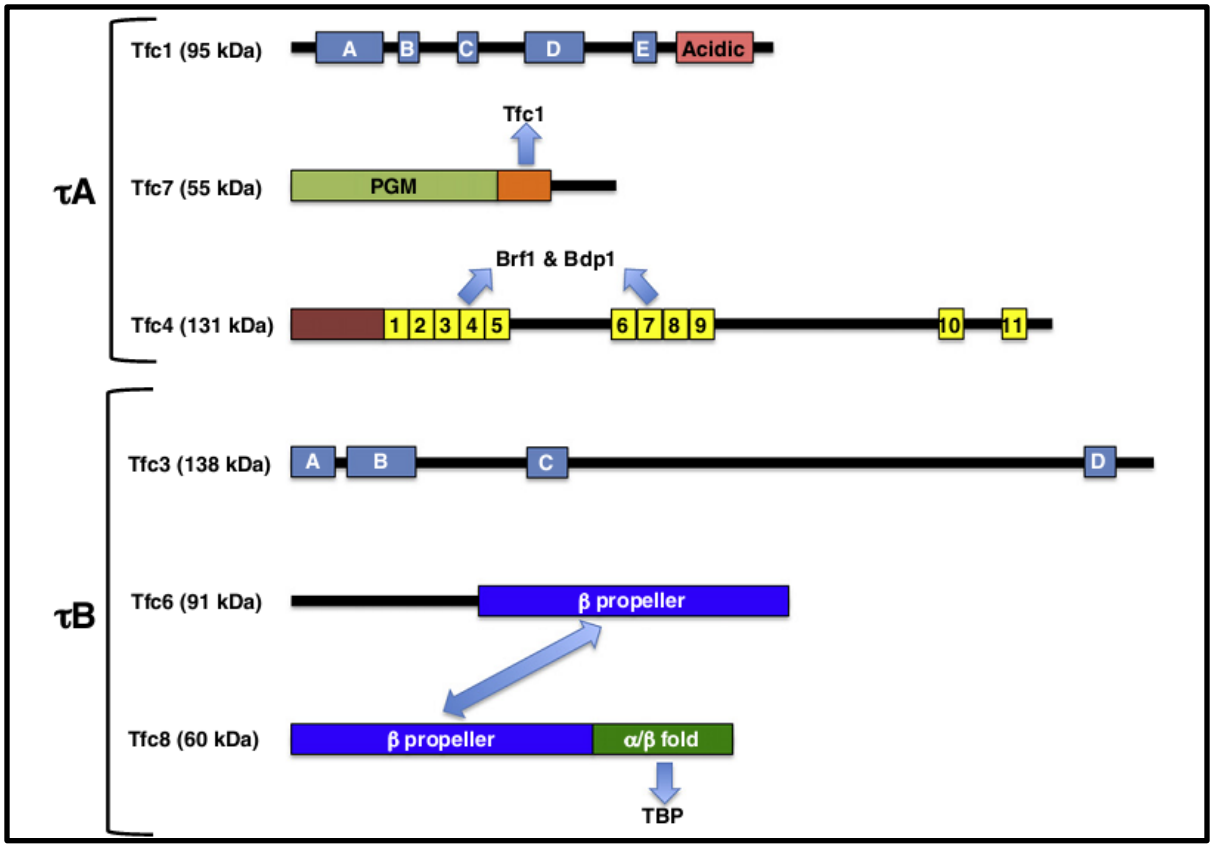


Figure 8: Schematic representation of six subunits of TFIIIC protein as solid bars with the type of domain in the sequence of the protein along with name of the subunits and molecular weight, being categorized at the right side of the figure (Acker *et al*., 2012).

2.3.7 Extra transcriptional activity of TFIIIC

TFIIIC is the largest complex of six subunits comprised of TFC1, TFC3, TFC4, TFC6, TFC7, & TFC8. TFIIIC complex binds to the DNA at both A-Box & B-Box elements, also known as internal control sequences (ICRs). The binding of the TFIIIC is, however, severely affected by the mutation in the conserved cytosine residue GGTTCGANYCY in the B-box consensus. Similarities can be found in the case of human as well. The binding of the TFIIIC is then followed by the binding of the TFIIIB which then recruits the Pol III to complete Pre-Initiation Complex (PIC).

Such type of binding by the TFIIIC thus creates a large stable complex (Nucleoprotein) creating a nucleosome free zone in the genome. Studies like genome wide binding profile of Pol III have pointed towards the fact that TFIIIC have binding sites other than just Pol III gene, such sites have been given a lots of names as Extra TFIIIC sites or ETC sites (Donze, 2012). Apart from forming just RNA, the Extra Transcriptional activity of the TFIIIC was first reported in 1990 when its role in retrotransposons was revealed (Chalker and Sandmeyer, 1990).

Soon after, another Extra transcriptional activity of TFIIIC was reported where the binding of the TFIIIC resulted in the increased in transcription of the neighboring Ty3 element, indicating the inhibiting effect on the Pol II transcription (Kinsey and Sandmeyer, 1991). Further study suggested that TFIIIC acts as a barrier, demonstrated by in vitro and mutational analysis by deleting B-box element (Simms *et.al*, 2004). Similar extra transcriptional activity of the TFIIIC includes the displacement of nucleosome as TFIIIC binds to the DNA, Direct regulation of transcription from Pol II by acting as a boundary element or best referred to as insulators or enhancer–blockers. TFIIIC has also been found to be involved in Cohesin, condensin, clustering and genome-wide organization (Donze, 2012) (Fig 9).

Also, Replication pause sites were found to be present in the tDNA region observed by 2-D gel electrophoresis, where bifurcated replication fork was detected (Deshpande and Newlon, 1996). Such type of pausing, of the replication fork was found to be dependent of at least binding of the TFIIIC, and later found out that such pausing of the replication fork is present in almost all the tDNA region as suggested by the genome wide CHIP experiments (Sekedat *et.al*, 2010).

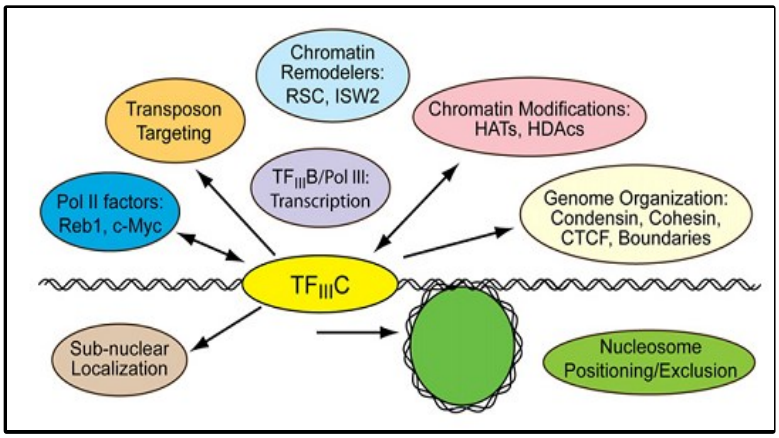


Figure 9: TFIIIC and its extra transcriptional role shown in graphical representation (Donze, 2012)**.**

2.4 TFIIIC in DNA Damage Response (DDR) Pathway

TFIIIC have not been reported to have any role in DDR pathway. The current study however was started following the large scale proteomics analysis previously performed in the lab. Large quantity of yeast was cultured and their cell lysate was used for proteomics analysis by Liquid Chromatography Mass spectroscopy (LCMS). The result showed surprising connection between TFIIIC and DDR pathway. The data showed interaction between TFIIIC to that of DDR proteins (Unpublished data, CCMB, 2014). Also, Mutant of TFIIIC containing mutation in Tfc3 subunit showed sensitivity to UV radiation and genotoxins in plate assay. Given the fact that TFIIIC is a DNA binding protein, it has the potential to be acting as a sensor for DNA Damage detection because most of the sensors are DNA binding protein. Apart from TFIIIC, similar function is exhibited by TFIIH in RNA Pol II transcription machinery have also been reported to have role in DNA damage as it helps to repair DNA via nucleotide excision repair (NER) also called Transcription Coupled Repair (TCR) (Hanawalt & Spivak, 2008).

2.5 Tfc3 mutant Background

Tfc3 is the largest subunit of transcription factor TFIIIC in yeast as mentioned above, is the subunit containing B box binding domain. A point mutation in the Tfc3 protein causing substitution from glycine to glutamine at 349th amino acid position was first made by using EMS (ethyl methane sulfonate) by J. R pringle, named tsv115 is the thermo sensitive mutant (Lefebvre, Rüth, & Sentenac, 1994). The mutant was created by segregation of SH518 having Tsv115 mutation & YPH499 named yOL8 (MAT a, ade1, tsv115, lys2-801, ura3-52, trpl-A63, and his3-Δ200). The mutation caused a defected binding of TFIIIC to DNA at higher

temperature and also binding was not strong as indicated by salt treatment experiment. Also, the mutation affected the 5s RNA synthesis in vitro. Even at 37 ˚C the protein binds to the DNA, but the binding strength is compromised in-vitro when properly folded TFIIIC having mutation was used and binding efficiency was checked (Lefebvre et.al., 1994). But in-vivo at 37˚C the protein levels start to decline after 2-3 hour and disappear after 8 hours as observed in the experiment done in the lab before. Probably due to the defect in the protein folding at 37˚C but the higher temperature do not seem to affect proteins already folded properly (Characterization of tfc3 mutant done previously in the lab).

2.6 Methodology adopted

Methodology for the current study has been adopted from number of works mainly from Papamichos-Chronakis & Peterson, 2008. In that paper role of Ino80 in the replisome stability and function have been studied. In general molecular biology work, where basic cellular process is investigated, focusing on any particular protein, the best way to determine the function and role in any given pathway could be determined by the mutant based study. Normally, to study the function of any protein in the cell, that particular protein is deleted and its effects are studied. But if the protein is essential protein, then mutant could be selected such as the one selected in this study, i.e. Tfc3 mutant (Yol8) with point mutation G349k and its a thermosensitive mutant. Now, to determine exactly which pathway is affected some background strain i.e. control (Wild type) strain is used against which the effect is compared. Then, in order to prove whether the effect observed is only due to mutation or deletion another strain with complemented copy of DNA (intact gene) is used to see does it manages to reverse the effect or not. All these methodology is quite common in this type of study but in current study most of the methodology is adopted from Papamichos-Chronakis & Peterson, 2008 including Pulse field gel electrophoresis for sensitivity assay, Protein level comparison and Fluorescence Activated Cell sorter (FACS) etc.

2.7 Some key DNA Damage Response (DDR) Proteins

2.7.1 H2As129-p (yH2Ax)

DNA in the nucleus is present in the form of chromatin. Chromatin is the structure formed by negatively charged DNA and Histone octamer (two copies each of histones H2A, H2B, H3, and H4). These chromatin structure plays a vital role in the regulation of genes also called epigenetic regulation which is affected by environmental factors as well. Normally, regulation is brought about by the modification of histones that can increase or decrease

chromatin folding thus regulating the genes as higher level folding of the chromatin results in transcription suppression of that particular gene(Moore, Yazgan, Ataian, & Krebs, 2007).

One of such modification is the phosphorylation of H2A S129 (H2AX S139 in mammals). This modification of H2A has been found to occur immediately after Double Strand Break of DNA strand (Rogakou *et al.* 1999; Downs *et al.* 2000; Paull *et al.* 2000). DNA Double Strand Break when detected by the sensor proteins results in the activation of Mec1p and Tel1P kinases (orthologs of mammalian ATR/ATM) which then plays a critical role in signal transduction. H2A S129 is also phosphorylated by these protein kinases upon the detection of damage. Once phosphorylated H2A S129 is then responsible for recruiting damage repairing proteins such as INO80, chromatin remodelers,Homologous recombination proteins & Non homologous end joining proteins (Moore *et al*., 2007).

2.7.2 H3K56 (acetylation)

Along with H2A, H3 is also modified by acetylation at lysine 56 which occurs in a newly synthesized DNA strand. Also, defects in the acetylation of H3K56 results in sensitivity to the DNA damage, suggesting its important role in the DNA damage response pathway. H3K56 is needed for the recovery of stalled replication fork and is usually taken as marker for completion of DNA repair as damaged DNA when synthesized by the repair machineries, this modification of histones occurs (Wurtele *et al*., 2012).

2.7.3 Rad53

Rad53 is an effector protein kinase that is activated (phosphorylated) by the Mec1p upon detection of the DNA damage. Activation of Rad53-P however, seems Rad9P dependent. Rad53 can auto phosphorylate once it is activated, thus amplifying the signal which results in the cell cycle arrest, activation of transcription, suppression of transcription, replisome stability, activation of DNA repair proteins etc. (Yeastgenome.org, 2015).

2.7.4 Rad9

Rad9 is the adaptor protein in S. Cerevisiae which is required for the activation of effector proteins. It serves as an amplifier of the signals generated in the cell due to DNA damage. Rad9-P is activated during normal cell cycle as well, but when there is DNA damage it is hyper-phosphorylated by Mec1-p and Tel1-p thus plays very important role in the DNA Damage response pathway (Yeastgenome.org, 2015).

2.7.5 Ddc2

Ddc2 also called LCD1 have been linked with the DNA Damage Respnose pathway due to sensitivity to DNA damage in its absence which is however corrected by increasing

nucleotide pool. Also, Ddc2 have been reported to interact with Mec1 in vivo (Rouse & Jackson, 2000). Later it was confirmed by another group that it is a homolog of ATRIP in yeast (Paciotti et.al, 2000). Its role in the DNA damage response pathway could be further validated by the finding that phosphorylation of Ddc2 takes place in late S phase and in G2 phase during an unperturbed cell cycle and is further increased in response to DNA damage (Paciotti et.al, 2000). But in order for Ddc2 to get phosphorylated it needs Mec1p indicated that Ddc2/Mec1 complex works together in the pathway (Paciotti et.al, 2000).

2.7.6 Mec1

Mec1 is an essential protein kinases similar to that of PI(3)K protein kinase that is required for the cell cycle checkpoint. Mec1p initiates a signal transduction cascade in response to DNA damage and replication blocks. Mec1p is responsible for the activation of Rad9p which amplifies and transduce signals to effector kinases as a part of the DNA Damage Response pathway. Mec1p is an ortholog of ATR in mammalian cells (Yeastgenome.org, 2015).

2.8 Effects of genotoxins Methyl Methane Sulfonate (MMS) and Hydroxyurea (HU)

2.8.1 Methyl Methane Sulfonate (MMS)

Methyl Methane Sulfonate is a alkylating agent that alkylates the DNA thus producing bulky DNA strands which has been found to be heat labile and also prone to strand breakage. Normally, MMS do not induce strand breakage directly, but breakage does occur due to stalling of replication fork at such site as mutilation makes it impossible for DNA polymerase to continue synthesis. Though what kind of damage does, it produces single or double strand breakage is not certain. It is thought that it ultimately ends in producing double strand breakage that is the reason cells lacking proper Homologous recombination are sensitive to MMS (Lundin *et al*., 2005)

2.8.2 Hydroxylurea (HU)

Hydroxyurea (HU) is an anti-cancer drug which acts by inhibiting RNR reductase required for the synthesis of nucleotides (dNTPS) for replication. Specifically, used for chronic myeloid leukemia (CML) it indirectly inhibits the DNA repair. As cancer cells are dividing continuously it needs to copy its DNA more frequently and also possibility of DNA damage is also high. In comparison, normal cell though, is also affected by HU do not divide frequently as cancerous cells (Hydroxycarbamide (Hydrea, 2015).

HU also induces the double strand break due to lack of dNTPs pool replication fork stops ultimately leading to single or double strand break (Nlm.nih.gov, 2015).

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

Most of the fine biochemicals were from Sigma and Merck. The protein pre-stained marker and 1kb DNA ladder used to be from Invitrogen. Zymolyase was from US Biologicals, Primary Antibodies were purchased from Millipore Abcam & Sigma Aldrich & secondary Antibodies was purchased from GE healthcare & Millipore. PCR components were purchased from Novagen (KOD DNA polymerase), Takara, and New England Biolabs (NEB).

3.2 Primers used in the study

All the primers used in this study were custom synthesized by Bioserve, Hyderabad. Source: 1: Wilhelm *et.al*, 2003; 2: CCMB; 4: Janke et.al, 2004

Table 3: List of the primers used in the study.

Gene/Ampl-icon	Forward primer	Reverse primer	Used for
RPS3-1	CGCTGACGGTGTCTTCTAC	CGGAAACAACAACTTCACAA (1)	Amplification of Rps3 gene, used in PCR.
RPS3-2	CCAACCAAGACCGAAGTTAT	GACAGCGGACAAACCA (1)	Amplification of Rps3 gene, used in qPCR.
TelVIR300	GGATTTTAGCAACGACTTCG	CTTGCACTTGAAAAAGCTGA (2)	ChIP
ARS305	TTTGGAGCTCAAGTGGATTG	CACACCGGACAGTACATGAAA (2)	ChIP
R53 s2		GCAAAATTGGACCAAACCTCAAAAGGCCCCGAGAATTTGCAATTTTCGCGTACGCTGCAGGTCGAC (3)	Amplification of Plasmid Cassette containing desired tag
R53 s3	CCATCTTCTCTCTTAAAAAGGGGCAGCATTTTCTATGGGTATTTGTCCTTGGTTAATCGATTGAATTCGAGCTCG (3)		Amplification of Plasmid Cassette containing desired tag
R53 Screen	ACGGGGAACATTTTGAAGAGAA (2)	R53 s3	Tagging confirmation by screeining PCR

3.3 Media and growth condition

Yeast extract and peptone used for preparing the media were purchased from Difco. Rich media used was YEPD, composed of 1% Yeast extract, 2% peptone, and 2% glucose. Cells were grown in an incubator with shaker at 30^0C for respective time intervals. Cells for protein interaction studies were usually harvested at A_{600} = 1.00 in Active sets. In Suppressed sets, cultures attaining the absorbance 1.00 at A_{600} were further kept for 2 hours in nutrient starvation condition, i.e., pelleting the cells from rich media and re-suspending them in 0.15X YEP (Yeast Extract, Peptone) without glucose.

Cells for Pulse Field Gel Electrophoresis (PFGE), Proteins level checking and FACS (Fluorescence activated cell sorter) were grown at 30^0C for overnight and then was diluted to get the O.D of 0.5 and was allowed to grow for an hour before splitting them into equally into two flasks, one was kept at 30^0C for 8hr and another was grown at 37^0C for 8hr after that genotoxins (MMS & HU) was added and then allowed to grow under genotoxins for 1hr in case of MMS & 2hr in case of HU. Cells were then harvested and processed.

For G1 arrest, cells were grown overnight, diluted in fresh medium to get O.D of 0.2, and then allowed to grow till O.D 0.4 and then α-mating factor was added, incubated for 2Hr then cells were washed, re-suspended in fresh medium, and harvested at different time interval.

3.3.1 Growth curve analysis of wild type, Tfc3 Mutant & Tfc3 complemented at 30 and 37 ˚C

Growth curve analysis has been carried out by first preparation of culture which was maintained at 0.2 O.D. by taking overnight culture as an inoculum. For analysis at 30˚C the culture was allowed to grow and O.D. Of the culture was taken every hour after that for several hours.

For Growth curve analysis at 37˚C the culture was first allowed to grow till 0.5 O.D. And then the temperature was shifted to 37˚C. O.D. Of the culture was then taken every hour for several hours.

3.4 Yeast strains used in the Study

This study is the mutant based comparative study mainly three types of strains were used.

3.4.1 Tfc3 Mutant

Yol8 strain of yeast having tsv115 (G349K) point mutation in Tfc3 subunit of TFIIIC. It is a thermosensitive mutant where protein folding is affected at 37˚C but is normal at 30˚C. Also, the strain contains empty plasmid (Appendix 2).

3.4.2 Tfc3 Complemented

It is also a Yol8 strain of yeast having tsv115 (G349K) point mutation in Tfc3 subunit of TFIIIC. But in this strain mutation in Tfc3 subunit have been corrected by inserting plasmid with correct copy if Tfc3 gene thus rescuing the mutation (Appendix 2).

3.4.3 Wild type Strain

As a wild type strain, normally used strains of yeast in the laboratory is used, namely BY4741, W3031a, S288C and closest strain to that of Tfc3 mutant i.e. YPH500 (Appendix 2).

3.5 Genetic manipulation of yeast

Genetic modification of yeast had been carried out using a PCR toolbox from Euroscarf (Janke *et.al*, 2004). To create a strain with N-terminal tag on a given protein, a cassette containing the tag as mentioned in the following description and selection marker was amplified using a pair of primers containing sequence of homology to the genomic target location at their 5'ends. Here, Plasmid selection depends on the kind of tag needed for experiments such as HA, Myc, FLAG & Fluorescence tagging. For example, in order to tag HA epitope PYM16 (4.4KB) plasmid is selected which contained 6X-HA epitope along with selection marker ampicillin for bacterial cloning and Hygromycin for yeast transformation in the plasmid. Construct of the Plasmid is shown in the Appendix 3 which is then amplified using S2 and S3 primers for N-terminal tagging as described by Janke *et.al*, 2004. This amplified PCR product (tag+selection marker) was then transformed into desired yeast strain so that it is integrated into the genome by homologous recombination, leading to N-terminal tagging of protein. As chimeric primers were used containing 50-55bp segment of DNA of Rad53. In forward primer 50-55bp upstream DNA, including stop coding and in

reverse primer from the stop codon plus 50-55bp downstream sequences were added as described by Janke *et.al*, 2004.

3.5.1 Plasmid isolation

Genetic manipulation of yeast viz., N-terminus tagging of the gene with an epitope, were carried out using plasmid-cassettes synthesized from DH5α ultra competent cells. 1µl PYM 16 plasmid was added to 50µl competent cells (carried out in ice) followed by a heat shock at 42°C for 90seconds. 700µl LB media was added to the above mixture and incubated at 37^{o}C for approx. 45mins. The media was removed by centrifugation and the cells were re-suspended in fresh LB media and spread on LB pates with ampicillin antibiotic as marker. The plates were kept at 37^{o}C overnight (Janke *et al*., 2004). Further, as colonies appeared on the plates, single colony was picked and inoculated in 20ml LB media and kept for overnight at 37^{o}C. Cells were pelleted by centrifuging at 10000g for 5 mins and supernatant was discarded. Pellet was re-suspended in Solution I and vortexed for 2 mins then Solution II was added and mixed gently, lastly Solution 3 was added. The mixture was then centrifuged at 15,500g for 30 mins at 4°C. Supernatant was recovered and 2.5x volume of Isopropanol was added and centrifuged for 30 minutes at maximum speed. The pellet recovered was washed with 70% ethanol and dried. Pellet was re-suspended in 100 µl TE RNase and stored at -20°C (Janke *et al*., 2004).

3.5.2 Polymerase Chain Reaction

PCR modules for C-terminal tagging were amplified using primers S2 and S3 (Table 3) with PCR tool box plasmids as a template. Plasmids were selected depending upon the tag required as HA or MYC and selection marker present in the plasmid. The plasmid used for this work was PYM16 which contains the HA tag and Hygromycin as the selection marker.

Table 4: PCR 'Reaction mix' for the amplification of DNA segment from Plasmid.

Reagents required	Volume
10X KOD buffer	10µl

S2 (forward primer) (10mM)	6.4µl
S3 (reverse primer) (10mM)	6.4µl
dNTP mix (10mM)	3µl
KOD taq polymerase (2U/ µl)	1µl
Template (plasmid pYM16) 1mg/ml	3µl
$MgCl_2$ (25mM)	4µl
MQ water(Nuclease free)	66.2 µl
Total Volume	100 µl

Following the program was set up in THemal Cycler (MJ Research, USA)

Table 5: PCR conditions for amplification of DNA from Plasmid

	STEPS	**TEMPERATURE**	**DURATION**
1.	Initial Denaturation	95°C	10 mins
2.	Denaturation	95°C	30 secs
3.	Annealing	50°C	30 secs
4.	Extension	72°C	2.40 mins
Go to step 2, 15 times			
5.	Denaturation	95°C	30 secs
6.	Annealing	65°C	30 secs
7.	Extension	72°C	2 min 40 sec

Go to step 5, 20 times			
8.	Final extension	72°C	10 mins
9.	Hold	4°C	10 mins

The tagging program completed in 3hr 20mins and the PCR product was checked on 1% agarose gel.

3.5.3 Yeast Transformation

Yeast transformation was done by Lithium Acetate - Poly Ethyl Glycol (LiAc-PEG) method. Cells from 5ml log phase culture were harvested and washed with MQ. The pellet was re-suspended in 1ml Mili-Q and 500µl was transferred to a fresh tube. Water was removed after spinning and the cells were re-suspended in 100 µl Lithium acetate, 50µl of PCR product and 10 µl of single strand denatured salmon sperm DNA, 700 µl of PEG solution. Heat shock was given at 42°C for 40 minutes and then it was incubated at 30°C for 30 minutes. After the heat shock, cells were pelleted and washed with Mili-Q, re-suspended in 5ml of YPD media and allowed to grow for 4-5hours before plating on to the respective antibiotic selection YPD agar plates (Janke *et al*., 2004).

Transformed colonies appeared on plates after 3-4 days. Colonies were screened for the insertion of PCR cassette. Replica plating was done for the selection of positive transformants. The success of integration was tested by immunoblotting and sequencing.

3.5.4 TCA (TriChloroacetic Acid) Method

Single colony was inoculated in 5ml media and incubated overnight. Cells were pelleted and washed with water. 20% TCA solution was added to the cell pellet according to the bed volume along with glass beads. This was vortexed for 20mins and the supernatant was transferred to a fresh vial. An equal amount of 5% TCA solution was added and kept at 4°C for 30 mins. Protein pellet was recovered by spinning at 3000 rpm for 10mins. Supernatant was discarded and 200 µl of the 2X sample loading buffer and 50µl Tris of pH8.8 were added to the protein pellet. Samples were then boiled, vortexed and spinned and then loaded on

8% SDS-PAGE and allowed them to run for 1:30 hrs at constant current (25mA) (Janke *et al*., 2004).

