



## Proteome analysis of *Saccharomyces cerevisiae* after methyl methane sulfonate (MMS) treatment

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### ABSTRACT

The treatment of methyl methane sulfonate (MMS) increases sensitivity to the DNA damage which, further leads to the cell death followed by a cell cycle delay. Delay in the cell cycle is because of the change in global transcription regulation which results into proteome change. There are several microarray studies on the transcriptome changes after MMS treatment, but very few studies are reported related to proteome change. The proteome analysis in this report identified subgroups of proteins, belonging to known cell cycle regulators, metabolic pathways and protein folding. About 53 proteins were identified by MS/MS and found that 36 of them were induced, 10 were repressed and few of them showed insignificant change. Our results indicated the change in the interactome as well as phosphorylation status of carboxy terminal domain (CTD) of RNA Polymerase II (RNAP-II) after MMS treatment. The RNAP-II complex was affinity purified and ~1640 peptides were identified using nano LC/MS corresponding to 27 interacting proteins along with the twelve RNAP-II subunit. These identified proteins participated in the repair of the damage, changes the function of the main energetic pathways and the carbon flux in various end products. The main metabolic enzymes in the glycolysis, pyruvate phosphate and amino acid biosynthesis pathways showed significant change. Our results indicate that DNA damage is somehow related to these pathways and is co-regulated simultaneously.

### 1. Introduction

Methyl methane sulfonate (MMS) is an alkylating agent as well as a potential carcinogen [1]. It methylates N3 and N7 position of deoxy-adenosine and deoxy-guanosine respectively as well as other oxygen and nitrogen atoms of the nitrogenous bases [2]. The modifications by MMS lead to double stranded DNA breaks or DNA damage. The presence of damage is signaled through specific phosphorylation pathways which further activate DNA damage response genes. Some of these pathways involve the yeast protein kinases Mec1 and Dun1 that eventually alter the activity of transcription factors [3,4]. These transcription factors regulate the whole genome expression. Due to changes in the activity of transcription factors set of genes are induced and repressed that rebuild and repair the damage [5,6]. The DNA damage produced by the MMS leads to cause cancer. So it is important to identify the global set of genes induced and repressed in response to damage for defining the cellular pathway involved in the recognition and repair of

damage. The study of DNA damage on cells can give us the understanding of the controlling factor which governs the whole process and the pool of the genes affected [7].

DNA damage and their governing factors have been studied in a number of ways. In *Saccharomyces cerevisiae* the transcriptional response at multiple time point has been assayed in presence of MMS using microarray [8,9]. Gasch et al studied transcriptional response in *S. cerevisiae* and reported more than 750 genes. His group identified cluster of genes co-regulated temporally in response to MMS [10,11]. In similar experiments Jelinsky et al reported 693 genes showed a significant transcriptional modulation in response to MMS [9]. In every new study additional genes have been identified as having a role in damage response. There is no single technique which can investigate the whole process so different approaches have been used.

There are some proteins which regulate the transcription by the phosphorylation of the carboxy terminal domain (CTD) of RNA Polymerase-II (RNAP-II). The CTD acts as a platform for the recruitment

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of transcription regulatory proteins [12–15]. The phosphorylation of the CTD exists in multiple forms [16]. Bensaude and colleagues first observed the accumulation of the phosphorylated I<sub>0</sub> form of the CTD in case of the heat shock. Further experiments in *Saccharomyces cerevisiae* show the rapid increase in Ser2 phosphorylation (Ser2P), but no change in Ser5 phosphorylation (Ser5P) in the heptad of RNAP II CTD [17]. Global changes in CTD phosphorylation are also brought about by DNA damaging agents [18]. The change in the CTD phosphorylation level brought together in several ways first may be the activation of the several kinases like Bur1 and Ctk1 in *Saccharomyces cerevisiae* which leads to Ser2P [19]. Increase in the Ser2P leads to the depletion of the RNAPII available for initiation so stress response leads to the reduction in gene expression. Finally, gene expression is controlled globally and at multiple levels in response to MMS stress. The relationships among these global regulations are still unclear.

Here in this study, we analyzed global regulation during MMS stress using proteomics approaches. We obtained a global overview of changes in proteome under MMS stress using two-dimensional gel electrophoresis (2-DE). We also checked the change in the CTD phosphorylation and the interactome of RNAP-II in MMS stress condition. Furthermore, the interactome of RNAP-II was identified by nano LC/MS using in solution trypsin digestion of the purified complex in normal as well as in MMS treated condition. The data indicate that in stress condition, there are changes in the proteome as well as in the proteins which are directly or indirectly interacting with the RNAP-II complex. The potential utility of this approach results in the detection of both known and novel DNA damage responsive proteins.

## 2. Materials and methods

### 2.1. Spot assay and growth curve

Spotting assay was done as described somewhere [20]. The *Saccharomyces cerevisiae* BY4741 strain was grown overnight in YPD at 30 °C on 200 rpm shaking. Secondary culture was done and cells were grown till OD<sub>600</sub> 0.7. For each condition equal numbers of cells were pellet and serial dilution of 10 fold was made till 10<sup>-5</sup> dilution. Then 10 µL aliquots from each of the dilutions were spotted on plates containing different concentrations of MMS (0.005, 0.001, 0.02, or 0.05%). The plates were incubated for 2–3 days at 30 °C and 25 °C. For growth curve, cells were grown in similar condition as mentioned and at every hour OD<sub>600</sub> was calculated and plot against incubation time.

### 2.2. Sample collection and sample preparation

The *Saccharomyces cerevisiae* BY4741 strain was grown in YPD medium till 0.7 OD<sub>600</sub> then 0.02% MMS treatment was done for 6 h. The cells were collected by centrifugation at 5000 rpm for 5 min and washed with phosphate buffered saline (PBS, pH7.4). Approximately 500 mg cells were suspended in 2.0 ml of protein extraction buffer (20 mM Tris, 4% CHAPS, 2.5% glycerol and 1X PIC). Cells were lysed using French press (30 Kpsi) and the supernatant was collected by centrifugation at 14,000 rpm for 30 min at 4 °C. The proteins in the supernatant were precipitated with 10% (v/v) TCA and incubated overnight at –20 °C. The precipitated protein was collected after centrifugation at 14,000 rpm for 15 min. The pellets were washed thrice with ice chilled acetone. Finally, the protein pellet was suspended in 300 µl of rehydration buffer (7 M urea, 2 M thio-urea, CHAPS 4%, DTT 10 mM, Tris 20 mM). Pellets were briefly sonicated in rehydration buffer and incubated for 2 h. Protein concentration was estimated and samples were stored at –80 °C. Approximately, 250 µl rehydration solution containing 250 µg protein was applied in rehydration tray and IPG strip (nonlinear, 11 cm, 3–10 pH range) was placed on rehydration solution mixture for strip rehydration. Samples were rehydrated passively for overnight in rehydration tray (GE healthcare).

### 2.3. IEF run and 2D electrophoresis

Rehydrated IPG strips were run on Ettan IPG phor3 (GE healthcare). After completion of IEF, the strips were equilibrated in equilibrium buffer (6 M Urea, 75 mM Tris-Cl (pH 8.8), 29.3% Glycerol, 2% SDS, 1% BPB). First equilibrium was performed with 100 mg DTT supplemented with 10 ml Equilibrium buffer followed by second equilibrium with the addition of 250 mg iodoacetamide in 10 ml Equilibrium buffer. Each equilibrium step was performed for 30 min. Equilibrated strips were placed at 12%, 18 × 16 cm acrylamide gel for second dimension gel electrophoresis. The gel was overlaid with 0.5% agarose prepared in 1X TGS buffer and was run at constant current of 40 mA for 6–7 h till dye reached to bottom. The gel was stained using coomassie brilliant blue R-250 (CBB R-250) solution (0.25% CBB R-250, 45% methanol, 10% glacial acetic acid) for overnight at room temperature and then destained in destaining solution (45% methanol, 10% glacial acetic acid). The gel was scanned with Labscan software (GE healthcare) and analysis was performed by Image Master™ 2D Platinum 7.0.