3.5.5 Western Transfer

The SDS - Polyacrylamide gel was then transferred to PVDF membrane. Wet Transfer was used for this in 1X western transfer buffer with 20% methanol. PVDF membrane and 2 filter papers were cut according to gel size. The PVDF membrane being hydrophilic is dipped in methanol for 5mins for enhancing the binding properties. The transfer was carried out at 200mA for 3 hrs at 4^{o}C in western transfer buffer through electrophoretic transfer (Biorad).

3.5.6 Detection by Western blotting

After the transfer, blots were incubated in 5% milk powder in 1X TBST for at least 1 hr, blots were then washed with 1X TBST and incubated with fresh primary antibody (anti HA, dilution ranges from 1:1000-1:15000) for 2 hrs at room temperature. Then blots were washed thrice with 1X TBST 10 mins each. Blots were incubated with secondary antibody conjugated with Horseradish Peroxidase for 40 mins. Secondary antibody can be either anti-mouse or anti-rabbit depending on the primary antibody (if primary antibody is raised in rabbit then secondary is anti-rabbit and if on mouse then anti-mouse secondary antibody is used) and its dilution ranges from 1:10000-1:15000. Blots were again washed thrice with 1X TBST 10mins each. ECL (Enhanced Chemi Luminescence) substrate which binds to secondary antibody and imparts chemiluminiscence was evenly added, blots were then viewed under Chemi- doc instrument (Vilber lourmat chemiluminiscence documentation), and the image was captured (Mahapatra et.al, 2011).

3.5.7 Genomic DNA Isolation

In 5 ml glucose containing media (YEPD) a single colony was inoculated and kept for overnight. Cells were pelleted and washed with water. To the cell pellet, 300µl of genomic DNA buffer was added and the glass beads were added according to the bed volume along with 30µl of PCI (Phenyl: Chloroform: Isoamyl alcohol) solution. The cells were vortexed for 2 minutes and the cell pellet was recovered by spinning for 15 mins at 15000 rpm. The above aqueous solution, containing nucleic acids was transferred to fresh vial. 100% ethanol

and Sodium acetate was added to the solution for the DNA precipitation and kept at 4°C for 15 mins. The tubes were centrifuged at 15000 for 15 mins to recover the DNA pellet. The recovered pellet was dried in vacuum spin and RNase treatment in TE was given at 45^{o}C for 1hr. The isolated DNA was run on 1% agarose gel and the bands were observed under UV Transilluminator.

3.5.8 Confirmation through Amplification of desired gene by PCR

Confirmation of tagging was done by PCR to check whether the 6X HA tag was inserted in our desired gene. Genomic DNA was isolated from the strain and used as the template. PCR reaction mixture was prepared as mentioned in table no. 4

Following program was used for Screening PCR

Table 6: PCR conditions for Screening of transformed colony.

	STEPS	**TEMPERATURE**	**DURATION**
1.	Initial Denaturation	**95°C**	**5 mins**
2.	Denaturation	**95°C**	**30 secs**
3.	Annealing	**50°C**	**45 secs**
4.	Extension	**72°C**	**1 min**
Go to step 2, 35 times			
5.	Final extension	**72°C**	**10 mins**
6.	Hold	**4°C**	**10 mins**

3.6 Immunoprecipitation (IP) and Co- Immunoprecipitation (CO-IP)

Immunoprecipitation technique is widely used to study protein-protein interactions in vivo. Doubly tagged strains (e.g. Protein A- FLAG tagged and Protein B- HA tagged) were inoculated in YEPD media and grown upto A_{600} =1.00 and harvested immediately by centrifuging at 4000 rpm for 1min at 4 °C. Cells of both the sets were stored at -80°C (Mahapatra *et al*, 2011).

3.6.1 Preparation of cell lysates

Cells stored in -80°C were thawed, re-suspended in 900µl of modified lysis buffer containing protease inhibitors and phosphatase inhibitors. Re-suspended samples were poured to 2ml screw cap tubes containing glass beads and fixed in bead beater at 4°C for a cycle of 30 seconds ON and 90 seconds OFF. 25-30 of these cycles were performed to obtain lysed cells (80-90% lysis). Cell lysates recovered after 21 cycles was checked under microscope (40X) to check the lysis and then centrifuged at 10000 rpm for 10 min. Supernatant was transferred to a fresh tube (Mahapatra *et al*, 2011).

3.6.2 Pull down of the proteins

From the cell lysate about 200ul of the lysate was taken; to it about 50ul of 50% slurry was added (antibody beads: MLB) and incubated in rotator overnight. The sample slurry mixture was washed with MLB three times and 5X laemmlli buffers were added (Mahapatra *et al*, 2011).

3.6.3 Detection by western

The sample was loaded in the SDS-PAGE (8%, 10% & 12% acrylamide gel) and run to resolve the protein accordingly, which was electrophoretically (wet transfer) transferred to PVDF membrane and was detected by Western blotting method (same as 3.5.6).

3.7 Genomic Integrity assays

For genome integrity assays mentioned above, sample preparation was done as follows: Tfc3 mutant and its respective wild type cells were grown up to OD 0.6 at 30 °C then shifted

to 37 °C for 8 hours. After this, cultures were treated with 0.1 % Methyl Methane Sulfonate (MMS) for 1hr. Cells were then pelleted and washed twice. Half of the cells were used for PFGE and the other half were used see the phosphorylation status and levels of some known DDR proteins to determine whether the pathway is being affected due to mutation (Mahapatra *et al*, 2011).

3.7.1 Pulse Field Gel Electrophoresis (PFGE) profile of wild type, Tfc3 mutant and Tfc3 complemented

3.7.1.1 Plugs preparation

For plugs preparation 0.4gm of LMP agarose (Seakem) was weighed and dissolved in 20 ml of 1M SCE buffer to make 2% LMP agarose solution, it was kept in the 60°C water bath. Yeast cells prepared, were re-suspended in 200 µl of 1M SCE buffer, 100 µl of 0.5M EDTA and 7 µl of β- Mercaptoethanol to make the final cell suspension close to ~350µl. This yeast cell suspension was thoroughly and quickly mixed with 350 µl of 2% LMP agarose and poured into molds to make the plugs. Five plugs for each sample were made and allowed to solidify at 4°C (Gardner *et.al*, 1993).

3.7.1.2 Lysis of yeast in agarose plugs

Plugs were transferred from molds to 10 ml screw cap glass bottles with help of tips and the bottles are labelled accordingly. 4 ml of 1M SCE buffer was added to each bottle along with 1U Zymolase (MP Bio) and 1mM β- Mercaptoethanol. These bottles were incubated at RT for 8-12 hours. After 12 hours plugs were washed with Wash Buffer (10mM Tris-HCl (pH 8), 50 mM EDTA). Plugs were then treated with 5 ml Lysis Buffer (10mM Tris-HCl (pH 8), 50 mM EDTA, 1% SLS) and 0.5/ml Proteinase K for 24 hours at RT.

After extensive washes with Wash Buffer (10mM Tris-HCl (pH 8), 50 mM EDTA), plugs were loaded on 1% agarose gel and sealed in the wells with a solution of 1% LMP agarose in 0.5X TBE. Gels were run at 14 °C at 160 V, with 19s to 160s for 19-26 hours using Bio-Rad CHEF Apparatus. Gels were stained with Ethidium Bromide, solution (Gardner *et.al*, 1993).

3.7.2 Comparison of DNA Damage Response (DDR) Proteins level

(a) H2S129-P and H3K56 levels in wild type, Tfc3 mutant and Tfc3 complement.

Western blotting was performed using Anti-H2s129s-P (abcam, cat no. ab15083) and anti-H3K56 (abcam, cat no. ab76307) antibodies. Anti- H2A and anti H3 (cat no. ab46765) antibodies were used as control respectively. SDS-PAGE gel was used with 12% concentration of acrylamide (Optimized Lab protocol, CCMB).

(b) Rad53 phosphorylation and Rad9 levels check in wild type, Tfc3 mutant and Tfc3 complement.

Rad9 was HA-epitope tagged with Wild-type, Tfc3 mutant and Tfc3 Complemented. Western blotting was performed using Anti-HA (Santa Cruz cat no. sc-805) and Anti Rad53-P antibodies. Anti- H3 antibody was used as a control. SDS-PAGE gel was used with 10% concentration of acrylamide for Rad53 and 8% concentration of acrylamide for Rad9 levels check (Optimized Lab protocol, CCMB).

3.7.3 Real-time PCR-based method for the comparison of genome sizes within wild type, Tfc3 mutant & Tfc3 complemented.

Absolute quantification of a single copy gene (*rps3)* in a genomic DNA sample *S. cerevisae* was done by Real Time PCR. The amount of sample used in this analysis is determined by UV-absorption spectrometry. The accumulation of PCR product was monitored in real time during the amplification process by measuring specific fluorescence signals, whose intensities were proportional to the amount of PCR product. The PCR process was followed using dsDNA specific SYBR Green dye (Wilhelm *et.al*, 2003).

3.7.4 Fluorescence Activated Cell Sorter (FACS) analysis of wild type, Tfc3 mutant & Tfc3 complemented.

For FACS analysis of the cells, cells were re-suspended in the 70% ethanol and was either stored or about 100-200µl of samples were transferred to new 1.5ml tube, was washed twice with 50mM Sodium citrate by adding about 1ml of Sodium citrate, vortexes & spun

for 5min at 2500rpm twice. Supernatant was discarded and 500µl of 50mM Sodium citrate was added to which 500µl of 20µg/ml PI in 50mM Sodium citrate was added so that the final concentration becomes 10µg/ml. FACS analysis of the samples was performed for asynchronous culture, G1 arrest cells (using mating factor of concentration of 10µg/ml) for wild type and Tfc3 mutant strain & of different samples taken after release from G1 arrest using the following settings;

FL2A; gain: 3.0, voltage: 840 volt. ; Rest as default (Optimized Lab protocol, CCMB, 2015).

3.7.5 Comet assay for wild type, Tfc3 mutant & Tfc3 complemented.

Comet assay, also known as a single cell gel electrophoresis, is a simple experiment developed, to detect DNA damage in the cell not in global level but in cellular level. Cells are embedded in agarose on a microscope slide, lysed, and electrophoresed. Broken DNA is drawn towards the anode, forming a 'comet tail', is stained with a DNA-binding dye and observed under fluorescence microscope. The protocol for the comet assay was adapted from the published article (Azevedo *et.al,* 2011).

3.7.5.1 Growth condition and preparation of cell for comet assay

Growth condition is similar to that of PFGE or Protein level comparison, but the only thing that differs here is that the cells are washed with a buffer 'S' before it was frozen.

Thus, frozen cells were first thawed on ice and were diluted to get the cell density of about 10^6 then it was washed with SCE buffer and was re-suspended in the same buffer (Azevedo *et.al,* 2011).

3.7.5.2 Preparation of slide for comet assay

i) Pre-coating of slide with agarose: Normal melting temperature agarose was prepared at the concentration of 0.5% w/v. It was then placed in a petri plate and then the slides to be pre-coated are cleaned first with methanol, then was dipped in the agarose and removed. The back side of the slide was wiped with tissue paper and was allowed to dry. Thus, prepared slides can be stored for a prolonged period of time at 4°C before being used.

ii) Micro gel preparation: After pre-coating about 200µl of the 1.5% low melting point (LMP) agarose was spread on the slide and coverslip was added, allowed to set at 4°C for 20-30 min then the coverslip was removed.

iii) Immobilization of cells: About 50-60µl of cells were suspended in SCE buffer and about 10µl of 12.5mg/ml zymolyase was added and was mixed with equal volume of 1.5% LMP agarose which was then quickly spread over the slide previously prepared. It was then covered with cover slip and allowed to set for 2-3 hr. Spheroplast were checked under the microscope and confirmed before moving to the next step.

iv) Lysis of cells: Lysis was performed using alkaline comet assay lysis buffer (Appendix 1). To the slide first cover slip was removed and lysis solution was added and incubated for 1-2 hr at 4°C.

v) Washings: Washings were done by the electrophoretic buffer three times, each of 20 min long at 4°C.

vi) Electrophoresis: Electrophoresis of the slides was performed at 25 volts for 10 mins in the electrophoretic buffer.

vii) Neutralization: Neutralization of the slide was performed in the neutralization buffer for 10 min at 4°C.

viii) Fixing: Fixing of the cells were done by dipping the slide in 76% & 96 % ethanol respectively for 10 mins. And was allowed to air dry, slides were immediately stained with PI in vectashield at a concentration of 10ug/ml or stored at 4°C and visualized later.

viii) Visualization: Visualization was done under fluorescence microscope at magnification of 400-500x (Azevedo *et.al,* 2011).

3.8 Chromatin Immunoprecipitation (ChIP)

A detailed account of the ChIP assay is discussed in the following sections.

3.8.1 Growth conditions and preparation of whole-cell extract

Yeast cells were grown to OD_{600} of 0.8 and fixed with formaldehyde (final 1% for 30 min). Excess formaldehyde was quenched with glycine (final 0.125 M). Cells were harvested, washed twice with Milli-Q water and the pellet was stored at -70^0 C until further use.

For bead beating, cell pellet was re-suspended in 1 ml of buffer L (50 mM HEPES-KOH, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate , 0.1% SDS plus

protease inhibitors) and lysed using bead beater until 90% lysis (30 sec ON, 3 min OFF, 20 cycles). Whole cell extract ~1.5 ml was recovered in a microcentrifuge tube (Dewari & Bhargava, 2014).

3.8.2 Fragmentation of chromatin by sonication

After bead beating, 500 µl aliquots of whole cell extract were processed for sonication in Bioruptor (Diagenode) to shear chromatin to mean-size 300-500 bp with the following settings: 30 seconds ON, 1 min OFF, total 30 cycles. 37^0 C. DNA size was checked on 1.25% agarose gel (Dewari & Bhargava, 2014).

3.8.3 Immunoprecipitation of chromatin fragments

Whole cell extracts containing sheared chromatin fragments was centrifuged at 10,000 g for 10 min at 4^0 C. A pellet containing debris was discarded and supernatant was recovered. Supernatant was pre-cleared by incubating with 50 µl of A+G Sepharose beads (pre-equilibrated with buffer L) for 3 h at 4^0 C. Beads were discarded and supernatant was divided into four aliquots of 300 µl each. 2 µg of antibody were added to each of the three aliquots (technical replicates) while to one aliquot 2 µg of IgG was added and kept as mock/control. Samples with antibody were incubated overnight at 4^0 C (Dewari & Bhargava, 2014).

Next day, 30 µl of A+G Sepharose beads (pre-equilibrated with buffer L) were added to each aliquot and incubated at 4^0 C for 3 hours. Immunoprecipitation complexes on beads were washed twice with buffer M_0 (20 mM Tris-Cl pH 8.0, 200 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), buffer M_1 (M_0 plus 500 mM NaCl), buffer M_2 (10 mM Tris-Cl pH 8.0, 250 mM LiCl, 2 mM EDTA 1% NP-40, 1% sodium deoxycholate) and buffer TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA); each wash for 5 min at room temperature. Bound complexes were then eluted by incubating beads with 500 µl of elution buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 1% SDS, 150 mM NaCl) at 65^0 C for 45 min, and supernatant containing immunoprecipitates was recovered. Supernatant and input samples (60 µl of original lysate) were treated with 50 µg of RNase A for 1 hr at 37^0 C, and cross-links were reversed by incubating samples at

65^0C for 10 hr. Samples were extracted once with PCI, DNA was precipitated with ethanol, dissolved in 100 µl of Milli-Q water, and quantified by real time PCR using SYBR chemistry (Dewari & Bhargava, 2014).

3.8.4 Real Time (Quantitative) PCR

DNA samples obtained from ChIP assay were analyzed using real time quantitative PCR with SYBR green chemistry. Sequences of the primer pairs used in this study are given in table 3.
For each Real-time PCR reaction, 3.5 µl of the template DNA and 2.5 Pico moles of each primer were used along with PCR master mix (SYBR green, hot start taq, 2 X taq buffers). Reactions were done in triplicates and the Ct (critical threshold) values which fell within 0.3 cycles were taken for calculating the mean Ct values (Dewari & Bhargava, 2014).

Table 7: PCR conditions for qPCR validation of TFIIIC ChIP in both active and suppressed condition

	STEPS	**TEMPERATURE**	**DURATION**
1.	Initial Denaturation	**105°C**	**5 mins**
2.	Denaturation	**95°C**	**30 secs**
3.	Annealing	**53°C**	**45 secs**
4.	Extension	**72°C**	**45 secs**
Go to step 2, 35 times			
5.	Final extension	**72°C**	**10 mins**
6.	Hold	**4°C**	**10 mins**

3.9 Experimental design

In order to determine the role of TFIIIC in DNA damage Response Pathway, Mutant based approach was taken. To validate the hypothesis, following experiments were performed to fulfill their respective goals,

3.9.1 Protein-Protein interaction study of TFIIIC with DDR proteins

In this experiment, interaction of TFIIIC with Ddc2 was re-validated by performing Co-IP with varying concentration of Methyl Methane Sulfonate (MMS). This experiment was performed to find out whether interaction between TFIIIC and Ddc2 is due to DNA damage or some other reason. If TFIIIC and Ddc2 interaction is a related to DDR then upon increasing the concentration of MMS, their interaction should also increase.

Also, to make sure TFIIIC functions with Ddc2 only in DDR pathway and not any other DDR proteins in signal transduction part, interaction of TFIIIC with other proteins in the pathway i.e. Rad53. Interaction study of TFIIIC and Rad53 was performed. The procedure for this experiment is adopted from Mahapatra *et al*, 2011.

3.9.2 Pulse field Gel Electrophoresis analysis

TFIIIC to have role in DDR pathway, its mutant should be sensitive to the DNA damage. In order to see that, Pulse field Gel Electrophoresis (PFGE) was performed to analyze the sensitivity of mutant towards DNA damage in presence of genotoxins such as Methyl Methane Sulfonate (MMS) and Hydroxyurea (HU). If TFIIIC has role in the DDR pathway it should show sensitivity towards genotoxins. The procedure for PFGE has been adopted from Gardner *et.al*, 1993.

3.9.3 Key DNA Damage Response (DDR) proteins level comparison

To validate role of TFIIIC in DDR pathway, level of several key DDR proteins were checked and compared between wild type mutant and complemented strains of yeast. If TFIIIC has role in DDR pathway then theoretically, activated protein level should be less than that of wild type in all condition. Here, as TFIIIC interact with Ddc2 (unpublished data, CCMB, 2014) it is suspected to be involved in DDR pathway (along with Ddc2 or before Ddc2). So, proteins like H2AS129, H3K56, Rad9 & Rad53 levels should be affected as they all lie below Ddc2 in the DDR pathway. For this experiment all the procedure was adopted from optimized protocol present in CCMB laboratory.

3.9.4 Cell Cycle progression check

If there is DNA damage in the cell then cells should get arrested in S-phase (normally) and normal cell cycling should get blocked. To check that, Fluorescence Activated Cell Sorter (FACS) analysis was performed. Propidium Iodide (PI) which stains DNA is used in FACS to determine total DNA content of the cell. Based on that data, cell cycle progression can be checked in FACS. For FACS of yeast to observe normal cell cycle progression, protocol was adopted & optimized mainly from the standard protocol followed in the CCMB laboratory but for mammalian cells.

FACS result was then further validated by yet two experiments,

3.9.4.1 Comet Assay

Comet assay is a standard technique to determine both single and double strand break in DNA. In Alkaline condition single strand breakage can be determined and in neutral condition double strand break can be determined. In this study comet assay was performed to determine single strand break which is also equivalent to stalled replication fork. Thus, if cells are in S-phase then it should show single strand break in comet assay. Comet assay protocol was optimized based on procedure described by Azevedo *et.al,* 2011.

3.9.4.2 Gene copy no. comparison using qPCR

If more no. of cells are in S-phase then copy no. of gene should be more than that of normally cycling cells. Because in S-phase, genome size is more than 'n'. So, mutant should show more copy no. of gene in qPCR than that of normal cell (wild type). Here, the protocol was adopted from Wilhelm *et.al*, 2003.

3.9.5 Chromatin Immunoprecipitation (ChIP)

ChIP of TFIIIC was performed to check the occupancy of TFIIIC in active and suppressed (nutrient deprived) condition to check how TFIIIC gets distributed in the genome in those two conditions. ChIP protocol was obtained and then optimized accordingly from Dewari & Bhargava, 2014.

CHAPTER 4

Results

4.1 Growth curve analysis of wild type, Tfc3 mutant and Tfc3 complemented

As mentioned in 3.4, experiment was carried out to compare the growth curve of test strain against wild type and complemented for any type of deviations. After taking O.D. of the culture both at 30˚C and 37˚C, graph of the readings were plotted against time,

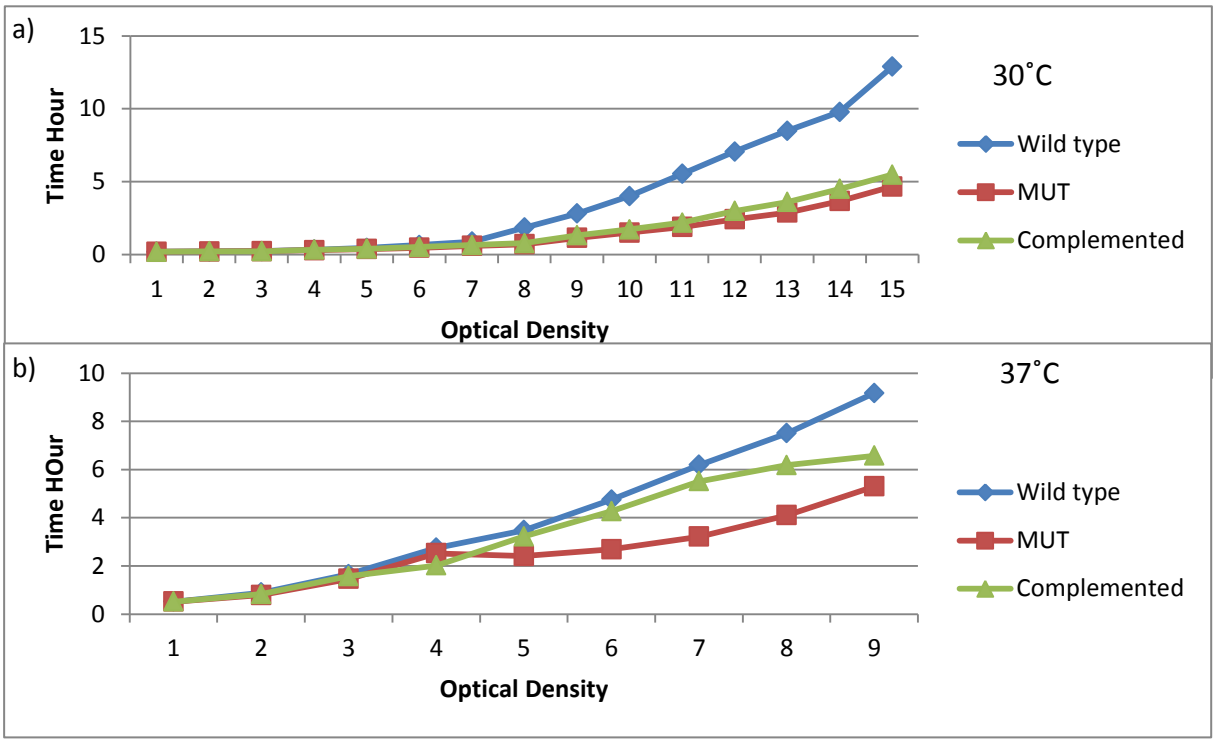


Figure 10: Growth curve of Tfc3 mutant (MUT), wild type, & Tfc3 Complemented (Complemented).Graph is plotted taking optical density at Y-axis and Time in hour at X-axis. a) Growth curve taken at 30˚C starting from O.D. of 0.2. b) Growth curve taken at 37˚C starting from OD 0.52.

From the simple experiment of growth curve analysis of fig. 10, it is clear that the growth of mutant is retarded in both 30 and 37 ˚C. At 30 ˚C, the growth rate starts to change which finally enters the log phase after 5 hours where wild type grows at a tremendous rate while Tfc3 mutant and Tfc3 complemented fails to grow at the same rate due to a mutation which causes stress and affects the protein folding. As the mutation is thermosensitive one, observation of the growth in cells was made after O.D. 0.5 which is the start point of log phase for Yeast. From the figure 10 it can be seen that all the strain grows incomparable rate till 3 hours. After that, the growth rate of mutant decline followed by the

complemented in 6 hours of the temperature shift (fig.10 b). This could be due to the degradation of Tfc3, as immediately after the temperature shift still there will be functional Tfc3 remaining which begins to decline from 3 hours as mutant's growth begins to slow down, but Tfc3 complemented manages to maintain similar growth rate which also fails at about 7 hours as it also fails to rescue completely. It is known fact that complemented strains rescues the cellular defect in some particular gene. Failure to rescue completely could be due to the presence of the defective gene in the genome and corrected gene in plasmid subjecting the cells to stress, also plasmid coded gene fails to correct the defect in many cases.

4.2 Strain construction

4.2.1 Plasmid Isolation

Genetic manipulation of Yeast (BY4741-background strain) was carried out by tagging the genome itself using the plasmid cassettes synthesized from DH5α ultra competent cells. pYM16 plasmid having 6X HA tag and Ampicillin selection marker was used in a vector. After transformation of DH5α cells, positive colonies obtained on the ampicillin plate was taken and the plasmid was isolated from an overnight culture which was run on 1% agarose gel (Janke *et.al*, 2004).