### 2.4. Identification of differentially expressed protein by MS/MS analysis

Differentially expressed protein spots from 2D gel were excised, chopped and destained. The gel pieces were dehydrated with solution A (2:1 mixture of acetonitrile (ACN): 50 mM ABC) and then rehydrated with 50 mM Ammonium Bicarbonate (ABC). Proteins in the gel pieces were reduced with 10 mM DTT (56 °C, 30 min in the dark), alkylated with 50 mM iodoacetamide (room temperature, 30 min) and digested with trypsin in 25 mM ammonium bicarbonate buffer (pH 8.0) at 37 °C for overnight. The digested peptides were extracted in extraction solution 50% ACN and 0.1% TFA in total volume 100 µl and speed vacuum to make the volume up to 5–10 µl. 0.5 µl of extracted peptides were spotted on the MALDI plate followed by 0.5 µl α-cyano-4-hydroxycinnamic acid matrix (10 mg/ml in 50% ACN and 0.5% TFA). Spots were dried completely and used for MS-MS on MALDI-TOF-TOF platform (model 4800, ABSciex, USA). The result obtained from MS/MS were analyzed by MASCOT search using NCBI nr databases with following parameter (fixed precursor ion mass tolerance of 20 ppm, fragment ion mass tolerance of 0.05 Da, calibration error of 0.005 Da, one missed cleavage, carbamidomethylation of cysteines and possible oxidation of methionine).

### 2.5. Protein identification using nanoLC/MS

TAP purification was done as described in Tardiff et al [21]. The in-solution trypsin digestion of the purified protein complex was done. Chromatographic separations of peptides were done on a nano-LC system consisting of a Nano-LC trap (3 µm, ChromXP C18CL, 120 Å, 0.5 mm × 350 µm (AB Sciex)) and a reversed-phase analytical (Chromolith Cap Rod RP-18e (150 × 0.1 mm) monolithic capillary column [22]. Tryptic digested samples were injected by auto sampler into the column. Washing of the trap column with solvent “1A” (2% ACN, 98% dH<sub>2</sub>O and 0.1% TFA) for 5 min at a flow rate of 10 µL/min was done for the de-saltification of the peptide mixture. Desalted peptide separation was achieved using solvent “2A” (2% ACN, 98% dH<sub>2</sub>O and 0.1% TFA) and “2B” (98% ACN, 2% dH<sub>2</sub>O and 0.1% TFA) with a flow rate of 800 nL/min in a linear gradient elution mode ramping from 0% to 90% “2B” over the course of 110 min. Peptide fractions were mixed online with α-cyano-4-hydroxycinnamic acid matrix solution (5 mg/mL in 50% ACN + 0.1% TFA) in a ratio 1:1 (v/v) and spotted onto a 384 wells containing stainless steel MALDI target plate (AB Sciex) [23]. Automatic spotting time was set from 5 min to 110 min, with 20 s drop intervals.

### 2.6. Mass spectral and data analysis

MS and MS/MS analysis were carried out on a 4800 MALDI-TOF/TOF mass spectrometer (AB SCIEX, USA) equipped with 355 nm Nd:

YAG solid state laser. Positive ion mass spectra were recorded in reflectron mode over a mass range of  $m/z$  800–4000 Da. External mass calibration was performed using TOF/TOF calibration mixture (des-Arg1-bradykinin,  $[M+H]^+ = 904.468$  Da; Angiotensin I,  $[M+H]^+ = 1296.685$  Da; Glu1-fibrinopeptide B,  $[M+H]^+ = 1570.677$  Da; ACTH (1–17 clip),  $[M+H]^+ = 2093.087$  Da; ACTH (18–39 clip),  $[M+H]^+ = 2465.199$  Da, and ACTH (7–38 clip),  $[M+H]^+ = 3657.9294$  Da). A maximum of twenty five precursors from each mass spectrum with a minimum signal/noise ratio of fifteen were selected for MS/MS analysis. Analysis of MS and MS/MS data and database searching were conducted against the NCBI database using ProteinPilot software (Version. 4.0, AB Sciex) with the MASCOT search engine using following search parameters (taxonomy was set to *S. cerevisiae*, trypsin as enzyme (one missed cleavage allowed), variable modification of Oxidation (M), precursor tolerance was set to 100 ppm and MS/MS fragment tolerance to 0.2 Da, and peptide charge was “1+”). Resulted proteins were considered at a significance level of  $p < 0.05$ .

### 2.7. CTD phosphorylation status and western blotting

To check the phosphorylation status of the CTD of RNAP-II the yeast strain was grown overnight in YPD broth. Then 500 ml YPD broth was inoculated with 1% of the overnight culture and grown at 30 °C and 200 rpm. After  $\sim 0.2$ – $0.3$  OD<sub>600</sub> culture was treated with 0.02% MMS. 50 ml of sample was collected at 0, 15, 30, 45, and 60 min time interval. After collecting the sample immediately centrifuge at 5000 rpm for 5 min and then pellet was washed with chilled millQ and store at  $-20$  °C. Now the pellets were collected in 1.5 ml microfuge tube and washed with 1X PBS and then suspended in 100  $\mu$ l 1X-PBS with 1X-PIC and 2 mM PMSF. Then samples were lysed using bead beater (20 s on bead beater and 3 min on ice, 3 shots). Now the lysed samples were collected in another 1.5 ml microfuge tube by pricking at the bottom of previous tube. Cell debris

was removed by centrifugation (5000 rpm, 5 min) and the lysate were collected in another microfuge tube. Samples were quantified using a standard Bradford method using 10 mg/ml BSA as standard. 100  $\mu$ g of protein was loaded on 8% SDS-PAGE. Then protein was transferred to nitrocellulose membrane and checked the rpb1 using anti-TAP (1:1000) and ser2, ser5, and ser7 phosphorylation was checked using 3E10 (1:1000), 3E8 (1:1000), 4E12 (1:1000). Secondary antibody anti mouse IgG HRP conjugated (1:5000) for anti TAP and anti-rabbit IgG HRP conjugated (1:5000) for phosphorylation [14,24].

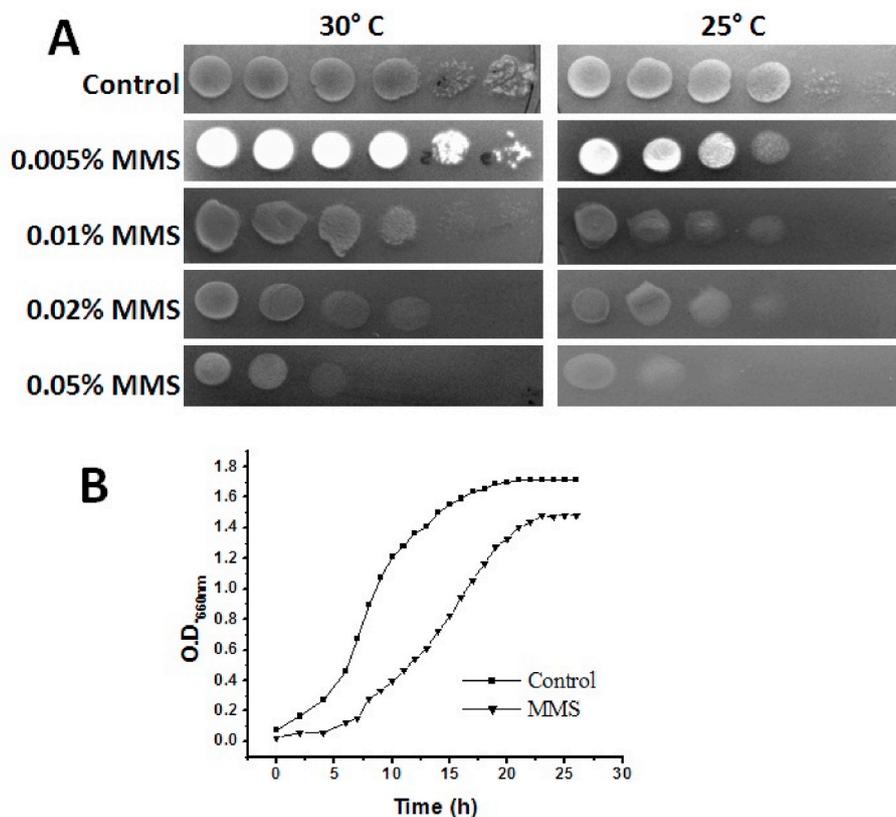
## 3. Results

### 3.1. Yeast sensitivity to MMS treatment

The sensitivity of the strain at different MMS concentration (0.005, 0.01, 0.02, 0.05%) was checked. The results showed that increasing concentration of MMS lead to decrease cell viability. Finally, the higher concentration of MMS became lethal to cell. The growth of the cells in 0.02% MMS was very slow and  $\geq 0.05\%$  MMS concentration was found to be lethal even at 25 °C (Fig. 1A). Furthermore, we checked the growth of the strain in 0.02% MMS concentration in broth and found that the growth was slow as compared to untreated sample. A shift in the growth curve at the initial phase was also observed. After initial slow lag phase as compared to the control, the growth was recovered at later stage (Fig. 1B). The results indicated that 0.02% MMS concentration was optimal for the proteomics analysis.