4.2.2 Tagging PCR

For genetic manipulation of Yeast, PCR based tagging approach was used, where epitope tagging of the desired protein (Rad53) was performed. For this purpose pYM16 (Janke *et.al*, 2004) plasmid was used and was amplified to produce the DNA to be integrated into the genome utilizing homologous recombination. Chimeric primers containing 50-55bp of genomic sequence & plasmid sequence were used to get the desired product for genomic tagging (6X-HA tag and Hygromycin selection marker). Primers used in this experiment were R53 s2 & R53 s3 (table 3) as mentioned in 3.5 which amplifies the section of cassette plasmid containing the tag and selection marker. After PCR, the product was run on 1% agarose gel and stained with Ethidium Bromide (EtBr). A PCR band of 2.1 kb, as expected, was observed, which was further used for transformation.

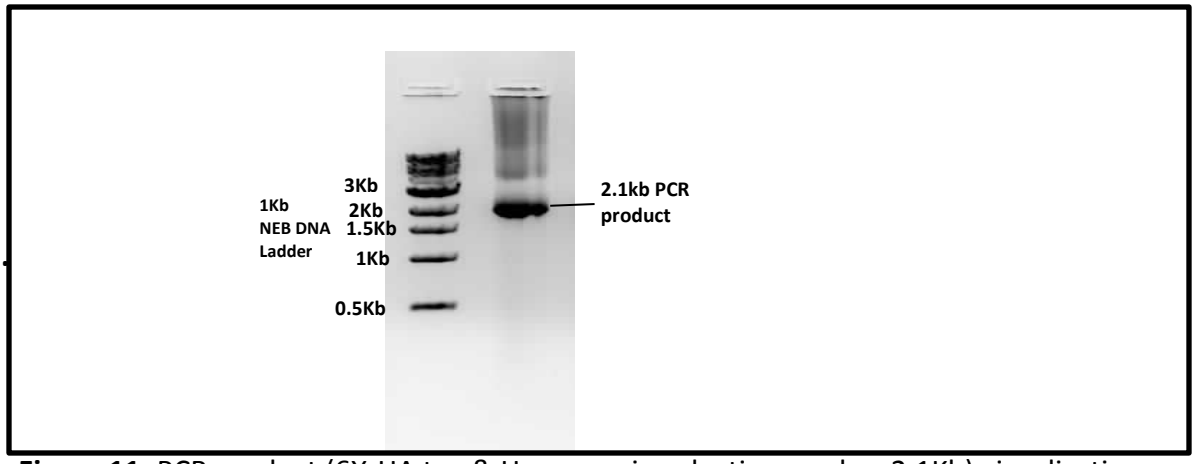


Figure 11: PCR product (6X-HA tag & Hygromycin selection marker-2.1Kb) visualization on 1% gel to be used for transformation.

4.2.3 Tagging confirmation by Western Blotting

After the transformation of the PCR product obtained from tagging PCR, positive colonies on the Hygromycin plates were taken and total protein extraction for the strains were done by TCA method. About 30-40 µl of the TCA precipitated sample in Laemmlli buffer was loaded at 8% SDS-PAGE and transferred to PVDF membrane. The membrane was then probed with anti-HA antibody and visualized under chemiluminescence documentation system.

The band was observed in the membrane that corresponds to the size of the Rad 53 (91kDa) protein plus molecular weight of 6X HA epitope which is about 10 kDa.

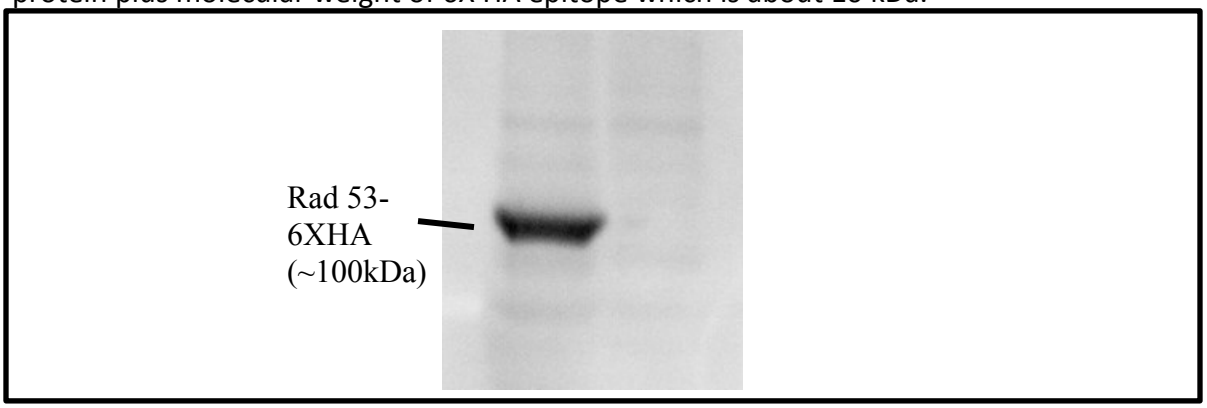


Figure 12(a): Western blots showing band for RAD53 in BY4741 background strain.

After confirmation by western blotting PCR was also performed to amplify Rad53 HA tagged gene (fig. 12-II) and which further confirmed the successful tagging of RAD53 with 6X HA epitope making a fusion protein.

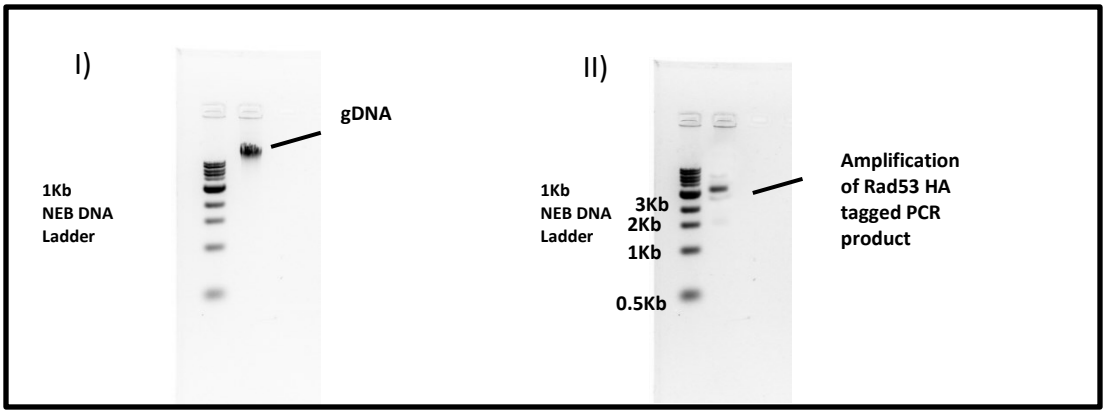


Figure 12 (b): Gel picture of strain construction of yeast for HA tagged Rad 53. **I)** Extracted gDNA from positive colony of yeast. **II)** Amplification of Rad53 HA tagged PCR product from positive colony after western for confirmation of tagging.

4.3 Protein-Protein Interaction studies

4.3.1 Ddc2 and Tfc1 interaction in increasing concentration of Methane Methyl Sulfonate (MMS)

Doubly tagged strain (Tfc1-73.5kDa FLAG-10kDa & Ddc2-86.4kDa HA-10kDa tagged) strain was used to study the interaction at different concentration of Methane Methyl Sulfonate (MMS) as described in 3.6. After lysis of the sample, lysate was used to detect the interaction using western blotting by specific FLAG and HA antibody in order to detect both Immunoprecipitation (IP) and Complex Immunoprecipitation (CO-IP) in the sample. The following results were obtained from the experiment;

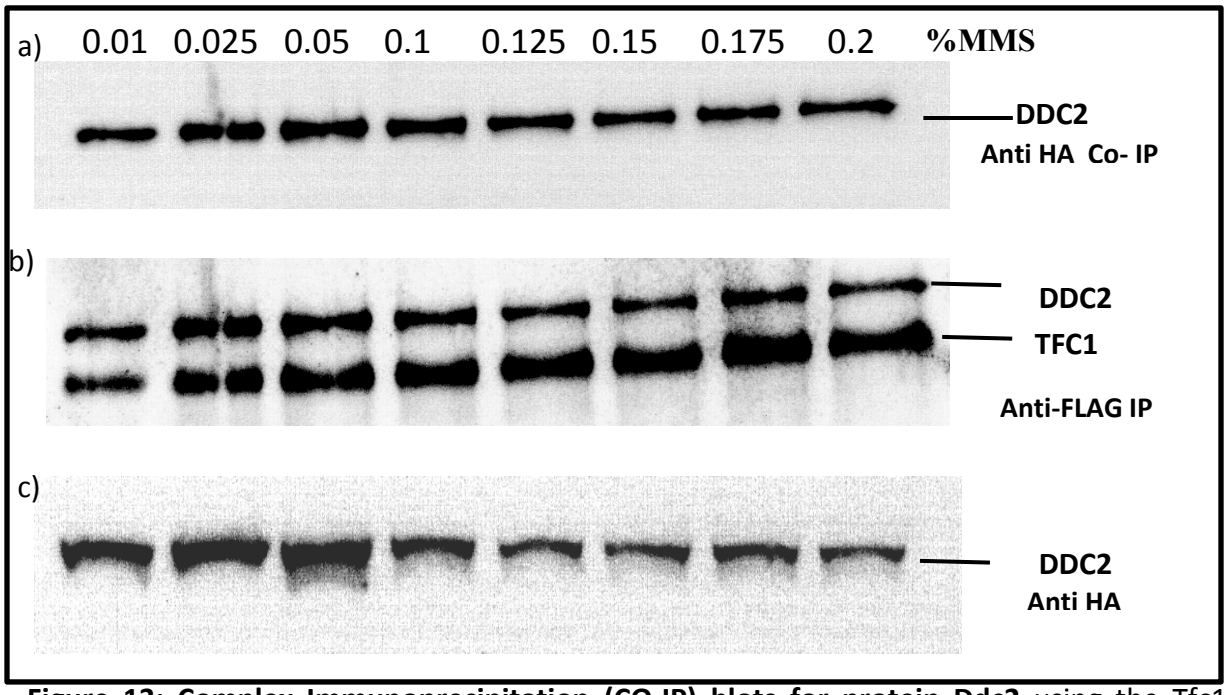


Figure 13: Complex Immunoprecipitation (CO-IP) blots for protein Ddc2 using the Tfc1 FLAG pulldown, **a)** Immunoprecipitation (IP) Blot probed with anti-FLAG antibody, **b)** On the

same blot showing bands of both Ddc2 and Tfc1 and at the bottom and **c)** Input of protein samples probed with Ddc2 anti-HA antibody.

From figure 13, at increasing concentrations of MMS Ddc2 pull-down was also found to be increased upto the concentration of 0.05% MMS after that, however the input and IP seems to be the same, but interaction is somewhat decreasing in higher concentration indicating that at higher concentration cells are unable respond properly. Thus a decrease in interaction is observed. The result suggests that the interaction of Ddc2 with Tfc1 which was not an artifact as here we can see a concentration dependent interaction of Tfc1 with Ddc2 upon exposure to the genotoxins.

4.3.2 Interaction of Tfc1 with Rad53

Constructed strain (4.2) was used to study the interaction between proteins using Immunoprecipitation technique to check the interaction between two proteins *in-vivo*. To confirm that TFIIIC works with only Ddc2 and not any other DDR proteins in the pathway along with Rad9 (Previously performed in CCMB lab) interaction with Rad53 was also performed. Doubly tagged strain was used to study the interaction between Rad53-91kDa and Tfc1- 73.5kDa where Tfc1 is FLAG tagged and Rad53 is HA tagged. In such a doubly tagged strain Tfc1 FLAG pull-down was performed and presence of HA (Rad53) was checked using specific anti HA antibody. The interaction result is described below:

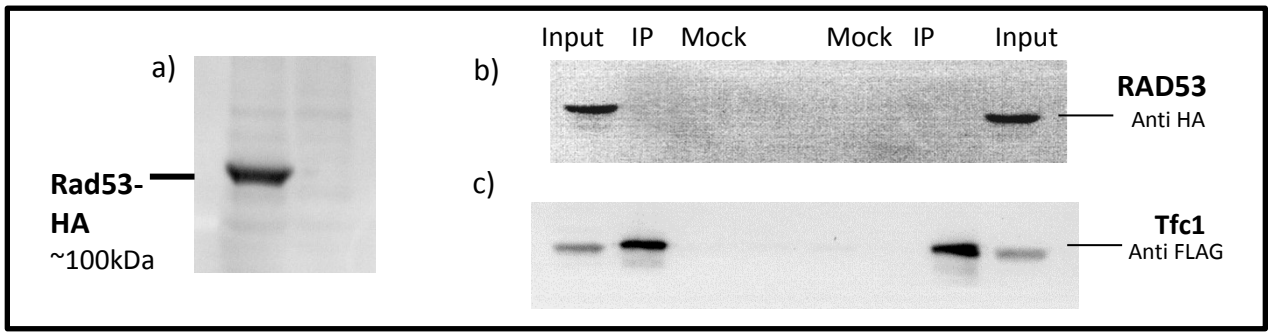


Figure 14: **Complex immunoprecipitation blot for protein Rad53** using Tfc1 FLAG pulldown **a)** Rad53 probed with anti-HA antibody and at right, **b)** Western Blot showing absence of band when probed with anti HA in IP plane where Input: sample before pull-down (antibody,-Beads), IP:Immunoprecipitation, Mock:sample+ agarose beads-antibody and **c)** presence of band when probed with anti-FLAG where, Input: sample before pull-down (antibody,-Beads), IP:Immunoprecipitation, Mock:sample+ agarose beads-antibody

Absence of bands in the IP lane when probing with anti HA signifies no pull down of the desired protein (Rad53) has occurred, same blot when probed with Anti FLAG antibody gives the band that shows that Anti FLAG pull down worked but Rad53 is absent in Tfc1

immunocomplex (fig. 14). Hence we can say that there is no direct interaction between Rad53 and Tfc1.

In budding yeast Rad53 is important for maintenance of genomic DNA as it is a part of signal transducers in the DDR pathway. It is required for cell-cycle arrest in G1/S, intra S and G2/M phase. It gets activated in the DDR pathway by Rad9 which then activates Chk1, an effector protein (WikiGenes-RAD53-Rad53p). We have observed that Tfc1 does not interact with Rad53 directly. Similar type of CO-IP previously performed in the lab indicates that Tfc1 interacts with Ddc2, which physically interacts with Mec1 (responsible for activation of Rad9), suggest that TFIIIC works upstream of the Rad 53 in the signaling pathway.

4.4 Genomic integrity test of wild type, Tfc3 mutant & Tfc3 complemented by Pulse Field Gel Electrophoresis (PFGE)

PFGE analysis of three strains was performed with different genotoxins with various concentrations at 30 & 37 ˚C.

4.4.1 BY4741, Tfc3 mutant, Tfc3 Complemented yeast strains 30 & 37 ˚C chromosomal profile

PFGE for the general chromosomal profile of three strains were performed at 30 and 37 ˚C to find out the general effect of temperature on the DNA damage in a global scale.

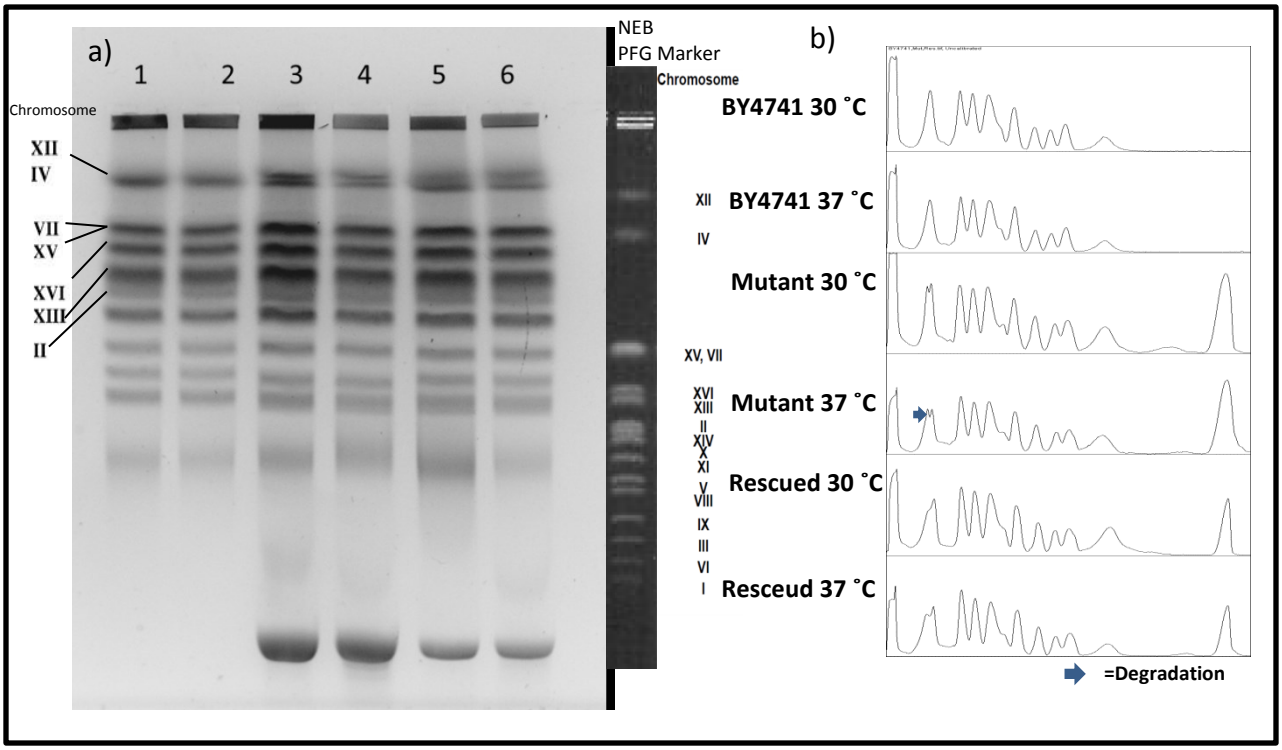


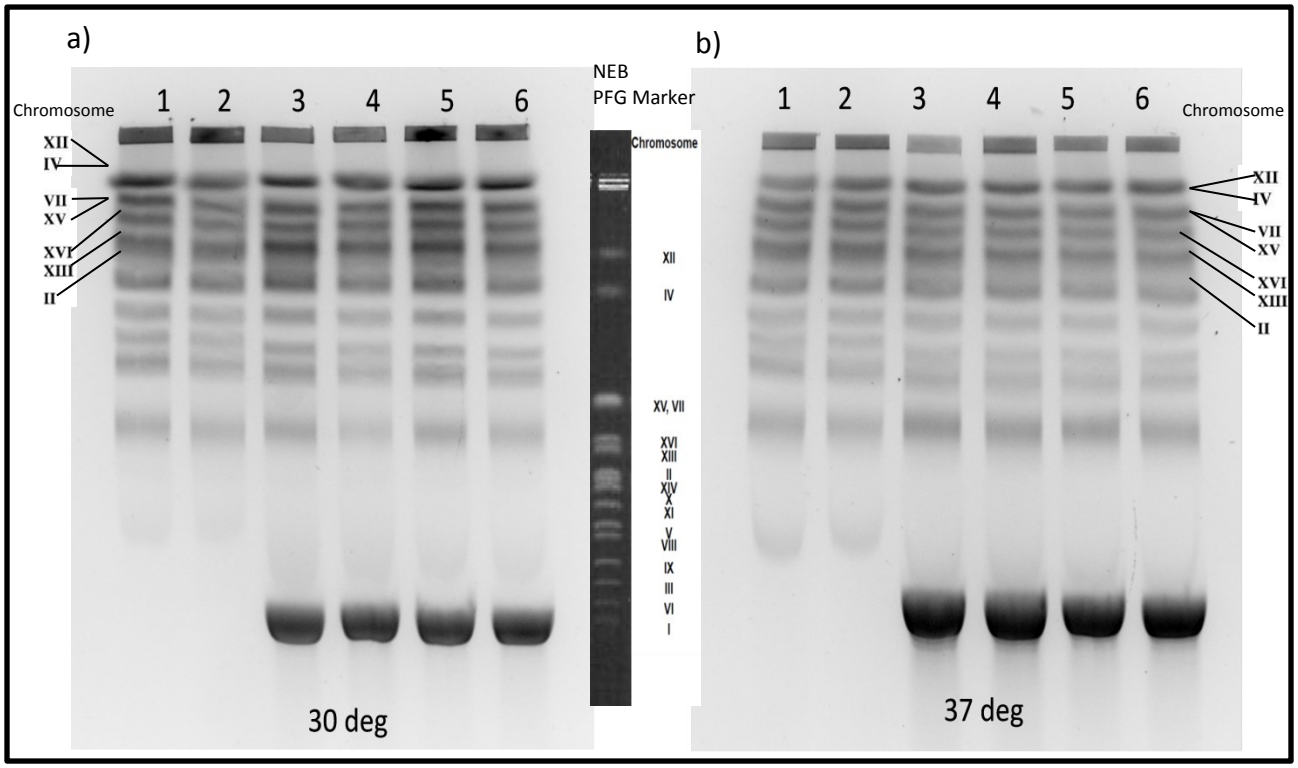
Figure 15: PFGE chromosomal profile (BY4741, Tfc3 mutant, Tfc3 Complemented) for detection of genomic integrity assessment a) General PFGE profile of three strains namely

BY4741, Tfc3 mutant and Tfc3 complemented loaded in gel as, lane 1:BY4741 30 ˚C, lane 2: BY4741 37 ˚C, lane 3: Tfc3 mutant 30 ˚C, lane 4:Tfc3 mutant 37 ˚C, lane 5: Tfc3 complemented 30 ˚C, & lane 6: Tfc3 complemented 37 ˚C **b)** Plots of 'a' for checking chromosomal integrity in order to determine the global DNA damage in 30 & 37 ˚C.

In PFGE, if the damage is mild and damaging agent is weak, then the damage will be visible only in large chromosome as it is more susceptible to damage than smaller chromosomes. Also, degradation in smaller chromosomes is visible only when extensive damage is present. For instance, to visualize damage visually from PFGE, at least 5Gy of X-ray radiation have to be given (Pinto, Newman, Prise, & Michael, 2000). From the PFGE profile obtained at 30 & 37 ˚C (fig.15), slight degradation of DNA was observed in 37 ˚C in all the strains (from plot), but there was more degradation in the mutant sample as there is very sharp decrease in the intensity of bands between 30 and 37 ˚C.

4.4.2 BY4741, Tfc3 mutant, Tfc3 Complemented yeast strains 30 & 37˚C chromosomal profile on treatment with Hydroxyurea (HU) at concentration of 200mM

PFGE profile of three strains (BY4741, Tfc3 mutant, Tfc3 Complemented) were checked at 30 & 37 ˚C but this time with a 2 hour exposure of genotoxin Hydroxyurea (HU). Genotoxin at concentration of 200mM was used for 2 hours of exposure to compare the sensitivity of Tfc3 mutant against wild type and whether complemented manages to rescue,



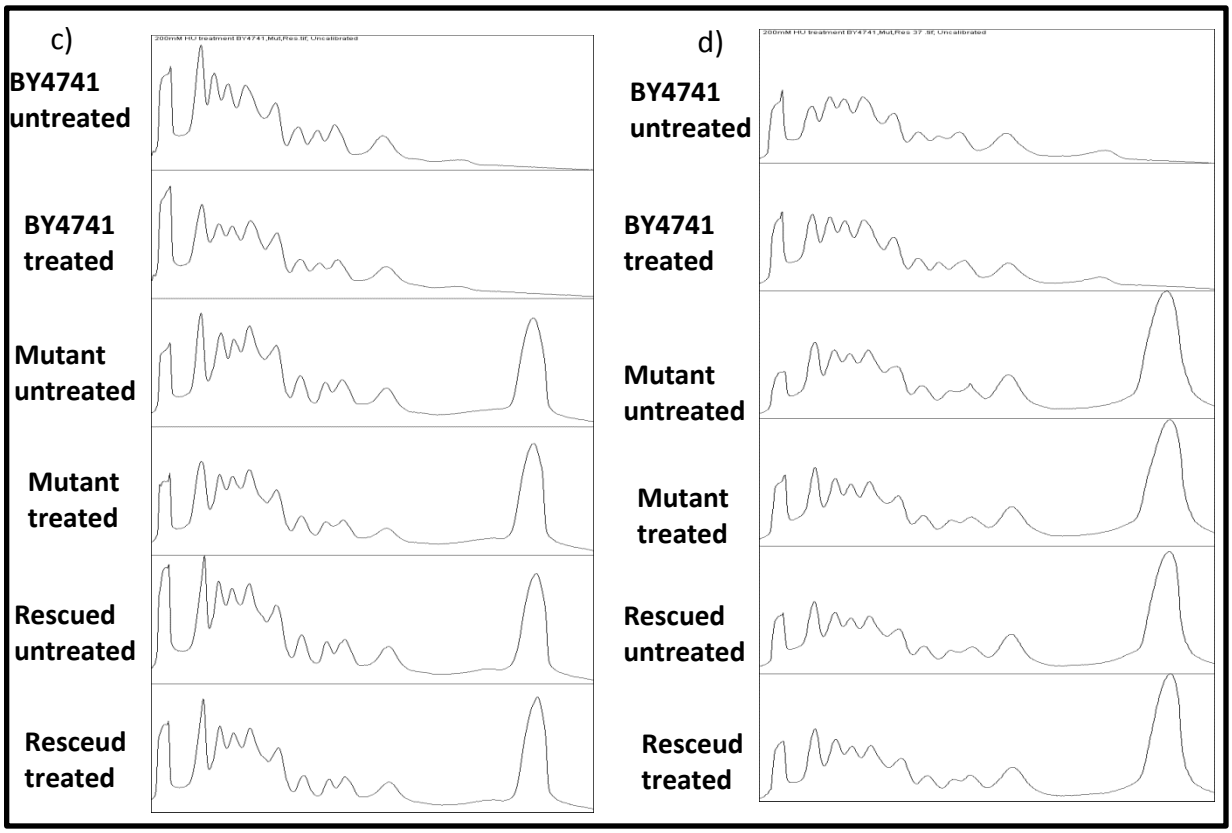


Figure 16: PFGE profile of three strains (BY4741, Tfc3 mutant, Tfc3 Complemented) for detection of genomic integrity on treatment with HU (200mM), **a)** PFGE profile of three strains of yeast namely BY4741, Tfc3 mutant and Tfc3 complemented loaded in gel as, lane 1: BY4741 untreated, lane 2: BY4741 treated, lane 3: Tfc3 mutant, lane 4:Tfc3 mutant treated, lane 5: Tfc3 complemented, & lane 6 Tfc3 complemented treated with HU (200mM) at 30 ˚C **b)** PFGE profile of three strains of yeast namely BY4741, Tfc3 mutant and Tfc3 complemented loaded in gel as, lane 1: BY4741 untreated, lane 2: BY4741 treated, lane 3: Tfc3 mutant, lane 4:Tfc3 mutant treated, lane 5: Tfc3 complemented, & lane 6 Tfc3 complemented treated with HU (200mM) at 37 ˚C **c)** Plot of fig. 'a' & **d**) Plot of fig. 'b' drawn using image-J software.