### 3.2. Proteome analysis after MMS treatment

To understand the functional proteomics of the yeast in DNA-damage response, the whole cell proteome analysis was performed by 2-D gel electrophoresis (2DE) and identification of the differentially expressed



**Fig. 1.** MMS sensitivity of yeast cells at different temperature. (A) The cultures were grown in YPD until the mid-log phase and a serial tenfold dilution were prepared and spotted onto YPAD plates containing different concentrations of MMS and incubated for two days. (B) Growth curve of yeast cell in the presence and absence of 0.02% MMS at 30 °C.



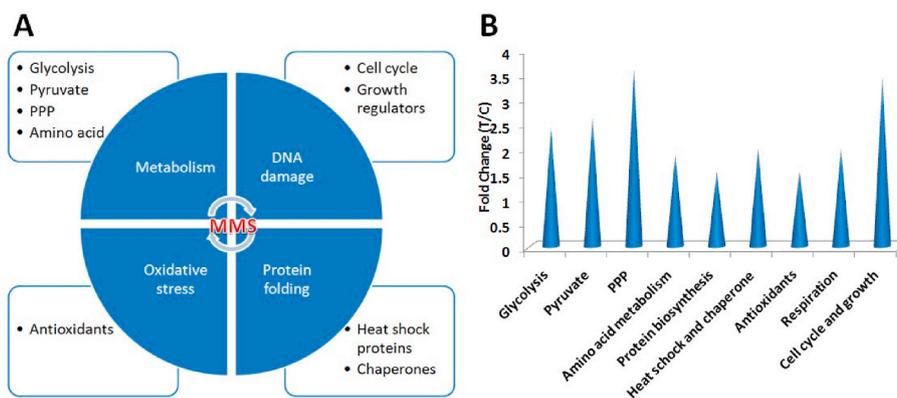


Fig. 3. Functional characterization of identifies proteins. (A) Diagrammatic representation of the identified proteins in 4 major metabolic pathways. (B) Average fold change in expression of the proteins related to a particular pathway (calculated by the average of fold change expression in total identified proteins in the specific pathway).