In the HU treatment at 200mM concentration there is no significant damage that can be seen visually on a global scale (fig. 16). It may be due to the fact that HU is a mild genotoxin that act indirectly and also the exposure time was of just 2 hours, which is a short duration for the effective action of HU.

4.4.3 BY4741, Tfc3 mutant, Tfc3 Complemented yeast strain chromosomal profile on treatment with Methyl Methane Sulfonate (MMS) at 30 ˚C

PFGE profile of three strains were checked, but this time genotoxin which alkyls the DNA strand, causing either a SSB or DSB i.e., Methyl Methane Sulfonate (MMS) was used at

varying concentration to check the DNA damage sensitivity of the mutant (Tfc3), wild type and complemented was performed at 30 ˚C.

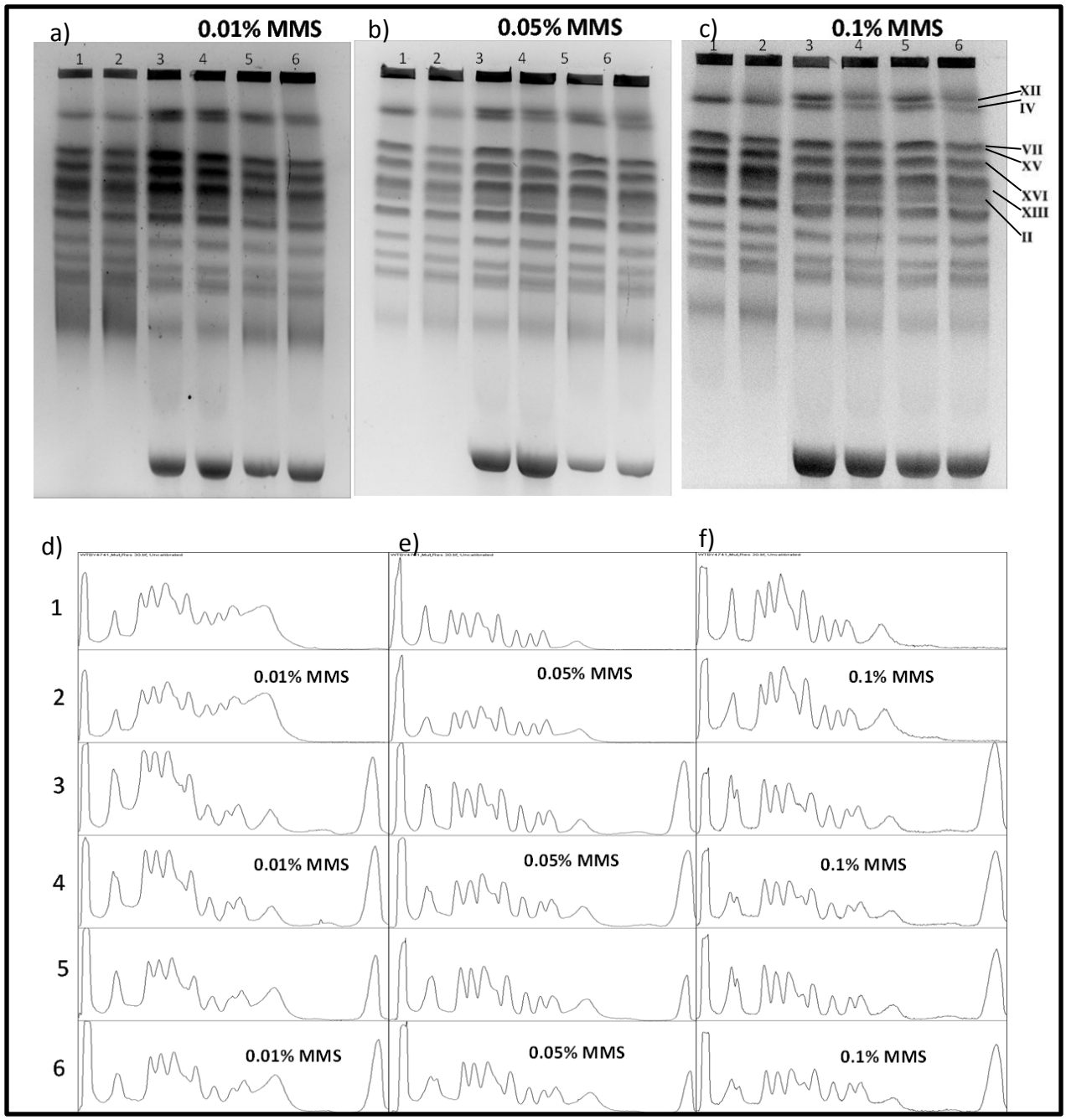
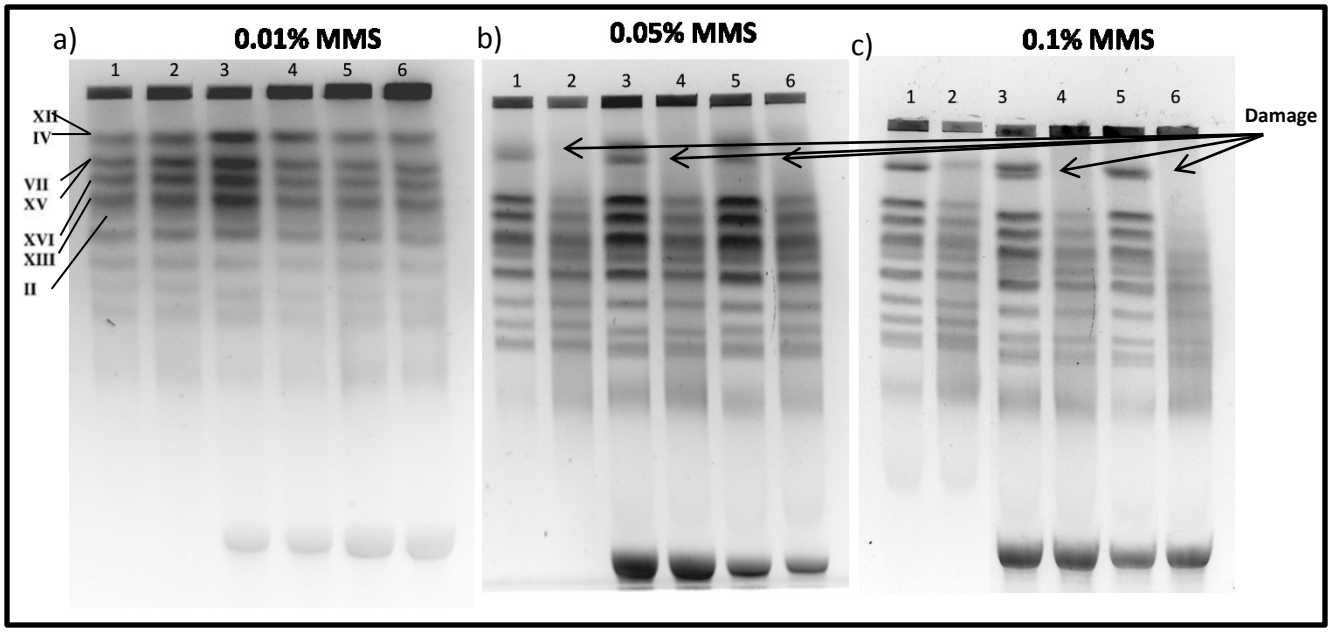


Figure 17: PFGE profile of three yeast strains (BY4741, Tfc3 mutant, Tfc3 Complemented for detection of genomic integrity assessment a) PFGE profile of three strains of yeast namely BY4741, Tfc3 mutant and Tfc3 complemented loaded in gel as, lane 1: BY4741 untreated, lane 2: BY4741 treated, lane 3: Tfc3 mutant, lane 4:Tfc3 mutant treated, lane 5: Tfc3 complemented, & lane 6 Tfc3 complemented treated with 0.01% MMS, **b)** PFGE profile of three strains of yeast namely BY4741, Tfc3 mutant and Tfc3 complemented loaded in gel as, lane 1: BY4741 untreated, lane 2: BY4741 treated, lane 3: Tfc3 mutant, lane 4:Tfc3 mutant

treated, lane 5: Tfc3 complemented, & lane 6 Tfc3 complemented treated with 0.05% MMS & **c)** PFGE profile of three strains of yeast namely BY4741, Tfc3 mutant and Tfc3 complemented loaded in gel as, lane 1: BY4741 untreated, lane 2: BY4741 treated, lane 3: Tfc3 mutant, lane 4:Tfc3 mutant treated, lane 5: Tfc3 complemented, & lane 6 Tfc3 complemented treated with 0.1% of MMS to check the sensitivity and DNA damage status on a global scale and plots of **d)** PFGE profile at concentration 0.01% MMS where lane 1: BY4741 untreated, lane 2: BY4741 treated, lane 3: Tfc3 mutant, lane 4:Tfc3 mutant treated, lane 5: Tfc3 complemented, & lane 6 Tfc3 complemented treated , **e)** PFGE profile at concentration 0.05% MMS where lane 1: BY4741 untreated, lane 2: BY4741 treated, lane 3: Tfc3 mutant, lane 4:Tfc3 mutant treated, lane 5: Tfc3 complemented, & lane 6 Tfc3 complemented treated & **f)** PFGE profile at concentration 0.1% of MMS where lane 1: BY4741 untreated, lane 2: BY4741 treated, lane 3: Tfc3 mutant, lane 4:Tfc3 mutant treated, lane 5: Tfc3 complemented, & lane 6 Tfc3 complemented treated, all at 30 ˚C.

4.4.4 BY4741, Tfc3 mutant, Tfc3 Complemented chromosomal profile on treatment with Methyl Methane Sulfonate (MMS) at 37 ˚C

PFGE profile of three strains were checked with Methyl Methane Sulfonate (MMS) at varying concentration to check the DNA damage sensitivity of the mutant (Tfc3), wild type and complemented was performed at 37 ˚C



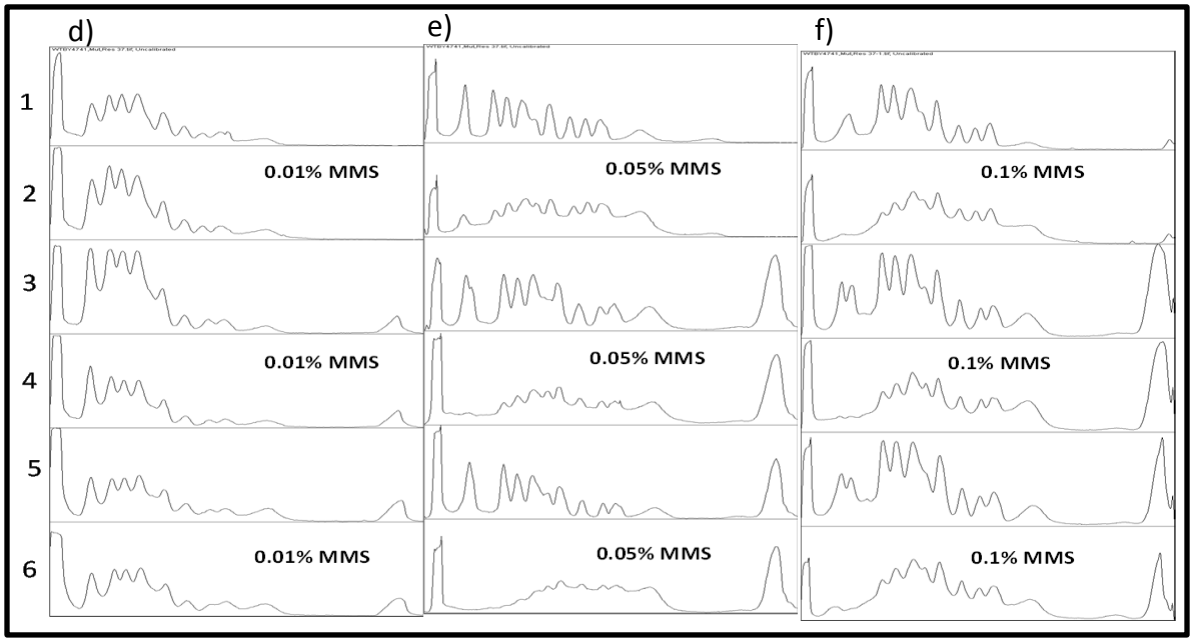


Figure 18: PFGE profile of three yeast strains (BY4741, Tfc3 mutant, Tfc3 Complemented) for detection of genomic integrity assessment a) PFGE profile of three strains of yeast namely BY4741, Tfc3 mutant and Tfc3 complemented loaded in gel as, lane 1: BY4741 untreated, lane 2: BY4741 treated, lane 3: Tfc3 mutant, lane 4:Tfc3 mutant treated, lane 5: Tfc3 complemented, & lane 6 Tfc3 complemented treated with 0.01% MMS, **b)** PFGE profile of three strains of yeast namely BY4741, Tfc3 mutant and Tfc3 complemented loaded in gel as, lane 1: BY4741 untreated, lane 2: BY4741 treated, lane 3: Tfc3 mutant, lane 4:Tfc3 mutant treated, lane 5: Tfc3 complemented, & lane 6 Tfc3 complemented treated with 0.05% MMS & **c)** PFGE profile of three strains of yeast namely BY4741, Tfc3 mutant and Tfc3 complemented loaded in gel as, lane 1: BY4741 untreated, lane 2: BY4741 treated, lane 3: Tfc3 mutant, lane 4:Tfc3 mutant treated, lane 5: Tfc3 complemented, & lane 6 Tfc3 complemented treated with 0.1% of MMS to check the sensitivity and DNA damage status on a global scale and plots of **d)** PFGE profile at concentration 0.01% MMS where lane 1: BY4741 treated, lane 2: BY4741 treated, lane 3: Tfc3 mutant, lane 4:Tfc3 mutant treated, lane 5: Tfc3 complemented, & lane 6 Tfc3 complemented treated, **e)** PFGE profile at concentration 0.05% MMS where lane 1: BY4741 untreated, lane 2: BY4741 treated, lane 3: Tfc3 mutant, lane 4:Tfc3 mutant treated, lane 5: Tfc3 complemented, & lane 6 Tfc3 complemented treated & **f)** PFGE profile at concentration 0.1% of MMS where lane 1: BY4741 untreated, lane 2: BY4741 treated, lane 3: Tfc3 mutant, lane 4:Tfc3 mutant treated, lane 5: Tfc3 complemented, & lane 6 Tfc3 complemented treated, all at 37 ˚C.

At a different concentration of MMS in 30 ˚C (fig.17) not very much subtle difference was observed, though slight degradation of the chromosomes could be observed, but that is not that significant as our mutant protein is not a dedicated player of DDR pathway and is not a central player which will affect global DDR mechanisms.

But at 37 ˚C (fig.18) there is a marked difference in damage between the treated and untreated one. The overall outcome of this experiment is that the mutant is sensitive and prone to the DNA damage, but the difference seems to be very mild as compared to wild type. In context of temperature, the mutant is sensitive to MMS at a concentration of 0.05% (fig.17 b & e) when the temperature is 37 ˚C but shows similar effect at 0.1% MMS (fig.18 b & e) when the temperature is 30˚C. Although, in a similar combination of temperature and concentration of MMS, some DNA damage was also observed in the wild type strain, but the effect is far too wise in mutant. In context of Tfc3 complemented strain the effect was comparable to the wild type strain.

4.4.5 PFGE profile of all Lab strains of yeast

Complete comparison of Tfc3 mutant with respect to all control was done to exclude any kind of possibilities of miss leading conclusion being drawn. To support this PFGE profiling of mutant with all the wild type laboratory strain present in the laboratory was performed.

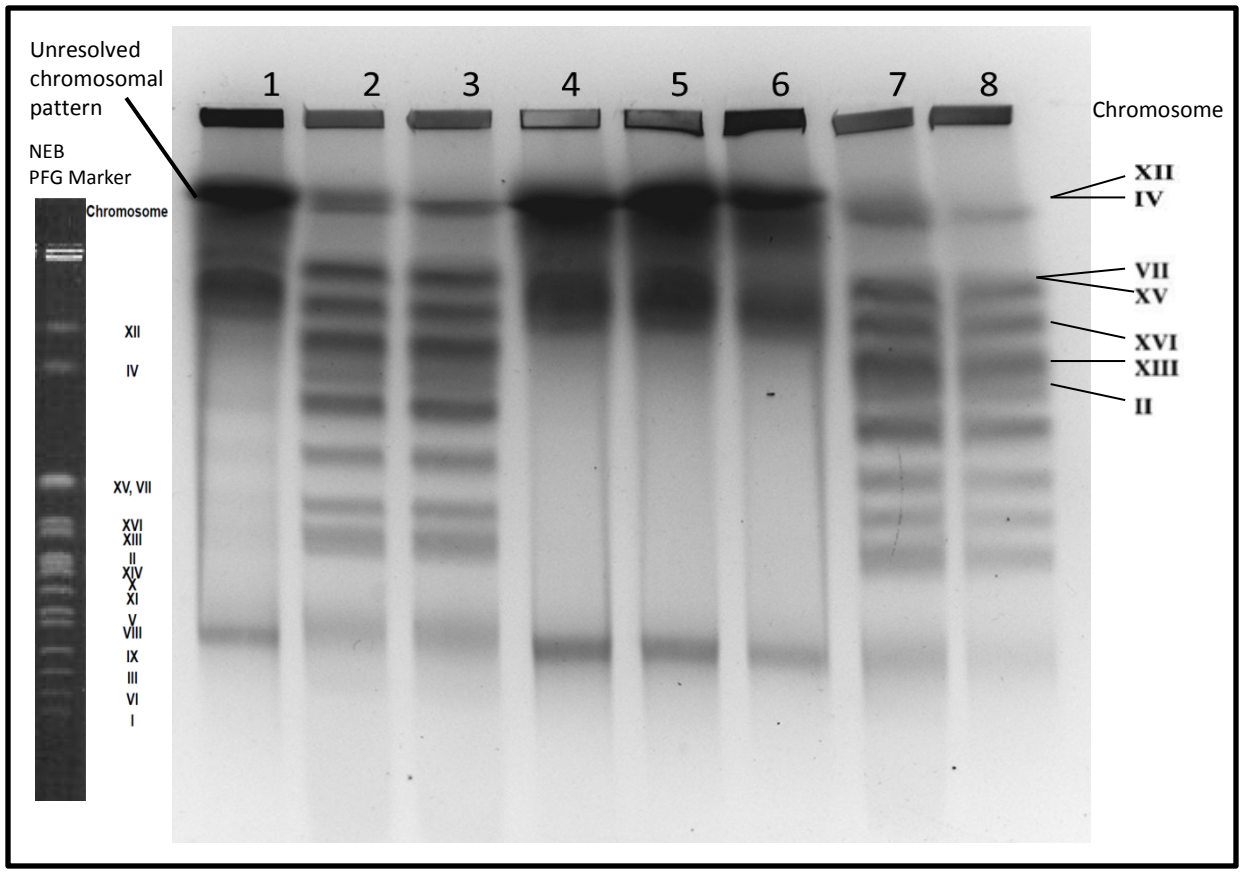


Figure 19: General PFGE profile of all laboratory strains of yeast loaded as lane 1: YPH500 strain, lane 2: Tfc3 mutant, lane 3: Tfc3 complemented, lane 4: S288C strain, lane 5: Haploid strain (euroscraf), lane 6: Diploid (euroscraf), lane 7:BY4741 strain & lane 8:W3031a strain for checking chromosomal pattern of Tfc3 mutant and Tfc3 complemented against all controls.

Laboratory strains of the wild type present was also processed to view the chromosomal profile by PFGE which gave the surprising and unexpected result as all the strains except BY4741, W3031a, Mutant, and Rescued did not give the expected proper chromosomal profile (fig.19). The cells, when in the dividing condition with the replication fork in place, then such kind of DNA remains stuck in the well and fails to give the proper profile, but the conclusion can't still be drawn based on the observation of this experiment alone.

4.4.6 YPH500, Tfc3 mutant, Tfc3 Complemented strain of yeast 30 & 37˚C profile

As YPH500 was the closest wild type strain to the Tfc3 mutant. Tfc3 mutant was created by segregation of SH518 (tsv115) x YPH499, PFGE profile against YPH500 was also performed under the same condition except the run time of 24 hours for complete separation of chromosomal DNA.

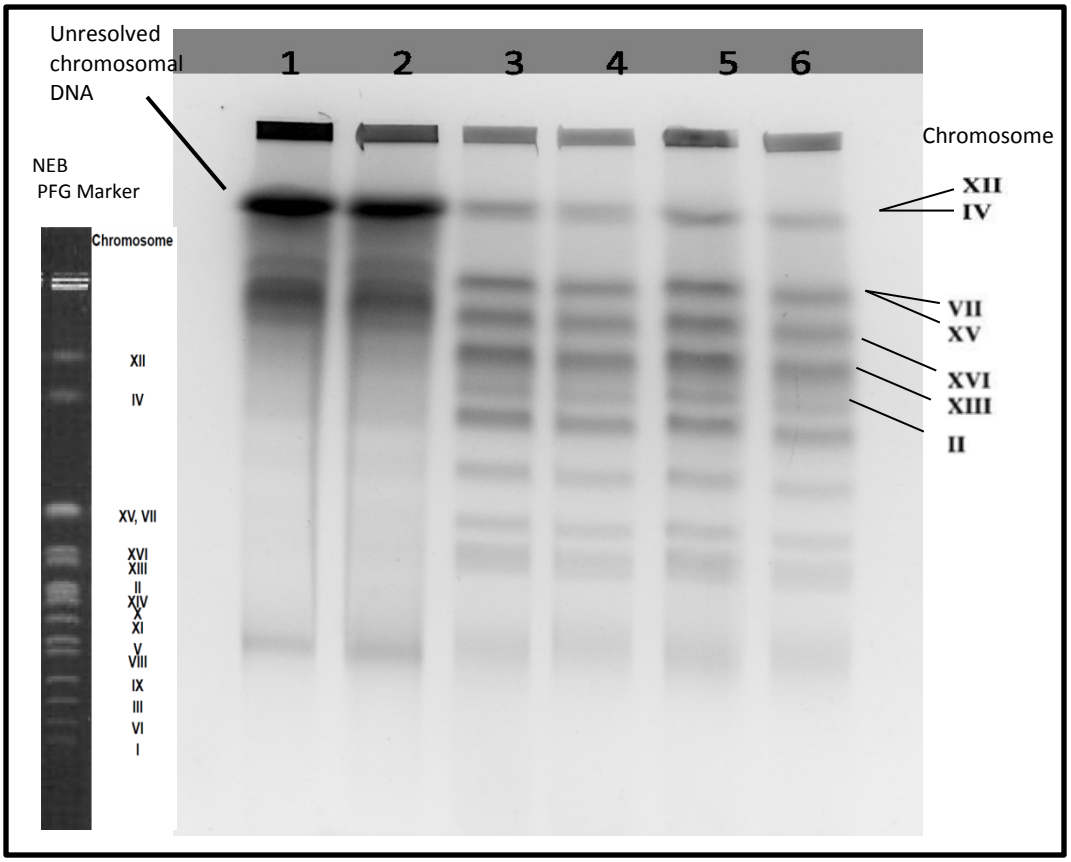


Figure 20: General PFGE profile of YPH500, Tfc3 mutant, Tfc3 complemented strains of yeast loaded as, lane 1: YPH500 strain at 30 ˚C, lane 2: YPH500 strain at 37 ˚C, lane 3: Tfc3 mutant at 30 ˚C, lane 4: Tfc3 mutant at 37 ˚C, lane 5: Tfc3 complemented at 30 ˚C, & lane 6: Tfc3 complemented at 37 ˚C to check the chromosomal integrity in order to determine the global DNA damage in 30 & 37 ˚C.

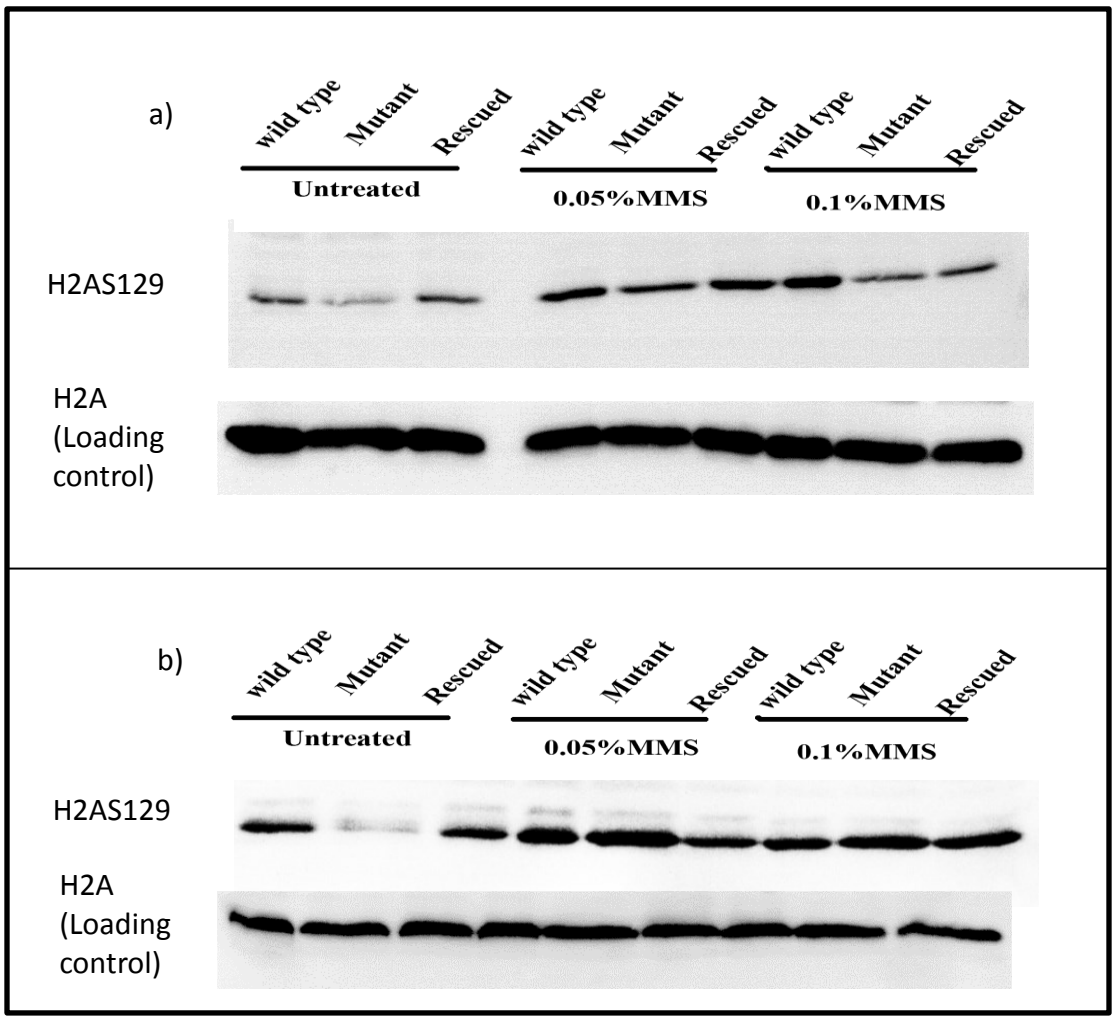
According to the figure 20, DNA damage in YPH500 is not visible at all visually as compared to mutant and rescued where largest chromosome seems a bit faint in the 37 ˚C as already observed in the other observations.

4.5 DNA Damage Response (DDR) protein levels in Wild type, Tfc3 mutant (mutant) & Tfc3 complemented (Rescued/Res)

Cells were lysed by bead beating in MLB (Appendix 1) with protease inhibitor in a bead beater which was then mixed with laemmlli buffer (80ul buffer in 200ul of sample) Western blotting was performed to check the levels of the DDR proteins.

4.5.1 H2AS129-P (yH2AX) levels

Levels of H2AS129-p also called yH2AX (~14kDa) levels were checked in the three strains without any treatment, with 0.05% MMS, 0.1%MMS at 30 & 37 ˚C which was normalized with H2A levels as an internal control.



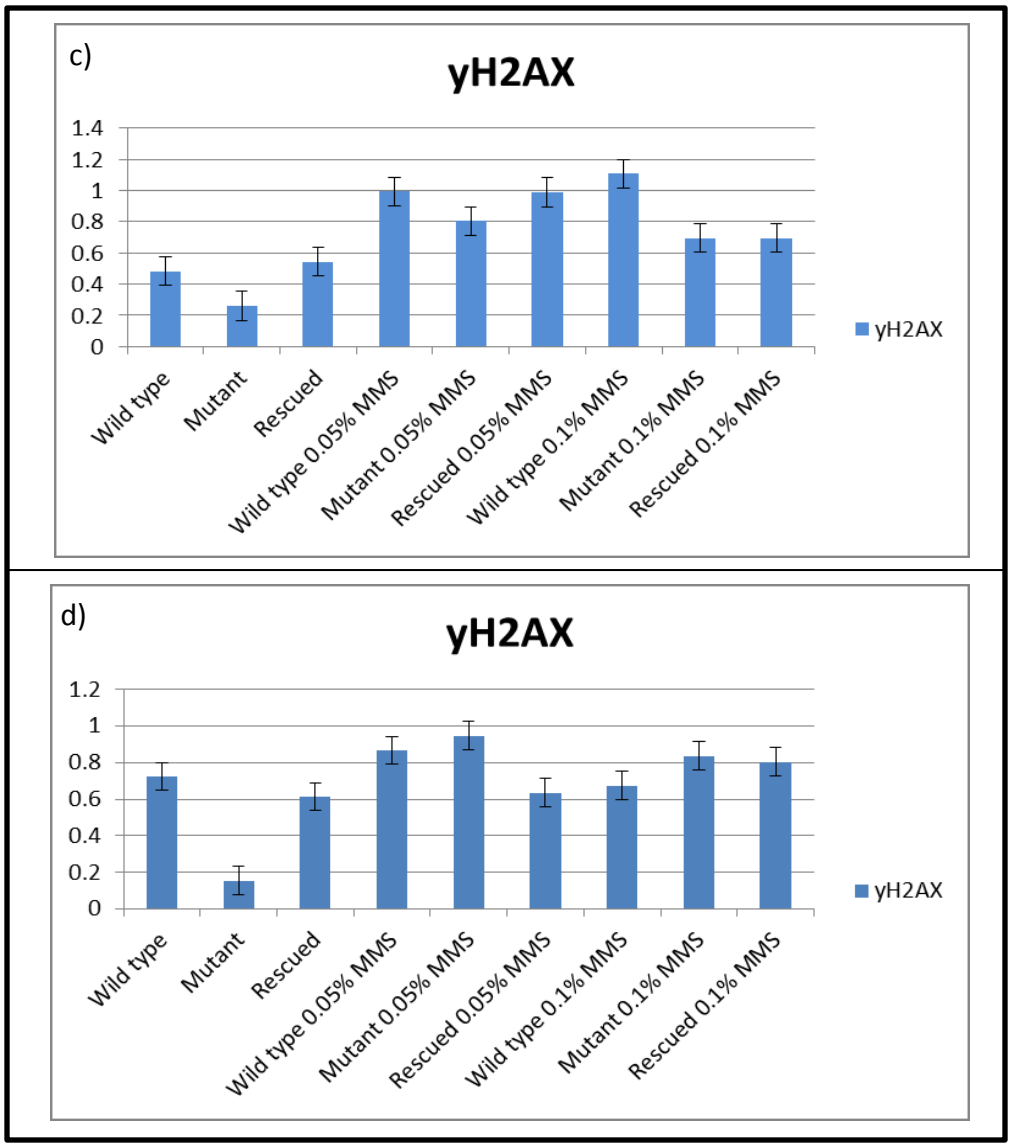
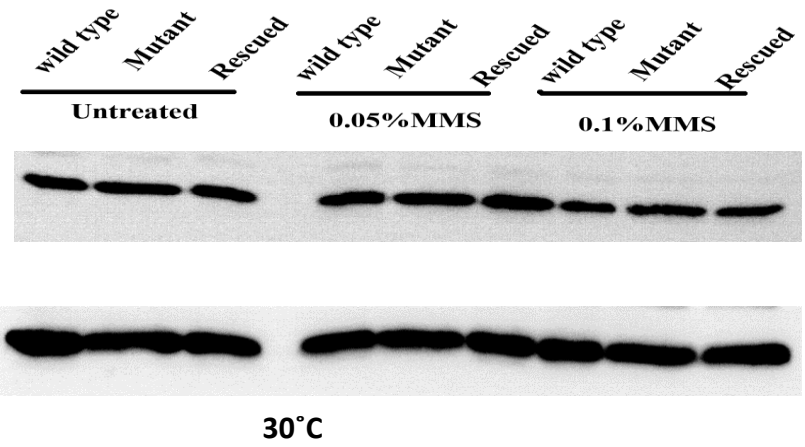


Figure 21: Western Blotting to check levels of H2AS129-P probed with anti- H2AS129-P **a)** Levels of H2AS129-P; 30˚C at the left side along with the H2A levels (Loading control) and **b)** Levels of H2AS129-P 37˚C at the left side along with the H2A levels (Loading control **c)** quantification of the 'a' on the basis of density of the H2AS129-P protein band and **d)** quantification of the 'b' on the basis of density of the band using image-j software.

4.5.2 H3K56 levels

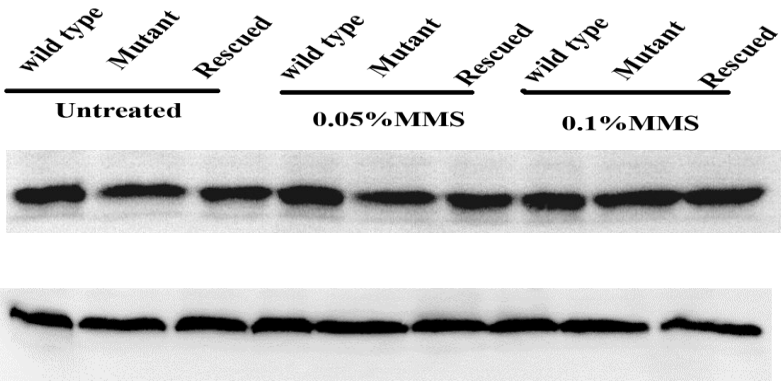
Levels of H3K56 (~15kDa) levels were checked in the three strains without any treatment, with 0.05% MMS, 0.1%MMS at 30 & 37˚C which was normalized with H3 levels as an internal control.

a)


wild type
Mutant
Rescued
wild type
Mutant
Rescued
wild type
Mutant
Rescued
Untreated
0.05%MMS
0.1%MMS
H3K56
H3
(Loading
control)

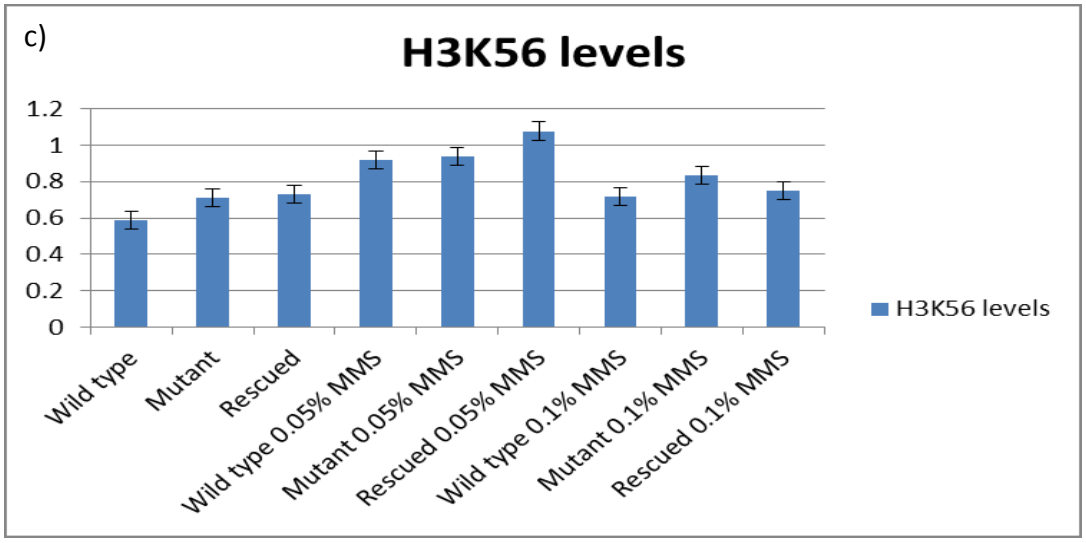
30˚C

b)


wild type
Mutant
Rescued
wild type
Mutant
Rescued
wild type
Mutant
Rescued
Untreated
0.05%MMS
0.1%MMS
H3K56
H3 (Loading
control)

37 ˚C

c)


H3K56 levels
1.2
1
0.8
0.6
0.4
0.2
0
Wild type
Mutant
Rescued
Wild type 0.05% MMS
Mutant 0.05% MMS
Rescued 0.05% MMS
Wild type 0.1% MMS
Mutant 0.1% MMS
Rescued 0.1% MMS
H3K56 levels

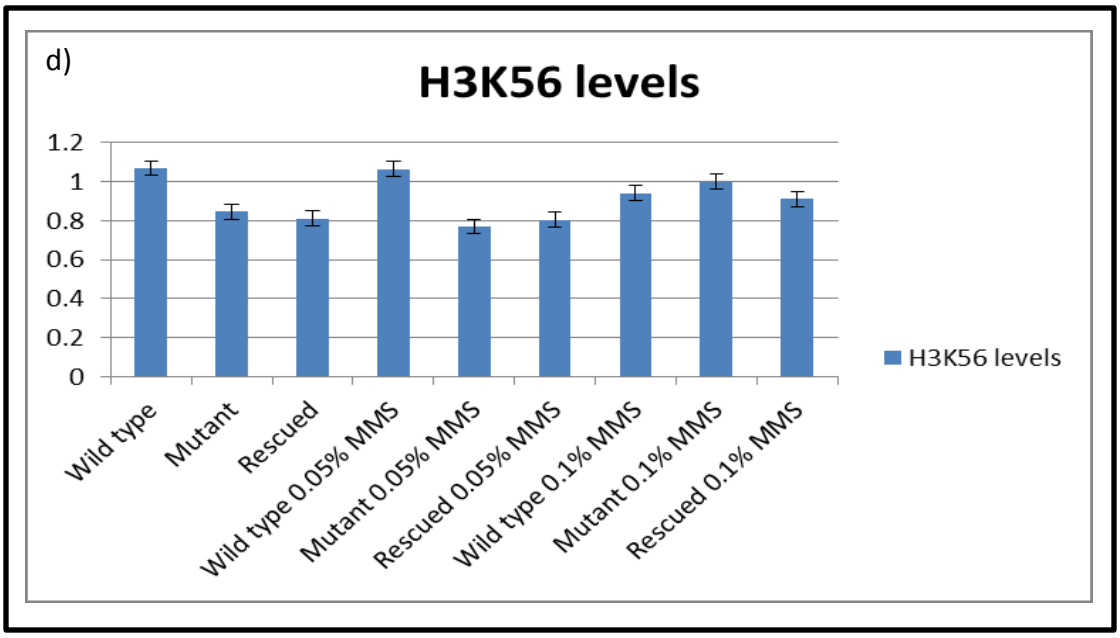
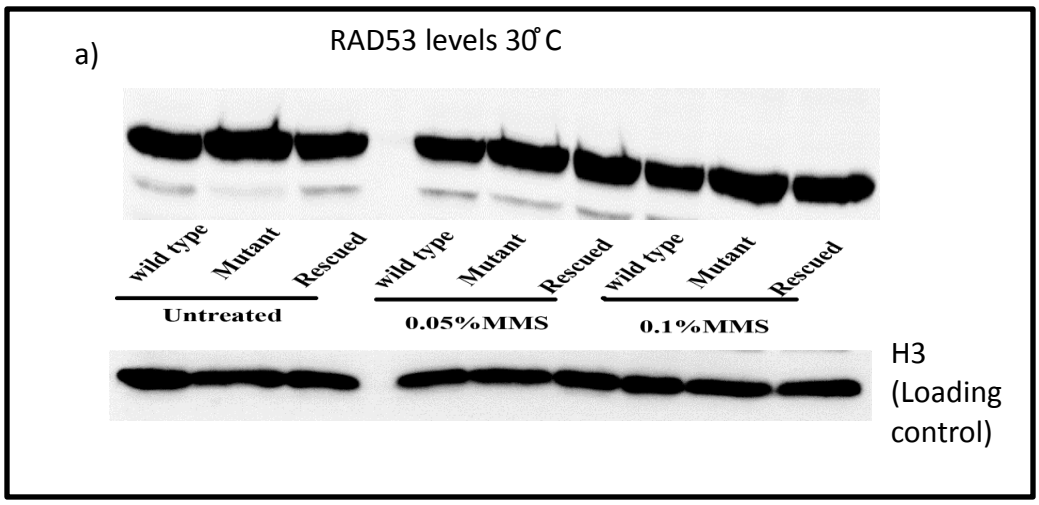
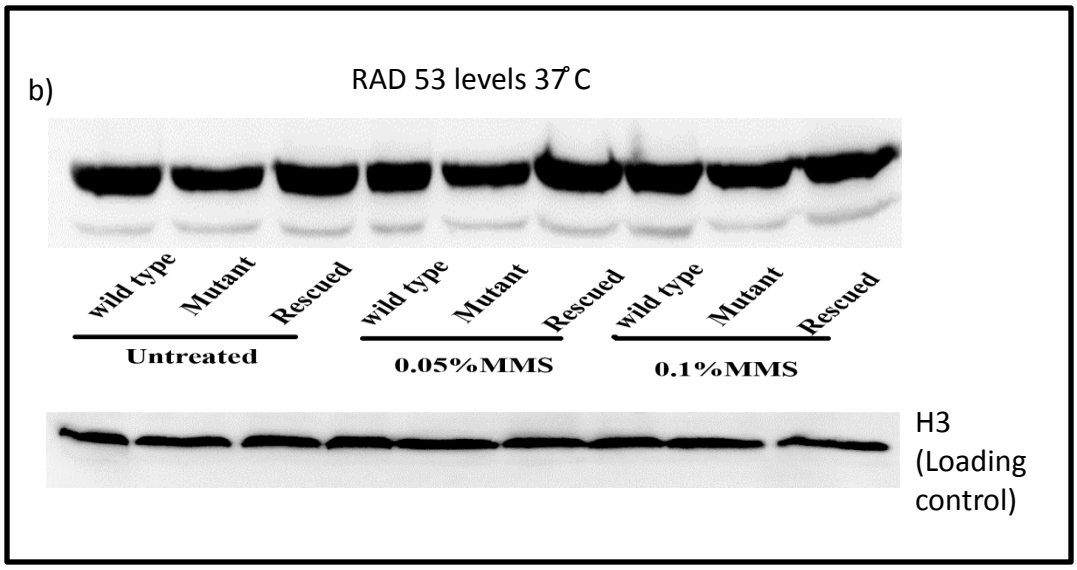


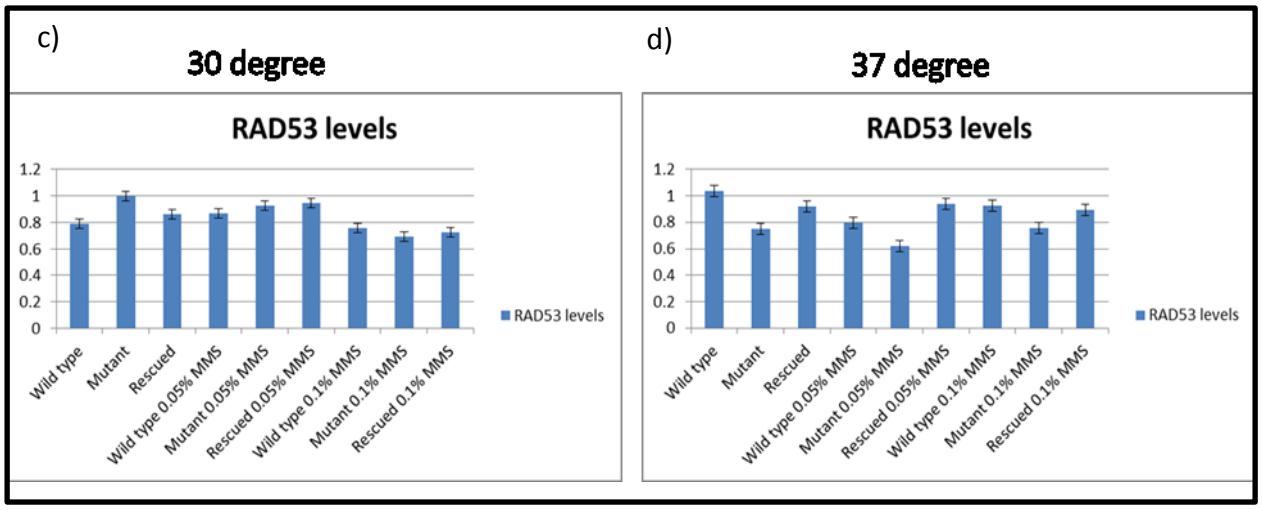
Figure 22: Western Blotting to check levels of H3K56 probed with anti- H3K56 **a)** Levels of H3K56; 30˚C at the left side along with the H3 levels (Loading control), **b)** Levels of H3K56 37 ˚C at the left side along with the H3 levels (Loading control), **c)** quantification of the 'a' on the basis of density of the band, and **d)** quantification of the 'c' on the basis of density of the band using image-j software.

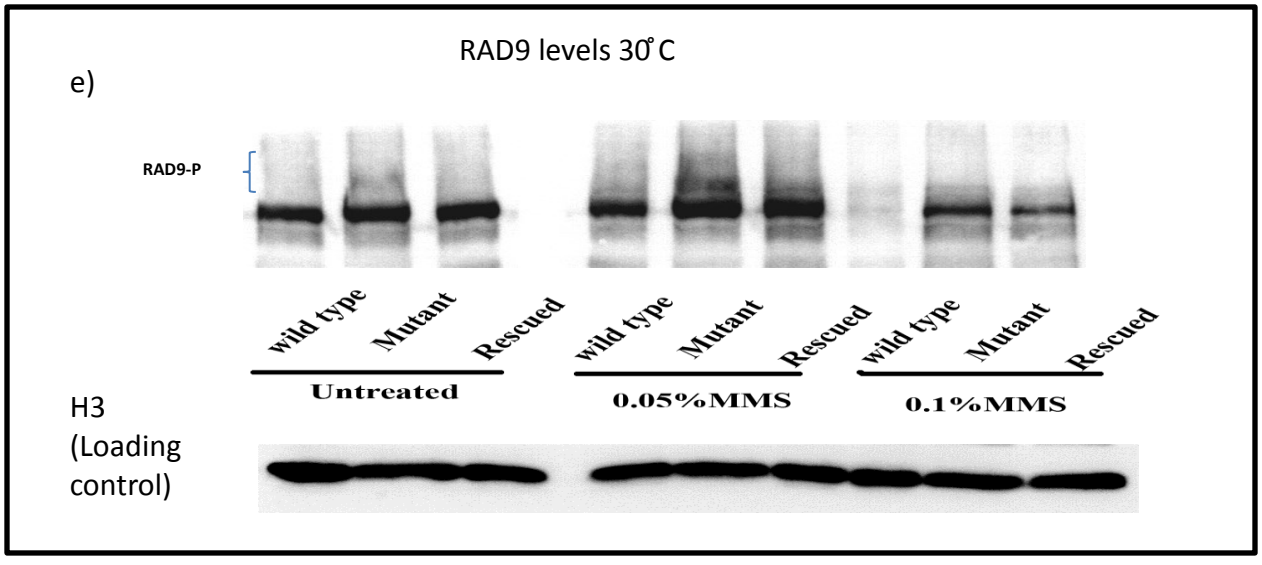
4.5.3 RAD53-P levels and Rad9 levels

Levels of RAD53-P (~91kDa) levels and Rad9 (~148kDa) levels were checked in the three strains without any treatment, with 0.05% MMS, & 0.1%MMS at 30 & 37 ˚C which was normalized with H3 levels as an internal control.




b)
RAD 53 levels 37° C
wild type
Mutant
Rescued
wild type
Mutant
Rescued
wild type
Mutant
Rescued
Untreated
0.05%MMS
0.1%MMS
H3
(Loading
control)


c)
30 degree
RAD53 levels
1.2
1
0.8
0.6
0.4
0.2
0
RAD53 levels
Wild type
Mutant
Rescued
Wild type 0.05% MMS
Mutant 0.05% MMS
Rescued 0.05% MMS
Wild type 0.1% MMS
Mutant 0.1% MMS
Rescued 0.1% MMS
d)
37 degree
RAD53 levels
1.2
1
0.8
0.6
0.4
0.2
0
RAD53 levels
Wild type
Mutant
Rescued
Wild type 0.05% MMS
Mutant 0.05% MMS
Rescued 0.05% MMS
Wild type 0.1% MMS
Mutant 0.1% MMS
Rescued 0.1% MMS


RAD9 levels 30° C
e)
RAD9-P
wild type
Mutant
Rescued
wild type
Mutant
Rescued
wild type
Mutant
Rescued
Untreated
0.05%MMS
0.1%MMS
H3
(Loading
control)