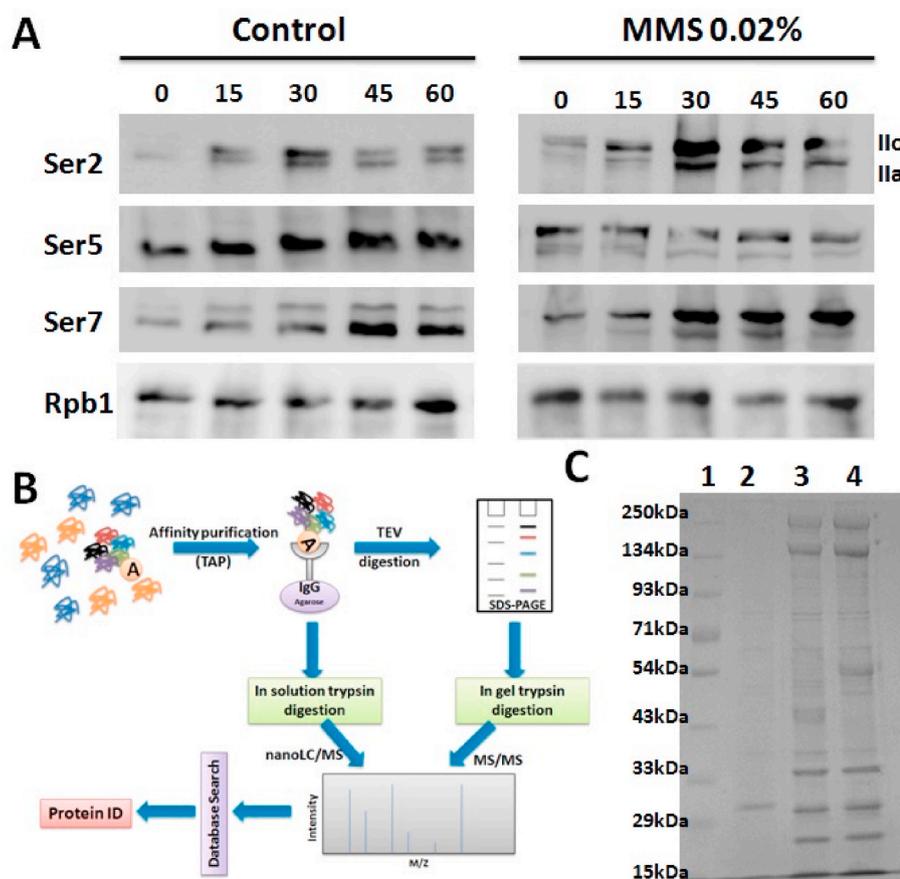


Fig. 4. CTD phosphorylation leads to change in the interactome of RNAP-II. (A) Cells were grown till 0.8 OD and treated with 0.02% MMS and the samples were collected at different time interval. Western blotting was done to check the Ser2, Ser5 and Ser7 phosphorylation status using 3E10, 3E8 and 4E12 antibody, respectively. Rpb1 level was checked using anti-TAP antibody. (B) Schematic representation of the strategies for the identification of the RNAP-II associated proteins. (C) 10% SDS-PAGE where Lane1 represent the ladder and lane2, 3 and 4 represent RNAP-II purification from JTY1, Y1056 and Y1088 respectively.

subunits along with few interacting proteins. In order to find out the change in interactome after MMS stress the culture with treated with 0.02% MMS at 0.7 OD<sub>600</sub> for 6 h and the complex was identified as described earlier. Approximately 1640 peptides were identified which correspond to RNAP-II subunits and 27 interacting proteins which are listed in the Table 1. The group of proteins included all core RNAP-II subunits as expected already identified by Tardiff et al [21]. More numbers of proteins were identified as compared to the earlier report (Table 1).

The identified proteins were analyzed by network mapping using the String 10 server [33]. The network map showed that most of the identified proteins were associated with the basal transcription factors and

RNA biogenesis (Fig. 5). The protein belonging to the basal transcription factors includes Tgf1, Tgf2, Spt5, Irw1 and Rtr1 while Rvb1, Rvb2, Nop58, Nop1, Cbf5 belongs to the RNA biogenesis. Some other factors (Ire1, Cad1, Lsm1 and Lsm7) were also identified irrespective of these categories. Basal transcription factors as well as RNA biogenesis factors are responsible for the global transcription regulation. Spt5 which is the component of DSIF complex was present in stress as well as control condition which functions in various aspects of transcription, chromatin maintenance, and RNA processing [34,35]. The attachment of the TFIIF with the RNAPII assembly was observed in case of treatment which was not present in the control sample.



**Table 2**

Comparison of the identified proteins with the earlier studies. The proteins shown in different proteomics analysis have been categorized as PA (the proteins which align with 2DE analysis data) and RNAPII (the proteins which align with RNAP-II interactome data).

This study		Chromatin enrichment Kim et al., 2011		Gasch et al., 2001		Jelinsky et al., 1999	
(PA)	(RNAPII)	(PA)	(RNAPII)	(PA)	(RNAPII)	(PA)	(RNAPII)
Ipp1, Tdh2, Tdh3, Fba1, YNL134C, Adh1, Tif1, Tdh2, Eno2, Gnd1, Eno1, Vma2, Cys4, Pdc1, Vma1, Ssc1, Tkl1, Tpi1, Adk1, Vma4, RPS0A, Spe3, Fba1, Pgl1, Eno2, Gdh1, Pgi1, Frs1, Bmh1, Rpp0, Stm1, Rnr4, Ilv5, Ura1, Cys3, Hsp60, Pdc1, Ssb2, Trr1, Tsa1, Pre9, Hxk2, Hsc82, Gpm1, Gpd1, Stil, Sse1, Oye3, Ssa1	Tcp1, Ssb2, Nop58, Tdh3, Tdh2, Adh1, Rpp0, Rtr1, Rps2, Sam1, Spt4, Spt6, Lsm1, Iwr1, Ssf2, Kch1, Npa1, Ire1, Adh2, Ssa2, Tef2, TFIIF, Tfg2, Tip49 (Rvb1), Ssb1, Kar2, Cad1, Cct7	Cys3, Ilv5, Stm1, Ura7, Vma2, Rnr4, Bmh1, Hsp60, Pdc1, Rpp0, Ssb1, Ssb2, Ura7, Rvb2	Rvb2 (Rvb1), Ssb1	Trr1, Oye2, Tsa1, Sti1, Hsc82, Kar2, Pre9, Sse2, Tps1, Gpd1, Hxk1, Gpm1, Rnr4	Kar2	Ssa1, Cad1, Rnr1, Rnr2, Oye3, Cct8, Gdh2, Pre3	Cad1, Cct8 (Cct7)