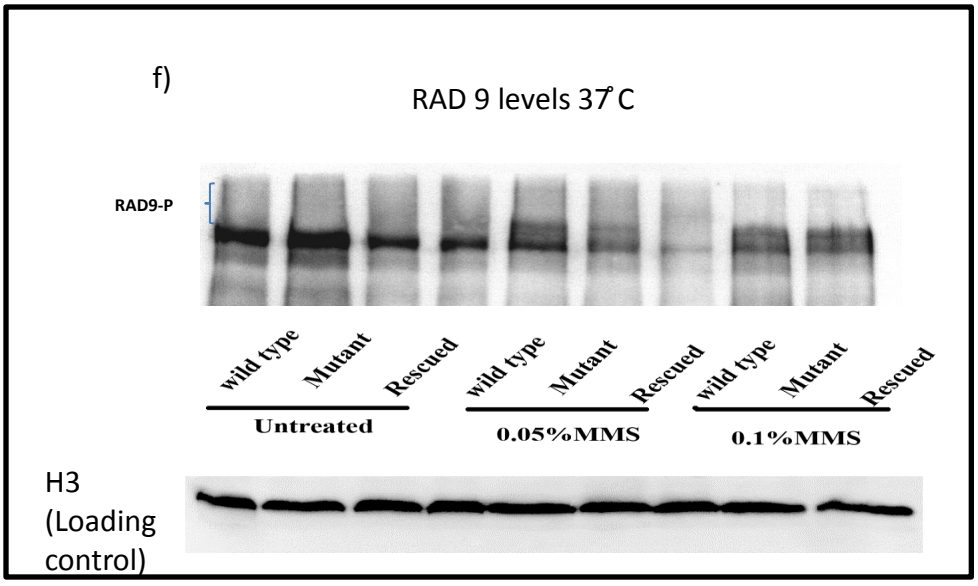
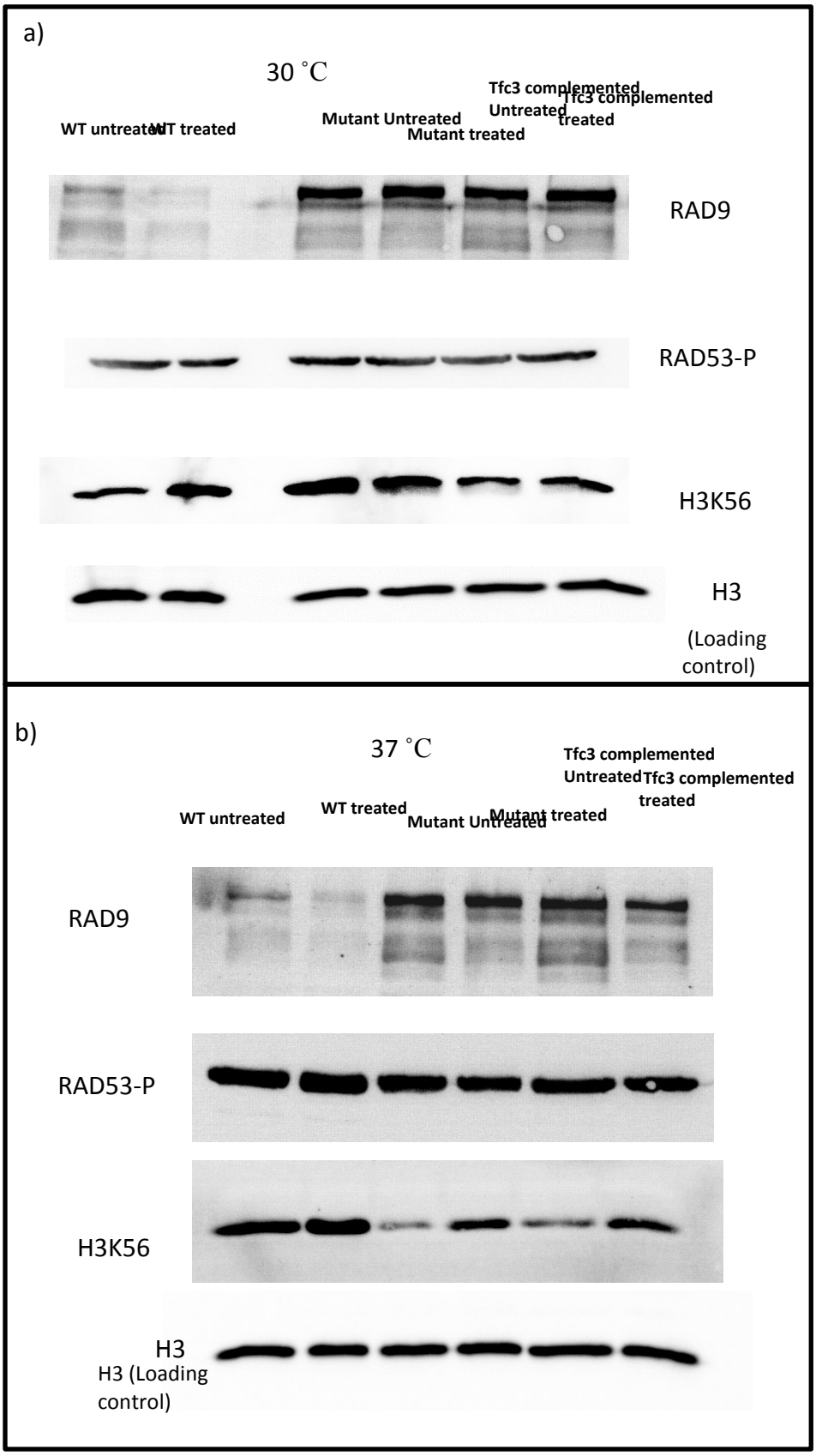


Figure 23: Western Blotting to check the protein levels of RAD53-P and Rad9 probed with anti-RAD53-P and anti-HA, respectively, **a)** Levels of RAD53-P; 30 ˚C along with the H3 levels (Loading control), **b)** Levels of Rad53-P at 37 ˚C along with the H2A levels (Loading control), **c)** quantification of the 'a' on the basis of density of the band and **d)** quantification of the 'c' on the basis of density of the band using image-j software. Also **e)** Levels of Rad9; 30 ˚C along with the H3 levels (Loading control); **f)** Levels of Rad9; 37 ˚C along with the H3 levels (Loading control).

In all above observation, the level of yH2AX (fig.20 a & c), H3K56 (fig.21 a & c) & Rad 53 (fig.23 a & c) at 30 ˚C were comparably different in wild type, Tfc3 mutant and Tfc3 complemented (rescued) stain. However, at 37 ˚C (fig. 20 b & d; fig.21 b & d; fig.23 b & d) the effect is not that uniform in case of MMS treated and untreated samples, indicating cells are already under stress, which upon exposure to the genotoxins becomes hyper-reacted and shows elevated levels of proteins. Despite the fluctuation in levels of other protein, Rad9 levels (fig. 23 e & f) are in a uniform pattern as we can see in the figure itself. Phosphorylation level of the Rad 9 is very high in wild type as compared to the mutant and Complemented, and the pattern is similar in all the conditions. This points out to the fact that TFIIIC might be affecting the activation of Rad9. Thus the possible DDR pathway, where TFIIIC is involved is the Mec1/Ddc2 dependent activation of Rad 9 levels. Though the pathway below Rad9 no distinct effect was observed.

4.5.4 Proteins levels upon treatment with Hydroxyurea (HU) at 30 and 37 ˚C

DDR protein levels were checked for the two hour exposure of HU at concentration of 200mM at two temperatures i.e., 30 & 37 ˚C.


a)
30 ˚C
WT untreated
WT treated
Mutant Untreated
Mutant treated
Tfc3 complemented Untreated
Tfc3 complemented treated
RAD9
RAD53-P
H3K56
H3
(Loading control)
b)
37 ˚C
WT untreated
WT treated
Mutant Untreated
Mutant treated
Tfc3 complemented Untreated
Tfc3 complemented treated
RAD9
RAD53-P
H3K56
H3
H3 (Loading control)

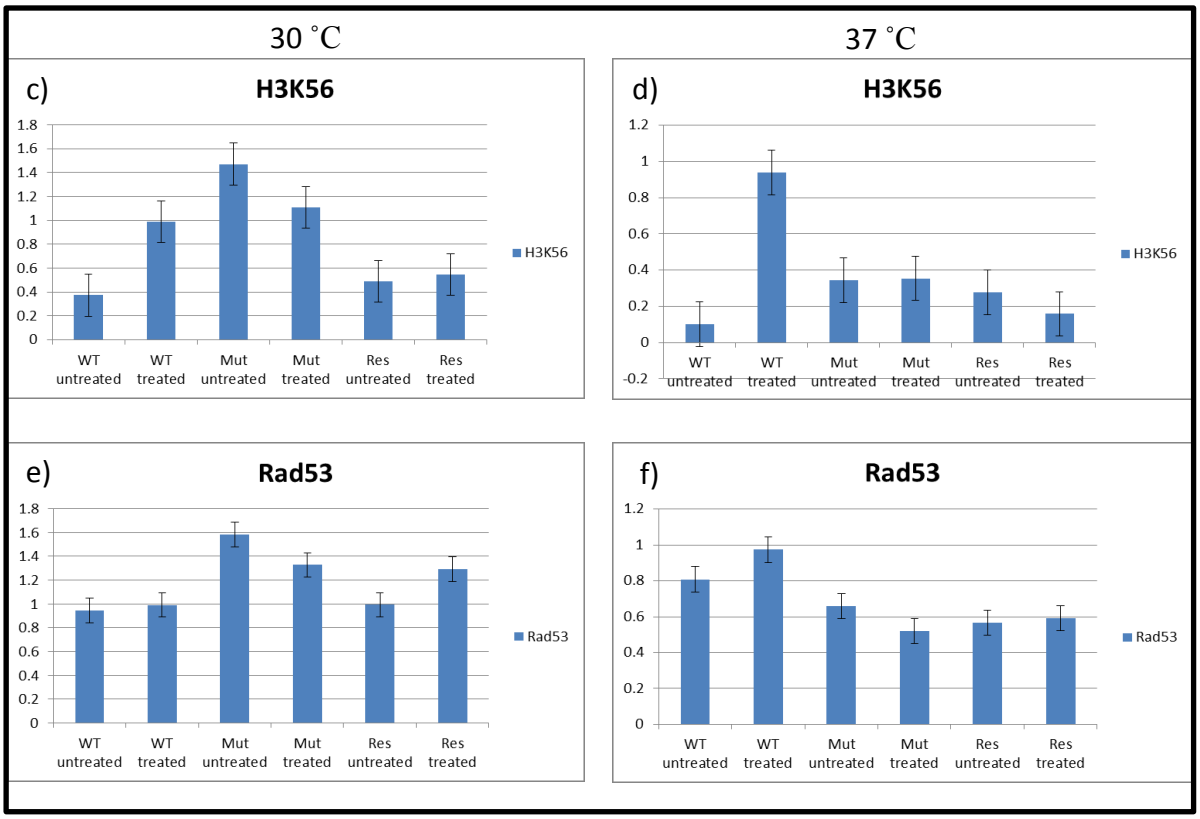


Figure 24: Western Blotting to check levels of DDR proteins, a) Protein levels of Rad9, Rad53, H3K56, and H3 checked at 30 ˚C, **b)** Protein levels of Rad9, Rad53, H3K56, and H3 checked at 37 ˚C, **c)** Quantification on the basis of densiometric analysis using image-J software of H3K56 at 37 ˚C, **d)** Quantification on the basis of densiometric analysis using image-J software of H3K56 at 30 ˚C, **e)** Quantification on the basis of densiometric analysis using image-J software of RAD53 at 30 ˚C, & **f)** Quantification on the basis of densiometric analysis using image-J software of RAD53 at 37 ˚C.

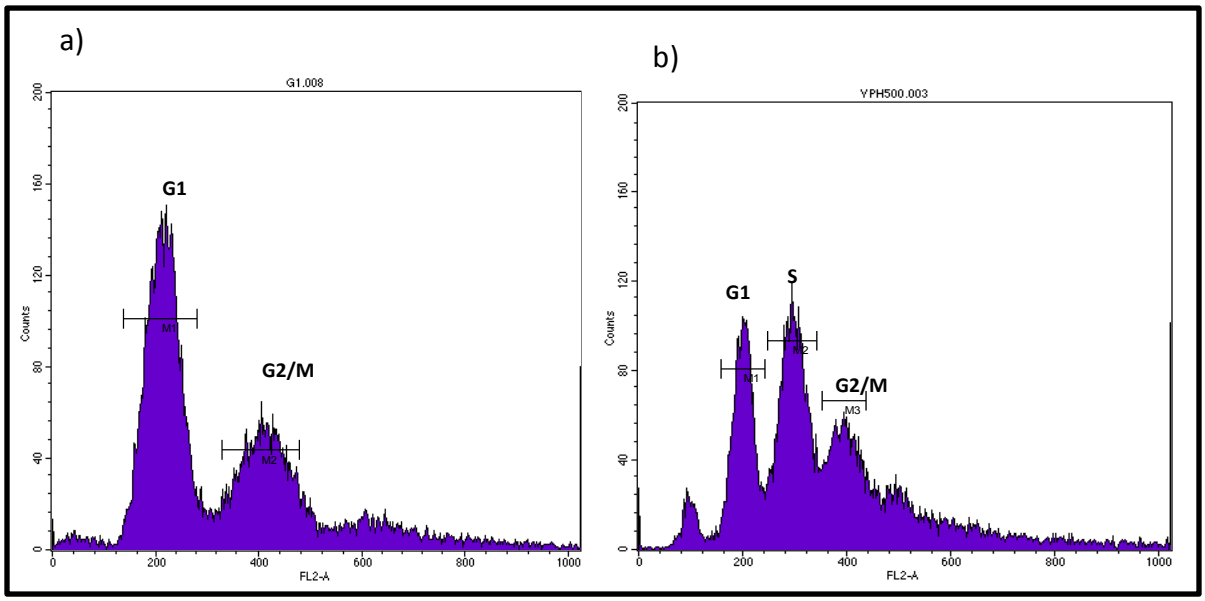
Proteins levels checked after exposure of HU at concentration of 200mM for two hour at 30 and 37˚C (fig.24). Rad 9 level changes in Wild type, Mutant, & Rescued (Tfc3 complemented) similar to that of MMS treatment but levels of H3K56 (fig24 c& d) does not vary at 30 ˚C much. The level at 37 ˚C is quite interesting as its level in wild type is much higher in treated and untreated as compared to the wild type visually. As Rad9 activation is being affected this could only occur, if proteins responsible for activation of Rad9 is not being activated. In this case as TFIIIC is found to be interacting with Ddc2, which along with Mec1 is responsible for activation of Rad9 itself. Rad53 however, could also get activated by the replicative stress via Mrc1 mediated activation (Alcasabas *et.al*, 2001) and Tel1 dependent independent pathway could also activate Rad53.The levels of H3K56, however it is quite interesting histone gets acetylated giving H3K56 only after DNA repair completion or after the replication where newly formed DNA gets acetylated (Wurtele *et al*., 2012).

4.6 Fluorescence Activated Cell Sorter (FACS) for cell cycle progression analysis

FACS analyses of Tfc3 mutant and Tfc3 complemented strains were performed along with some wild type laboratory strains of yeast; BY4741, W3031a, & YPH500 in different conditions. FACS was done by using PI (Propidium iodide) as a dye which stains the DNA. Cells were subjected to RNAse treatment before the experiment in order to remove the unspecific binding by PI. Total DNA content was checked based upon plots plotted by software (FACS Calibur, USA) showing the cell cycle progression of the cells.

4.6.1 FACS for asynchronous cultures of wild type, Tfc3 mutant & Tfc3 complemented

Asynchronous cultures prepared along with the G1 arrested wild type were taken. Cells were fixed with 70% ethanol and stained with PI, after washing with 50mM sodium citrate. A graph was obtained from these samples showing the population of cells at different stages of the cell cycle.



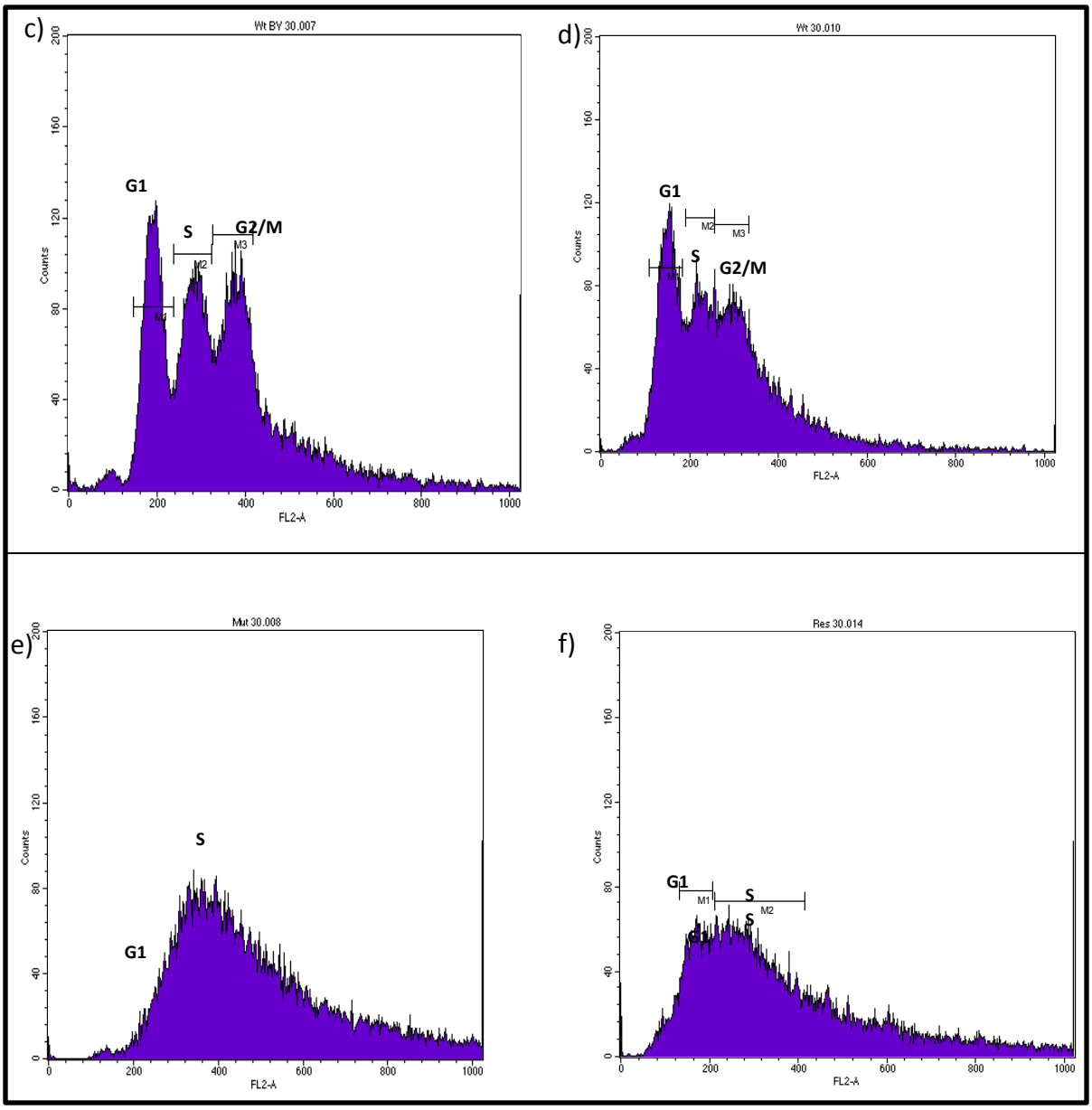


Figure 25: FACS plots of different strains of yeast to check cell cycle progress, histogram plot of all the samples drawn against counts vs log of fluorescence used(FL2-A), **a)** YPH500 G1 arrest, **b)** YPH500 (asynchronous), **c)** BY4741 (asynchronous), **d)** W3031a (asynchronous), **e)**Tfc3 mutant (asynchronous) & **f)** complemented (asynchronous) at 30 ˚C.

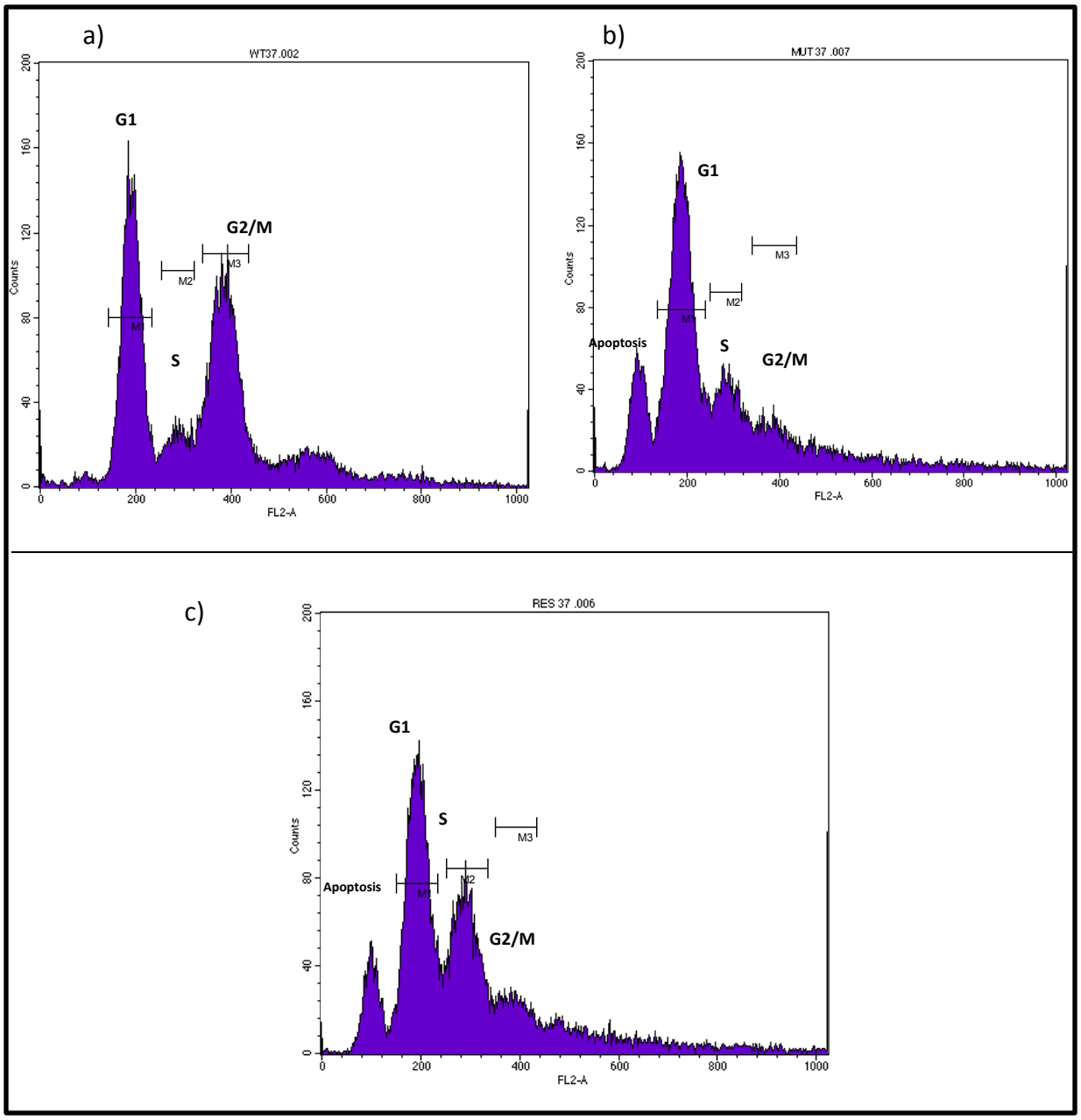


Figure 26: **FACS plots of different strains of yeast** to check cell cycle progress, histogram plot of all the samples drawn against counts vs log of fluorescence used(FL2-A), **a)** BY4741 strain, **b)** Tfc3 mutant strain & **c)** Tfc3 complemented strain at 37 ˚C of asynchronous culture; Showing cells at different stage of cell cycle and apoptosis.

4.6.2 FACS for synchronous cultures of wild type, Tfc3 mutant

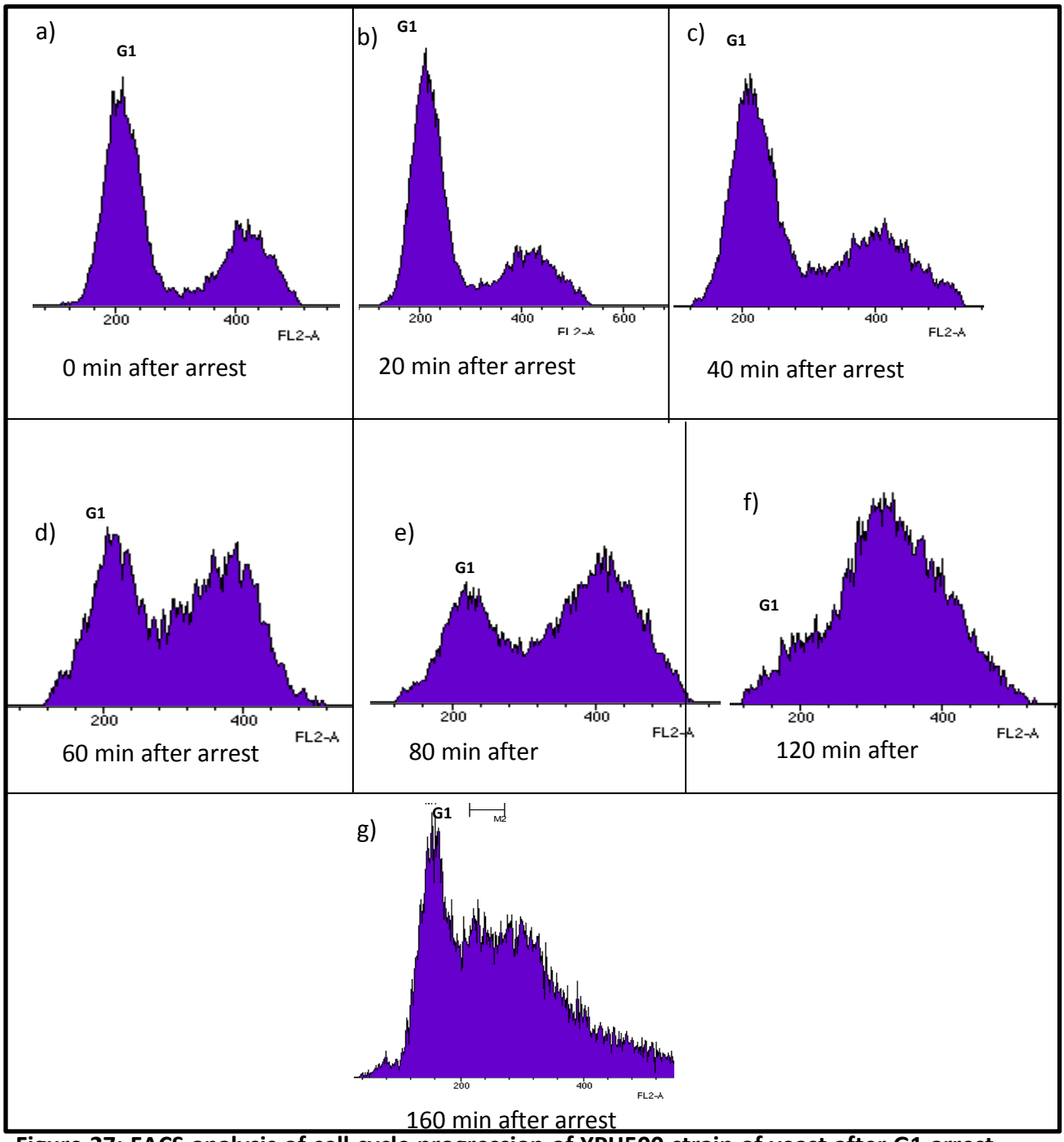


Figure 27: FACS analysis of cell cycle progression of YPH500 strain of yeast after G1 arrest (using α-mating factor), histogram plot of all the samples drawn against counts vs log of fluorescence used(FL2-A), a**)** sample taken after G1 arrest, **b)** sample taken after 20min, **c)** sample taken after 40 min, **d)** sample taken after 60 min, **e)** sample taken after 80 min, **f)**

sample taken after 120 min, & **g)** sample taken after 160 minutes respectively after its release from G1 arrest.

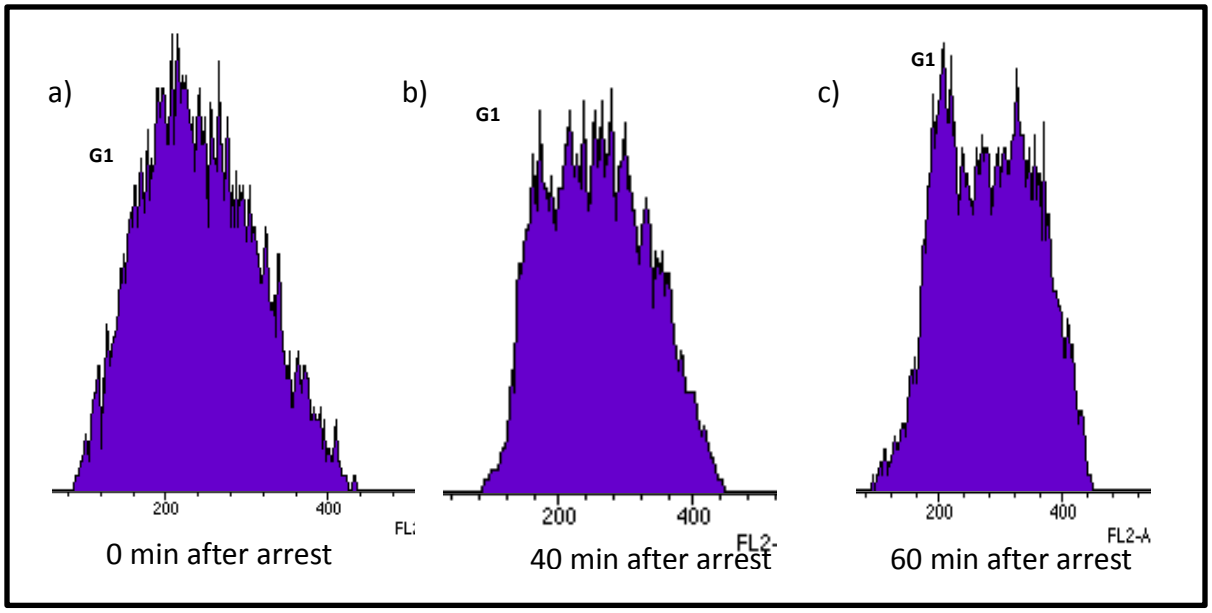


Figure 28: FACS analysis of cell cycle progression of Tfc3 Mutant after G1 arrest (using a-mating factor), histogram plot of all the samples drawn against counts vs log of fluorescence used(FL2-A), **a)** after G1 arrest, **b)** samples taken at 40 minutes **c)** samples taken at 60 minutes after its release from G1 arrest.

From the FACS result, it can be said that mutants have a very unusual cell cycle progression profile as most of the cells seems to be stuck in the S-phase (fig.25). But in G1 arrest cells, it can be seen that mutant does pass through a normal cell cycle (fig. 27) but still it has the longest period of cell cycle compared to wild type.

The majority of the cells being in the S-phase (about 43-50%: Appendix no.5) signify having difficulty in replication of DNA and also taking longer time than usual to replicate DNA. Other reasons for this observation could be damaged DNA due to various cellular stresses and replicative error, most probably stalled replication fork at tDNA sites. This might be causing SSB or DSB to arrest cell at S-phase and spending more time in the S-phase giving the profile as observed by FACS above.

4.7 Comet Assay of yeast

Comet assay of wild type (BY4741, YPH500), Tfc3 mutant (mutant) & Tfc3 complemented (rescued) strains using the protocol as described by Azevedo *et.al,* 2011. Prepared slides were visualized under axiom fluorescence microscope and analyzed using image-J software (open comet plugin).

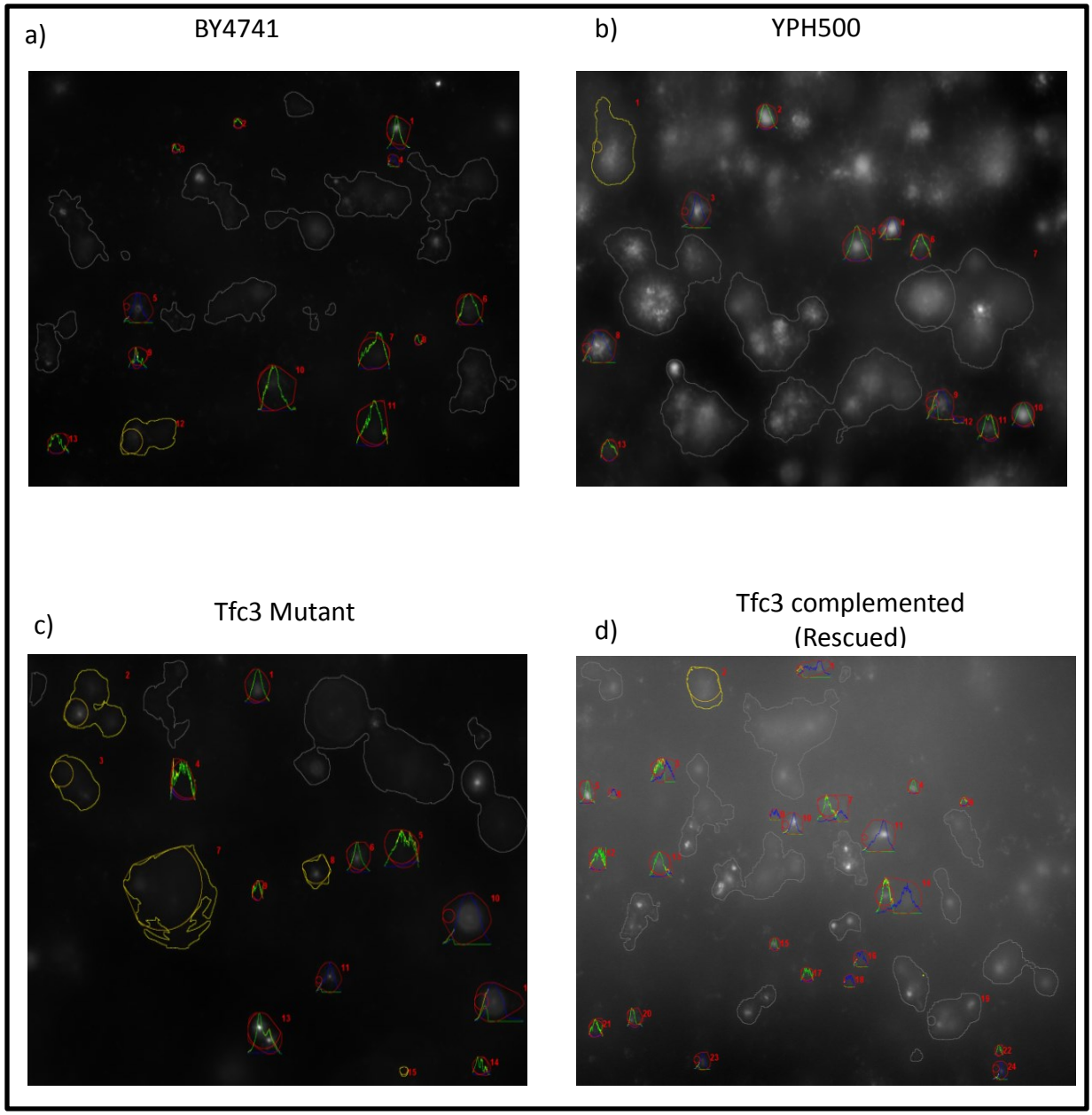
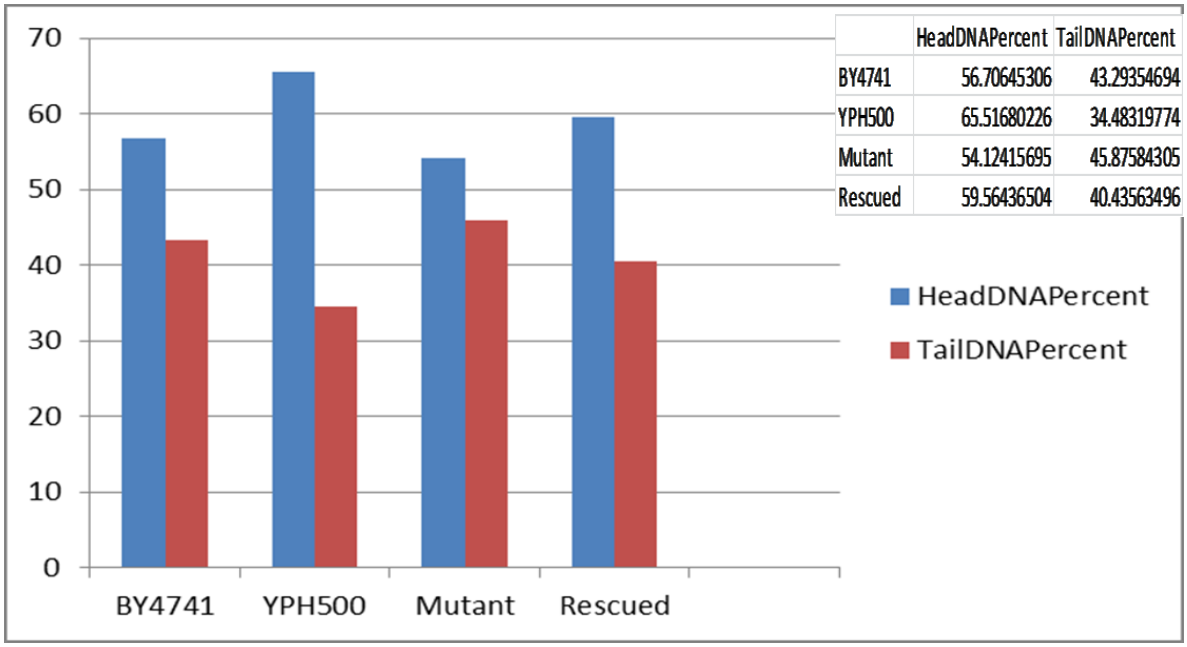


Figure 29: Comet assay showing SSB in cells as a trail of comet performed on different yeast strains **a)** Control BY4741 strain, **b)** Control YPH500 strain, **c)** Tfc3 mutant and **d)** Tfc3 complemented after automatic analysis using image-J plugin (Open comet).



	HeadDNAPercent	TailDNAPercent
BY4741	56.70645306	43.29354694
YPH500	65.51680226	34.48319774
Mutant	54.12415695	45.87584305
Rescued	59.56436504	40.43563496

Figure 30: Quantification of all comet assay slide obtained from microscopy using Image-J (open comet plugin which automatically quantifies the head and tail DNA).

Comet Assay was performed to control and test strains of yeast. As control two strains were used BY4741 and YPH500, which was visualized under fluorescence microscope after PI staining using vectashield as a mounting medium.

After quantification with automated software, mutant showed high amounts of tail DNA in comparison with control and rescued (fig. 30). Control BY4741 showed higher amounts of tail DNA than that of rescued. Mutant showed higher amounts of tail DNA than all other strains, suggesting more SSB is present in the mutant strain. SSB could be taken as a quantification of stalled replication fork. Thus, the result of the comet assay in agreeing with FACS result.

4.8 Gene copy no. comparison of Tfc3 mutant, Tfc3 complemented with various laboratory wild type strains, using qPCR based method

Genomic size were compared using qPCR based method (Wilhelm, Pingoud, & Hahn, 2003) where Ct (critical threshold) values were compared after taking equal amount of the DNA i.e., equal mass of DNA is taken as a template of about 35ng. Dilution curve was also plotted with different copy no. of one wild type strain whose genomic size was already known (copy no. were calculated using Avogadro's no. and formulas related to it).

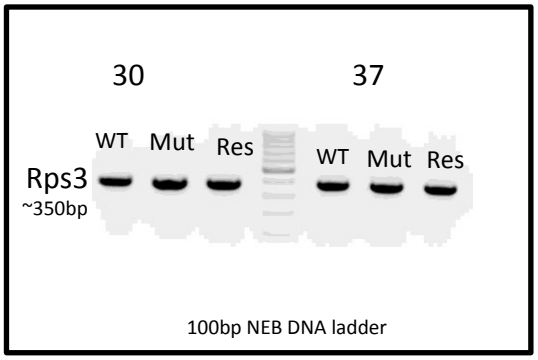


Fig. a) PCR product with same 10ng DNA from wild type (WT), Tfc3 Mutant (Mut) & Tfc3 complemented (Res)

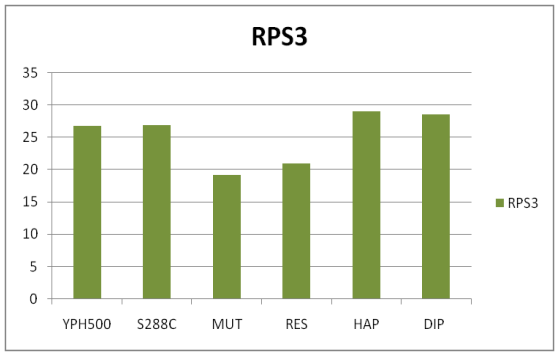


Fig. b) Real time Ct value for RPS3 gene from 35ng gDNA

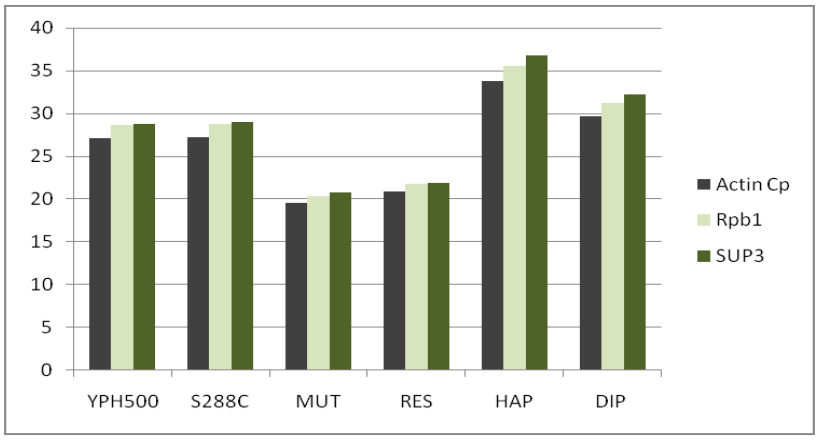


Fig. c) Real time Ct value from 35ng gDNA

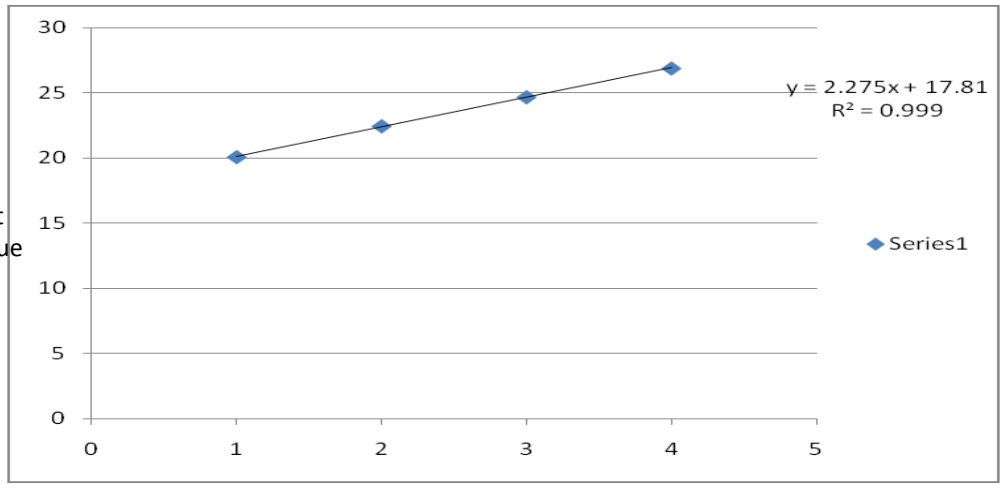


Fig. d) Real time Ct value from serial dilution of gDNA from S288C with 10 fold increase in copy number of gene (Calibration Curve for Ct value).

Strain	Actin Ct value	RPS3 Ct value	Rpb1 Ct value	SUP3 Ct value
YPH500	27.08666667	26.78	28.63333333	28.81333333
S288C	28.71333333	28.00666667	29.86	31.10333333
MUT	19.58333333	19.25	20.3	20.81333333
RES	20.84333333	21.01	21.76	21.88666667
HAPLOID	33.81666667	29.05	35.57	36.79
DIPLOID	29.65333333	28.6	31.18	32.26333333

Fig. e) tabulated Ct values of fig 'c'

Figure 31: **qPCR experiment to compare genomic size of different strains of yeast**, **a)** PCR product of wild type Tfc3 mutant & Tfc3 complemented for Rps3 gene using culture grown at 30 and 37 ˚C. **b)** Bar diagram plotted for comparative study of difference in Ct values of different strains against Tfc3 mutant and Tfc3 complemented of Rps3 gene; **c)** Bar diagram plotted for comparative study of difference in Ct values of different strains against mutant and complemented of different genes, **d)** Ct value curve of various dilutions with a different copy number of S288C from 1 to 4 on X-axis, copy number of 10^9,10^8,10^7, 10^6 with mass of DNA 13260, 1326, 132.6 & 13.26 ng respectively. Showing Ct value difference of about 2.2 cycle upon the increase of template concentration by 10 fold, & **e)** tabulated Ct values of fig 'c'.

For the comparison of gene copy no. in the cells, Rps3 a single copy gene, which is also single copy even in the diploid strains, was taken as a standard whose Ct value was further validated using some housekeeping genes as a control (actin, Rpb1 & SUP3) genes (fig.31). The result was, however uniform as mutant, and rescued showed the low CT value as compared to the wild type, indicating these strains had greater copy no. of gene in comparison. This could be due to the genomic rearrangement or due to more no. of cells in S-phase. In S-phase the copy no. of the gene will be greater as there will be both parent and daughter DNA molecule as well.

4.9 ChIP (Chromatin Immunoprecipitation) for occupancy checking of TFIIIC

Chip was performed using Tfc6 FLAG tagged strain of yeast; using anti-FLAG coupled agarose beads in active (nutrient rich) and suppressed (nutrient deprived) condition. Occupancy was checked on Upstream (UP) location, TATA box and at A/B- box region using specific primers.

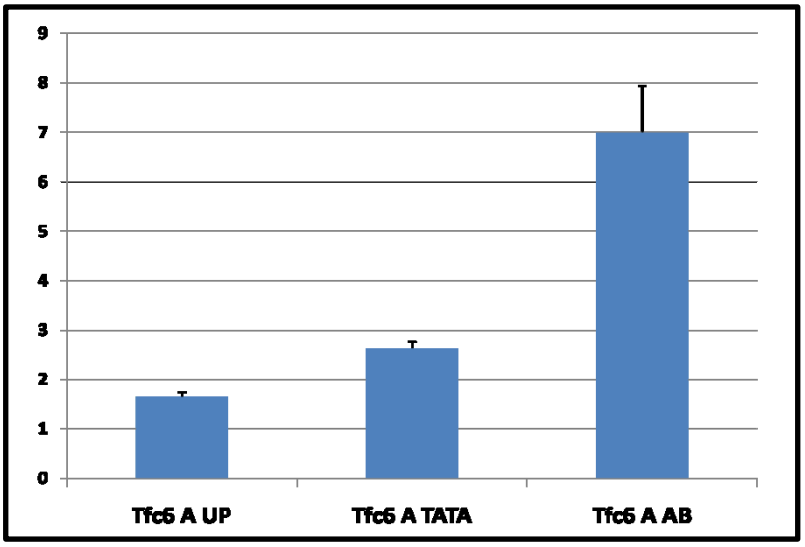


Figure 32: Tfc6 FLAG Tagged ChIP in Active conditions. ChIP result of TFIIIC performed by pull-down of Tfc6 anti-FLAG antibody in active condition calculated using fold increment method.

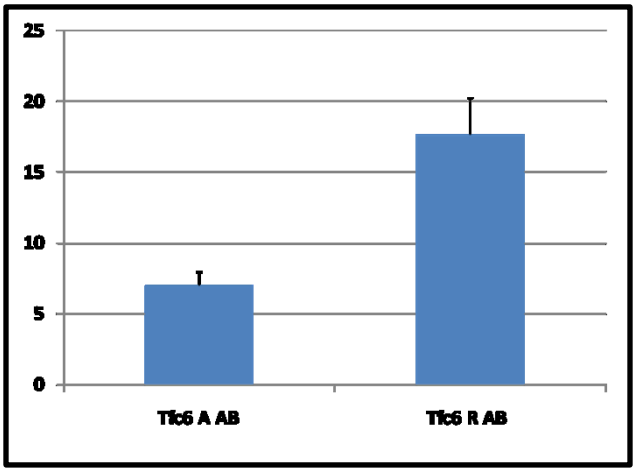


Figure 33: Tfc6 FLAG Tagged ChIP in both Active and suppressed conditions. ChIP of TFIIIC using Tfc6 anti-FLAG antibody and occupancy of TFIIIC in A/B box region calculated using fold increment method.

As expected occupancy of TFIIIC is higher in the A/B box region of the gene as it contains an A/B box binding domain (fig.32 & fig.33), whereas it showed the occupancy upstream of gene body and at the TATA box region as well (fig.32). The occupancy of TFIIIC is very low in the upstream region than that of the TATA box and A/B box region (fig.32). Occupancy of TFIIIC in TATA box is higher than that of upstream as TFIIIB binds to TATA box which is

recruited by TFIIIC. Thus, in the TATA box region TFIIIC interacts with TFIIIB causing it to precipitate. Thus, high occupancy in the TATA box than upstream region is observed.

In another experiment as shown in the figure no. 33, TFIIIC showed elevated occupancy in suppressing condition. But TFIIIC remains bound to the A/B box region even in the absence of RNA Pol III transcription PIC. TFIIIC showing high occupancy in suppressing condition signifying its role in RNA Pol III suppression as it is a well-known fact that RNA Pol III is suppressed in nutrient deprived condition. So, in contrast to that TFIIIC, being a part of RNA Pol III transcription machinery, its occupancy should decrease.

CHAPTER 5

Discussion

In this study, Yeast has been used as a model organism to study the DNA damage response of TFIIIC using thermosensitive mutant of TFIIIC subunit Tfc3 mutant called tsv115 (containing empty plasmid). A comparative study was done using mutant as a test strain, which was compared against different wild type strains (BY4741, YPH500) and Tfc3 complemented (Rescued) with a correct copy of Tfc3 gene present in the plasmid. Also, a genomic tagging of Rad53 with 6X HA epitope was performed on By4741 background strain for protein-protein interaction study.

5.1 Growth curve analysis

In a very first experiment (fig. 10), growth curve analysis of all three yeast strains, Tfc3 mutant showed a retarded growth rate compared to wild type. Rescued (Tfc3 complement) also showed retarded growth while the rate is slightly higher than that of mutant. The result suggests that mutation could be affecting proper cellular function resulting in retarded growth in the mutant. At 37˚C (fig. 10 b) Complemented have managed to grow at a comparable rate with that of wild type while mutant growth rate was retarded. Here, the most probable cause of the result could be the design of the experiment as at 37 ˚C, where the analysis was done only after attaining O.D. Of 0.52 which lies above lag phase (Asaduzzaman, 2007). Analysis done at 30˚C however, was carried out from O.D. From 0.2 onwards, which is considered to be the margin of lag and log phase in Yeast. But the most important result has been the fact that mutant and complemented showed the retarded growth rate at both temperatures suggests TFIIIC mutation is affecting the growth rate of cells.