conserved C-terminal domain (CTD), which is composed of repeated unit of Y<sub>1</sub>S<sub>2</sub>P<sub>3</sub>T<sub>4</sub>S<sub>5</sub>P<sub>6</sub>S<sub>7</sub> consensus sequences. The CTD undergoes multiple posttranslational modifications which results in the recruitment and dissociation of a plethora of proteins to the CTD, essential for the active transcription [12–14,28]. RNAP-II responds to DNA damage by increasing the CTD phosphorylation, especially Ser2 and factors that mediate or respond to this phosphorylation controlling the survival under DNA damage conditions [17,18]. The results indicated that the MMS treatment leads to changes in Ser2 as well as Ser5 and Ser7 CTD phosphorylation which lead to the hyper-phosphorylation of the CTD (Fig. 4A). The phosphorylation and dephosphorylation of CTD regulates the association of other transcriptional regulatory proteins to the RNAP-II as well as the transcription initiation, elongation, termination, mRNA-splicing and mRNA-export [16,17,52,53]. It means this epigenetic modification on the CTD regulates the global gene expression. This is why the MMS treatment leads to the up/down regulation of some metabolic pathways, oxidative pathway and DNA damage response pathway to protect the cell.

#### 4.5. CTD phosphorylation leads to interactome change of RNAP-II

Although many of the proteins that comprise the initial response to MMS have been identified but, the complete repertoire of downstream DDR events induced by MMS especially with respect to the RNAPII remains poorly understood [12–15,54]. Comparison of fractions associated with RNAP-II in MMS treated sample indicate proteins that are differentially regulated and have a greater degree of association with RNAP-II in response to the MMS during active transcription. The whole cell proteomics approaches even after chromatin enrichment was able to identify very few proteins related to RNAP-II (Table 2, Kim et al., 2011). The approach using in solution digestion of proteins associated with RNAP-II and its subsequent analysis by nanoLC-MS/MS resulted in the identification of twenty seven RNAPII associated proteins after the 6 h of MMS exposure (Table 1). The network mapping of the RNAPII associated proteins using the string 10 showed that the identified proteins belong to the basal transcription factors and RNA biogenesis (Fig. 5). The MMS mediated hyperphosphorylation of the CTD led changing of the interactome which further govern the global gene regulation. In addition to these, the result identified the interaction of phosphatase (Rtr1) and kinase (Ire1) to the RNAP-II. Rtr1 is phosphatases which help in the dephosphorylation of the Ser5 position of the CTD which help in the transcription elongation and termination [55]. Irw1 was identified in the complex which involved in the transport of the RNAP-II complex to the nucleus where it further replaced by the transcription factors [56]. The Rtr1 have role in the CTD modification while the role of Ire1 in CTD modification is still unknown. The Ire1 have been identified alleviate endoplasmic reticulum stress by orchestrating the unfolded protein response [57]. The recruitment of these proteins might leads to the activation of the global transcription which may further regulate the

variety of genes participates in oxidative stress produced by MMS.

## 5. Conclusion

The comparison of our proteome data with transcriptome data from the literature suggests that genes are regulated at different level; some are regulated at the transcriptomic level, whether some are regulated at proteomic level. The heat-shock proteins and chaperone proteins were significantly more abundant in the stress responses including oxidation and replication stress. To begin a characterization of proteins associated with active transcription in stress condition, we purified tagged RNAPII from *S. cerevisiae*. Several categories of transcription relevant proteins were co-purified. In the MMS treated condition we found approximately 27 proteins which were interacting with the complex. In case of treatment the phosphorylation status of the CTD changes and leads to increase in Ser2 phosphorylation, which may lead to the interaction of other proteins to RNAP-II. We have investigated the general protein composition in MMS stress condition in *Saccharomyces cerevisiae* which may leads to the identification of the potential targets and analysis of their role in cellular processes. This will not give only a global overview of the cellular changes elicited by stress, but also provide the framework for understanding the mechanisms behind MMS stress response in yeast.

## Declaration of competing interest

The authors declare that they have no competing interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2020.100820>.

## Author's contribution

APB participated in conceptualization, data curation, analysis, investigation, methodology, data validation and writing of the original draft. SK has done the formal analysis, validation of the data and writing the draft. MSA conceived the idea and participated in formal analysis and editing of the draft.

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## Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

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