5.2 Protein –Protein interaction

In the current study, which was started from the observation of the preliminary interaction of TFIIIC a transcription factor of RNA Pol III with Ddc2, confirmed by Complex Immunoprecipitation (COIP) experiments with Tfc1 pull down was performed in different concentration of Methyl Methane Sulfonate (MMS) to check interaction with Ddc2 (fig. 13) which has been found to be positive and had linear relation for upto the concentration of 0.1% of Methyl Methane Sulfonate (MMS). Furthermore, the interaction of TFIIIC with Rad53, checked with TFc1 pull down gave negative results along with Rad9 (unpublished, manuscript in preparation, CCMB, 2014) telling a fact that TFIIIC might act before Rad9 and probably above Mec1/Ddc2 complex. The result points towards the probable role of TFIIIC

in the DNA Damage Response (DDR) pathway, the part of DDR pathway where Mec1/Ddc2 complex is involved.

The tRNA genes are known to be difficult to replicate. tRNA, 5sRNA genes are transcribed by RNA Pol III (Dalgaard, Godfrey, & McFarlane, 2011). Replication of these genes is difficult probably due to presence of transcription Pre-initiation Complex (PIC) assembled in the genes. RNA Pol III PIC is assembled in the genome all the time which acts as a replication barrier resulting in pausing of the replication fork (Donze, 2012). Such kind of event results in the activation of replication stress pathway. Which then suppresses RNA pol III via Mrc1/Mec1 dependent activation of Rad53 (Nguyen *et al*., 2010; Enserink, 2011). Though suppression of RNA pol III is brought about via Maf1 mediated suppression, Nguyen.C Vesna *et.al,* suggests that suppression of RNA pol III is not solely due to Maf1 activity and it does not plays a central role. The fact that tRNA genes are difficult to replicate and PIC acting as a replication barrier resulting in the replication fork pausing, Probable collision of PIC with replication fork or RNA polymerase itself possess risk of DNA damage in the cell (Donze, 2012; Dalgaard *et al*., 2011; Sabouri *et.al*, 2012).

5.3 Pulse Field Gel Electrophoresis (PFGE) for genomic integrity assessment

To check sensitivity of Tfc3 mutant towards DNA damage, genomic integrity test in presence and absence of genotoxins at varying concentration (MMS & Hydroxyurea) was performed. Result of PFGE was not that straight forward as this technique cannot detect DNA damage which is less than the damage caused by 5 Gy of radio (Pinto *et.al*, 2000). As TFIIIC is a transcription factor which have been reported to show activities other than just transcription (Donze, 2012) and not a professional DDR protein, expectation of very large DNA damage was not there. Which was consistent with the result as the damage was there in the control as well but the extent of damage was a bit more in mutant than that of control and rescued (Tfc3 complemented). In a simple 30/37 ˚C profiling (fig.16) along with mutant, control also showed some degradation but degradation in mutant was higher visually, but in samples treated with genotoxins i.e., MMS, mutant showed marked difference only at concentration of 0.05% at 37 ˚C (fig.18) but tolerated the MMS up to 0.1% at 30˚C (fig.17). In both conditions, wild type showed very little degradation, though degradation was there but in Tfc3 mutant almost complete degradation in largest chromosome at 37˚C & partial degradation at 30˚C could be observed which suggests sensitivity of Tfc3 mutant towards genotoxins. The result suggests that mutant is more susceptible to DNA damage than that of control. But in HU treatment the effect was not visible as the exposure was of only 2 hours, which might not be enough to cause substantial damage.

5.4 Analysis of DNA Damage response (DDR) Protein levels

In conquest to check the genomic integrity, along with DNA damage assessment DDR proteins were also checked: Rad9, Rad53, yH2AS129-p, H3K56. All proteins mentioned are involved in DNA damage repair pathway which ultimately leads to activation of DNA damage repair along with cell cycle arrest, transcription activation etc. These proteins were chosen based on the type of genotoxins used (both induce DNA strand breakage) and role of TFIIIC is also suspected to be in DNA strand breakage (Probably strand breakage due to replication fork stalling and continuous transcription where single strand DNA is more prone to recombinational mutation or breakage (Enserink, 2011; Dalgaard *et al*., 2011).

The study of protein levels showed quite different result, except for Rad9 all other proteins showed different results. Level of yH2AS129-p (fig.21) matched expectation in samples without treatment high in control and complemented but low in mutant. MMS treated samples at 30˚C (fig. 21 a & c) followed the expected pattern, but at 37 ˚C (fig.21 b & d) mutant showed elevated levels in mutant as compared to untreated one. For H3K56 also results were different at 37 ˚C (fig. 22 b & d) followed the pattern, but not 30 ˚C (fig.22 a & c) which is explainable as at 30 ˚C protein (TFIIIC) is inefficient but functional (thermosensitive mutant). Even H3K56 showed a fluctuation at 0.1% MMS. A similar pattern was found in case of Rad53 as no distinct pattern could be observed.

Interesting result was levels of Rad9 (fig.23), result was consistent with all condition and treatment, control showed very high level of activation and mutant showed low activation as compared to control and Tfc3 complemented showed some improvement in activation of Rad9 (phosphorylation of Rad9) in contrast to Tfc3 mutant. These results suggest that the DDR pathway affected is the activation of Rad9 which occurs via Mec1/Ddc2 complex. This result correlates with protein-protein interaction result, as TFIIIC showed interaction in-vivo with Ddc2 which is known to interact physically with Mec1 (Zhou & Elledge, 2000). Activation of Rad53 however can also be achieved by the MRX/Tel1 mediated activation upon DNA strand breakage independent of Rad9 though Rad9 remains a major activator of Rad53, also Rad53 can be activated in absence of Mec1p (Gilbert, Green, & Lowndes, 2001). This can in some extend explain the observation as Rad53 levels and downstream protein levels showed altered levels as the DDR pathway affected due to mutation is not the only available pathway for activation of Rad53. Which can activate DNA damage repair pathway, but Rad53 cannot phosphorylate H2AS129 which showed altered levels at 37 ˚C. Apart from Mec1/Ddc2 mediated phosphorylation of H2A, in yeast another pathway for H2A phosphorylation is via Tel1 which could be the explanation for the observed result as at 37 ˚C cells are already under stress suffering from damage which upon genotoxin treatment

boosts DNA damage resulting in elevated levels of H2AS129 in Tfc3 mutant but not in control which showed consistent levels as that of at 30 ˚C.

5.5 Fluorescence Activated Cell Sorter (FACS), qPCR based genomic size comparison, Comet assay, & ChIP analysis

FACS result showed Tfc3 mutant could be struggling with either DNA damage repair or in replication of DNA as it is stuck in the S-phase for prolong period of time. At 37 ˚C, high level of apoptosis in Tfc3 mutant and Tfc3 complemented is observed. At 37 ˚C there were more cells in G1 and S along with high quantity of apoptotic body. The profile of mutant and rescued looks more like G1 arrested. Higher level of apoptosis could also explain the irregular levels of DDR proteins that was observed in the westerns performed. Although, comet assay showed accurate quantitative data, showing Tfc3 mutant is suffering from more DNA damage than that of controls and Tfc3 complemented. Gene copy no. comparison performed using Real time PCR based method showed mutant to contain more copy number of most of the genes supporting the FACS data. ChIP data, however, supported already characterized data of probable TFIIIC distribution in the tRNA genes (Donze, D., 2011). Chip showed increased occupancy of TFIIIC in suppressing condition than that of active condition. RNA Pol III transcription is suppressed during nutrition deprivation where RNA Pol III transcription is suppressed, suggesting TFIIIC could be having a role in RNA Pol III suppression as well.

5.6 TFIIIC in DDR Pathway

From the previous work by Nguyen. C Vesna *et.al*, replication stress suppresses RNA Pol III via Mec1 dependent activation of RAD53 & Maf1 (a well-known regulator of RNA Pol III). Role of Maf1 was not central as described by Nguyen *et al*., 2010 therefore, a step in transcription that is affected by Maf1 such as polymerase recycling or chromatin reorganization that affects pre-initiation complex (PIC) assembly or stability, might be regulated by replication stress pathway. Also, pausing of the replication fork at the tRNA genes. That ultimately results in activation of replication stress pathway. This then results in suppression of RNA pol III and disassembly of the PIC. So that, the replication fork could resume and finish the replication.

TFIIIC consists of 6 subunits and have been found to be involved in many other functions other than just transcription as described by David Donze,2011 where the extra transcriptional activity of TFIIIC have been well illustrated. Adding to the function of TFIIIC, Interaction of TFIIIC with Ddc2, sensitivity to genotoxins observed from PFGE & comet assay and protein levels all points out to the fact that TFIIIC might be involved in the replication fork resuming and probably in the regulation of RNA pol III, as suggested by Nguyen *et al*.,

2010, as Mec1 can activate TFIIIC via Ddc2 interaction. ChIP result is also in agreement with possible function of TFIIC in DDR pathway and RNA Pol III suppression. The possible mechanism by which TFIIIC might be involved in DNA damage is by disassembling RNA Pol III PIC upon replication stress signals via Mec1, making way for replication fork and avoiding damage due to prolonged replication fork pause (Lambert & Carr, 2011). FACS result also supports the inference as cells are stuck in the S-phase because replication fork could be stalled at tRNA genes due to failure to remove PIC and repress RNA pol III completely, causing replication very difficult for the cells. Comet assay on the other hand was performed using an alkaline lysis method that is useful in detecting single strand breaks. Replication forks stalling usually results in a single strand break or equivalent to single strand breaks, which in time results in double strand breaks upon collapse. High damage detection in Tfc3 mutant in comparison could be related to paused fork in Tfc3 mutant, which is more than that of control.

Form the results obtained in this study, the possibility of TFIIIC activating DDR protein upon DNA damage cannot be denied. Rad9 activation seems to be affected which has a major role in the DDR pathway upon DNA damage. But the finding is still preliminary & needs further validation probably via in-vitro experiments.

From previously known facts and already characterized pathway, TFIIIC (based on current evidences) could be responsible for resuming paused replication fork. Mec1/Ddc2 complex (replication stress pathway) upon activation of replicative stress pathways removes the PIC (pre initiation complex) via TFIIIC. After that RNA Pol III transcription is suppressed by major regulator of RNA Pol III, Maf1 via Mec1 dependent activation which was also stipulated by the former worker Nguyen et al. in 2010.

CHAPTER 6

Summary & Conclusion

6.1 Summary

Under this study, possible role of TFIIIC in DDR pathway in mutant based comparative study. Budding Yeast was used as a model organism to study the basic cellular process of the DNA damage response pathway. Where strain, having point mutation on Tfc3 subunit of TFIIIC, Wild type, mutant, and Tfc3 complemented (with Plasmid having corrected copy of the gene) was used to execute the comparative study. Different assays as genetic integrity assay with and without genotoxins exposure, DDR protein level assay, protein-protein interaction, qPCR based genomic size estimation for genomic rearrangement assessment & FACS for cell cycle progression assessment of all three strains followed by few CHIPS experiments were carried out.

Role of TFIIIC in DDR pathways was there, but extension of its genome guarding functions is somewhat limited. As only Rad9 activation was also directly affected from the existing pathway knowledge about the budding yeast DDR pathway, it appears that Ddc2/Mec1 complex is involved in release of replication stress caused by stalling of fork in a tRNA gene which has been found to interact with TFIIIC in-vivo and the interaction was very strong. Also, mutant is struggling to replicate DNA as suggested by the experiment of qPCR based genomic size estimation supported by PFGE and FACS experiments. Still, TFIIIC working with RNA Pol III to detect damage in DNA and activating DDR pathway via Mec1/Ddc2 complex remains a possibility.

6.2 Conclusion

The present research study carried out on yeast as a model organism, which is a mutant based study of a subunit of TFIIIC, using a strain yOL8 having point mutation tsv115 (G349K), revealed some surprising and some expected results. TFIIIC is a transcription factor of RNA Polymerase III, showed role in the DNA damage response pathway as one of its many extra transcriptional functions. This study suggests TFIIIC could have role in resuming paused replication fork. The Mec1/Ddc2 complex upon activation of replicative stress pathways removes the PIC (pre initiation complex) via TFIIIC and helping in suppression of RNA pol III to exclude the possibility of replication fork and transcription machinery collision. Thus, prevents the deleterious effect of fork collapse. So, TFIIIC could be involved in DNA Damage Response (DDR) pathway, Where TFIIIC helps to resume paused replication fork by disassembling PIC.

However, TFIIIC having a role in DDR pathway other than the one mentioned above needs to be validated as there are not enough evidences in this study to support a direct link of TFIIIC in DDR pathway.

Recommendation

DNA damage is the key cause of various diseases, mutations, and probably cancer. Much is still unclear regarding the mechanism of how cells maintain the genomic integrity, detection mechanism, various proteins involved in the DDR responses, mostly upstream proteins like sensors, etc. From this study one such unknown protein, TFIIIC was detected through further validation is required for complete elucidation of the complete pathway involved.

Future recommendation of the study of TFIIIC in DDR pathway could be:

1. Replication forks stall assay for comparison of replication fork stalling in control and mutant.
2. In-vitro experiments to validate the DDR response of TFIIIC.
3. Nature of interaction of TFIIIC with Ddc2.
4. Potential connection of TFIIIC mutation to cancer.
5. Validation of pathway of TFIIIC in DDR in higher organism.

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Appendices

Appendix 1: Commonly used Buffers

Table 8: Commonly used buffers in the study

(Sources: Molecular-biology-protocols.readthedocs.org, My-whiteboard.com, Covachem.com)

1. 5X TGS running buffer (per litre) Tris 15.14g Glycine 93.83g SDS 5g	**2. 10x TBE Buffer (per litre)** Tris 108g Boric acid 55g 0.5M EDTA 40ml
3. 5X Western transfer Buffer (per litre) Tris 5.8g Glycine 14.4g SDS 0.4g Methanol 200ml	**4. 10x TBST buffer (per litre)** Tris 12.11g Tween20 5ml NaCl 87.66g
5. Modified Lysis buffer (for 50ml) K HEPES(7.5) 50mM NaCl 150mM $Mgcl_2$ 2mM NP40 0.01% Glycerol 10% 0.5M EDTA 1 mM	**6. Laemmli sample loading buffer (for 20ml)** Tris-Cl pH 6.8 60mM Glycerol 10% B-mercaptoethanol 5% SDS 2% 1%Bromophenol blue 0.01%
7. Alkali Lysis buffer 1 (Solution I) Glucose 50mM EDTA pH 8.0 10mM Tris pH 8.0 25mM	**8. Alkali Lysis buffer 2 (Solution II)** SDS 1% NaOH 0.2N
9. Alkali Lysis buffer 3 (Solution III) KOAc 60ml glacial acetic acid 11.5ml	**10. TE Buffer** Tris-Cl pH 8.0 10mM EDTA pH 8.0 1mM
11. Protease cocktail Inhibitor NaF 10mM	**12. Genomic DNA Buffer** Triton X-100 2%

Na_2VO_3 2mM PMSF 2mM Protease Inhibitor Cocktail 1 X	SDS (10%) 1% NaCl (5M) 100mM Tris Cl pH 8.0 10mM EDTA (0.5M) 1 mM
13. Lysis buffer (PFGE) Tris pH 8 10 mM EDTA 50mM SLS 1% Proteinase K 1 U/ml	**14. SCE buffer (1 Litre) (pH 8)** Sorbitol 1 M EDTA 60 mM Sodium citrate 100mM
15. ChIP Lysis Buffer K HEPES(7.5) 50mM NaCl 140mM $Mgcl_2$ 2mM Sodium Deooxycholate 0.1% Triton X-100 1% 0.5M EDTA 1 mM SDS 0.1% **17. Buffer M1** Tris-Cl(pH 8.0) 20mM NaCl 500mM EDTA 2mM Triton X-100 1% SDS 0.1%	**16. Buffer M0** Tris-Cl(pH 8.0) 20mM NaCl 200mM EDTA 2mM Triton X-100 1% SDS 0.1% **18. Buffer M2** Tris-Cl (pH 8.0) 10mM LiCl 250mM NP-40 1% EDTA 2mM

19. Elution Buffer Tris-Cl(pH 8.0) 10mM NaCl 150mM SDS 1% EDTA 1mM	

Appendix 2: Yeast Strains used in this study

Table 9: Yeast Strains used in the study

Name	Genotype	Parent strain	Used for
TFC1 FLAG (transf)	MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 bar1Δ	W303-1A	Strain construction
Tfc3 mut	MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 bar1 *asf1*::KanMX	YYK9	Genomic integrity test
Tfc3 rescued	MATa ura3-52 trp1-Δ63 his3-Δ200 leu2::PET56 HIS3locus:int pJD30 -10IME2-Sc4251, LYS2 locus:int pJD52 X-Sc4251/LYS2 Asf1-18Myc:TRP1	JDY51	Genomic integrity test
Yph500	MATa hta1-htb1D::LEU2, hta2-htb2D::TRP1, leu2-D1, ura3-52, trp1-D63, his3-D200/ pJH23 (FB1251) HIS3 CEN ARS HTA1, FLAG-HTB2		Genomic integrity test
By4741	MAT a hta1-htb1D::LEU2, hta2-htb2D::TRP1, leu2-D1, ura3-52, trp1-D63, his3-D200/ pJH23 (FB1251) HIS3 CEN ARS HTA1, FLAG-HTB2 RPC160-9Myc:hph ASF1-6HA:KanMx	FY406	Genomic integrity test
S288c	MATa hta1-htb1Δ::LEU2 hta2-htb2::TRP1 leu2-Δ1 ura3-52 trp1-Δ63 his3-Δ200 *asf1*::KanMX pJH23(FB1251) HIS3 AMP CEN HTA1 FLAG-HTB2	FY406	Genomic integrity test
HAP	MAT a ade2-1 trp1-1 his3-11,15 ura3-1 leu2-3, 112 can1-100 brn1::BRN1-GFP-KanMX6	GA180	Genomic integrity test
DIP	MAT a ade2-1 trp1-1 his3-11,15 ura3-1 leu2-3, 112 can1-100 brn1::BRN1-GFP-KanMX6 *asf1*::hph	GA2663	Genomic integrity test
DDC2 HA	MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5+ ISW1-3FLAG:kanMX	YTT166	COIP
Wt Rad9	MAT a hta1-htb1D::LEU2, hta2-htb2D::TRP1, leu2-D1, ura3-52, trp1-D63, his3-D200/ pJH23 (FB1251) HIS3 CEN		Genomic integrity

HA	ARS HTA1, Rad9-6HA:KanMx		test
Tfc3 mut Rad9 HA	MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 bar1 *asf1*::KanMX; Rad9-6HA: HTB2	yOl8	Genomic integrity test
Tfc3 res Rad9 HA	MATa ura3-52 trp1-Δ63 his3-Δ200 leu2::PET56 HIS3locus:int pJD30 -10IME2-Sc4251, LYS2 locus:int pJD52 X-Sc4251/LYS2 Asf1-18Myc:TRP1; Rad9-6HA: HTB2	yOl8	Genomic integrity test

Appendix 3: Map of the Plasmid used in strain construction

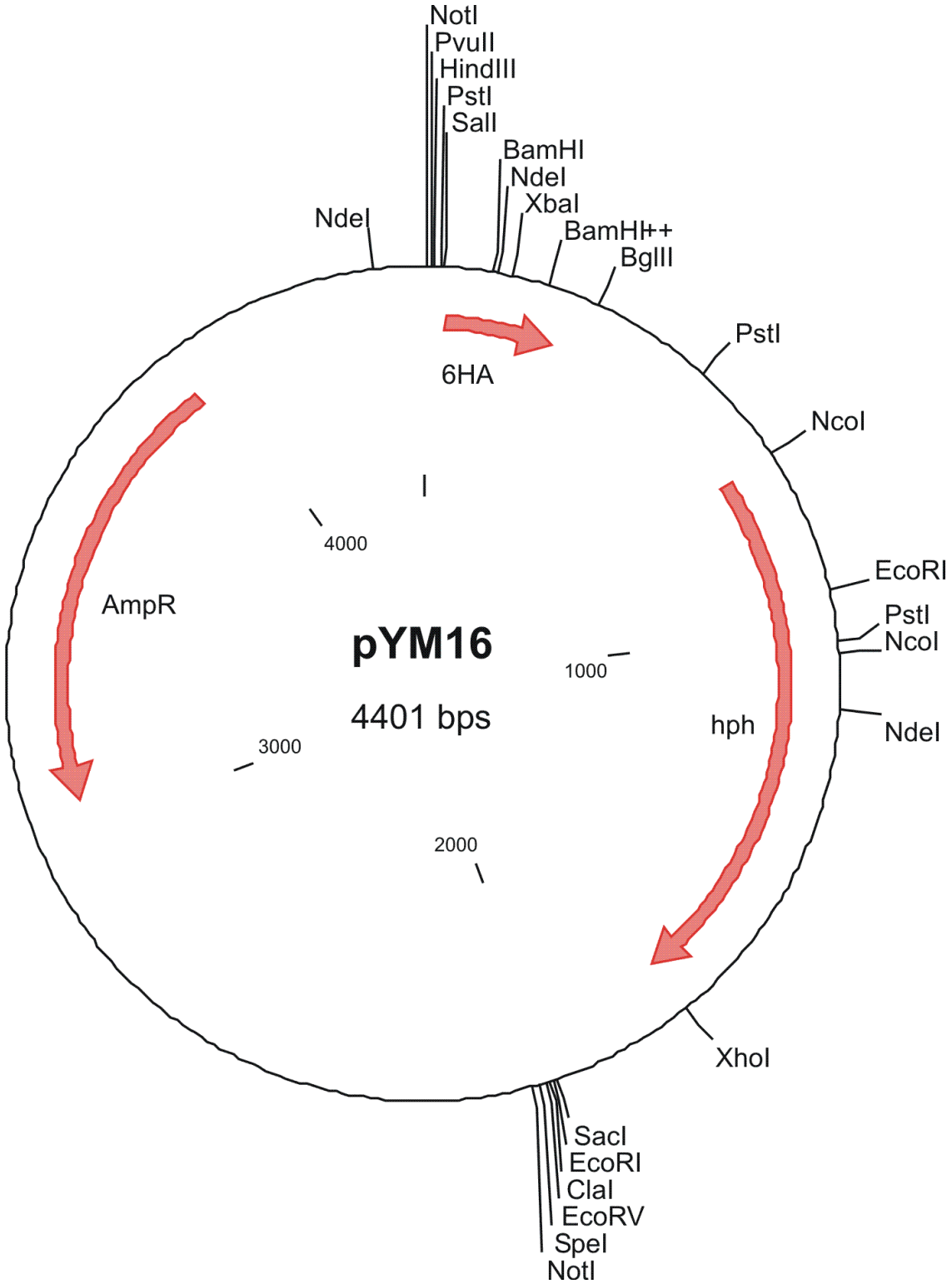

Figure 34: Map of the pYM16 plasmid used in the construction of Rad53-HA strain (Janke *et. al,* 2007) (plasmid sets from EUROSCARF-PCR Toolbox).

Appendix 5: Supplementary data for Fluorescence Activated Cell Sorter.

Table 10: Histogram statistics of fig 25. b

Histogram Statistics

File: YPH500.001 — Log Data Units: Linear Values
Sample ID: YPH500 — Patient ID:
Tube: — Panel:
Acquisition Date: 15-Jan-04 — Gate: No Gate
Gated Events: 20000 — Total Events: 20000
X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	20000	100.00	100.00	372.30	326.18	50.16	326.00	1023
M1	169, 254	4486	22.43	22.43	210.46	209.54	9.33	210.00	202
M2	256, 351	6098	30.49	30.49	306.29	305.35	7.77	307.00	321
M3	374, 459	3409	17.04	17.04	414.85	414.19	5.64	415.00	417

Table 11: Histogram statistics of fig 25. a

Histogram Statistics

File: G1.008 — Log Data Units: Linear Values
Sample ID: G1 — Patient ID:
Tube: — Panel:
Acquisition Date: 30-Dec-14 — Gate: No Gate
Gated Events: 20000 — Total Events: 20000
X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	20000	100.00	100.00	330.38	291.33	52.51	253.00	1023
M1	138, 281	10732	53.66	53.66	213.12	210.99	13.97	213.00	220
M2	330, 480	5345	26.72	26.72	407.90	406.05	9.45	409.00	404

Table 12: Histogram statistics of fig 25. e

Histogram Statistics

File: Res 30.013 — Log Data Units: Linear Values
Sample ID: Res 30 — Patient ID:
Tube: — Panel:
Acquisition Date: 30-Dec-14 — Gate: No Gate
Gated Events: 20000 — Total Events: 20000
X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	20000	100.00	100.00	377.15	315.09	61.95	305.00	1023
M1	133, 208	3766	18.83	18.83	171.90	170.59	12.26	172.00	172
M2	213, 417	8691	43.45	43.45	299.63	294.29	19.04	292.00	240

Table 13: Histogram statistics of fig 25. f

Histogram Statistics

File: Mut 30.013
Sample ID: Res 30
Tube:
Acquisition Date: 30-Dec-14
Gated Events: 20000
X Parameter: FL2-A (Linear)

Log Data Units: Linear Value
Patient ID:
Panel:
Gate: No Gate
Total Events: 20000

Marker	Left,	Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0,	102	20000	100.00	100.00	377.15	315.09	61.95	305.00	1023
M2	213,	41	8691	43.45	43.45	299.63	294.29	19.04	292.00	240