Abstract

Meiosis is a complex process committed to generate the gametes and disseminate the genetic information to the daughter cells through two consecutive rounds of cell divisions. In meiosis I, homologous chromosomes segregate followed by the sister-chromatid separation in meiosis II thereby, resulting in haploid gamete formation from diploid parental cell. Cell cycle regulators such as Polo-like kinases (PLKs) play an important role in the chromosome dynamics and cell division during mitosis and meiosis. PLKs are profound members of serine/threonine (ST) kinases, which consist of conserved kinase domain (KD) at the N-terminus, and a hallmark polo-box domain (PBD) at the C-terminus. There is a single PLK, Cdc5 in Saccharomyces cerevisiae, which is indispensable for both mitotic and meiotic divisions.

During meiosis, Cdc5 regulates multiple events, including exit from pachytene stage of meiosis I prophase, mono-orientation of homologs and meiotic divisions. Cdc5 promotes pachytene exit by activating resolution of recombination intermediates called joint molecules (JMs) between homologous chromosomes as crossovers (COs) and breakdown of synaptonemal complex (SC). However, the molecular targets of Cdc5 in these processes are not known. The present study was undertaken to identify the potential protein substrates of budding yeast PLK, Cdc5 during meiosis with emphasis on pachytene exit during meiosis I prophase. Tandem affinity purification (TAP) system was used in this study to pull-down and identify the protein targets of Cdc5 kinase. Towards this goal, an estrogen inducible TAP system for expression of Cdc5-TAP during meiosis was constructed. Two yeast strains were constructed CDC5-IN (ndt80∆ pGAL1-CDC5-TAP) and Cdc5-kinase inactive mutant (ndt80∆ pGAL1-cdc5-N209A-TAP-IN) were constructed through genomic integration approach. Haploid parental yeast strain (ndt80Δ-CDC5-TAP) was transformed with SnaBI digested linearised plasmids, pMJ830 and pMJ840 containing pGAL1-CDC5 and pGAL1-cdc5-N209A, respectively. The haploid transformants were selected on hygromycin selection media and verified by PCR using four different sets of primers resulting in AS1 (pGAL1-CDC5-TAP) and AS2 (pGAL1-cdc5-N209A-TAP). Two diploid strains, ASd1 (Cdc5-TAP-IN) and ASd2 (cdc5-N209A-TAP-IN) were generated by crossing with widtype parental strain of opposite mating type containing Gal4:Estrogen receptor (ER) transcription factor (Gal4:ER) (Mat α), and verified for inducible expression.
To induce meiosis, cells were transferred to sporulation medium. After 7 h expression of Cdc5-TAP and cdc5-N209A-TAP was induce with the addition of estradiol (1 μM ED). The inducible expression of the Cdc5-TAP and cdc5-N209A-TAP fusion proteins in both ASd1 and ASd2, respectively, was confirmed by Western analysis using anti-bodies specific to the TAP affinity tag. Specific bands of size ~ 101 kDa, corresponding to the expected size Cdc5-TAP and cdc5-N209A-TAP were detected in ASd1 and ASd2, respectively, upon addition of ED (1 μM). These bands were absent in the uninduced (-ED) samples. The functional validation of Cdc5-TAP induced resolution of meiotic recombination intermediates (JMs) was confirmed by Southern analysis using probe specific to the recombinant inserts.

Further, the ability of inducible expression of Cdc5-TAP to phosphorylate its substrates was analyzed by Western blotting using antibodies specific to phospho-threonine. Western analysis with p-Thr antibodies detected a band of ~ 101 kDa protein for both 8 h and 9 h samples in the presence of ED, which was absent in the uninduced (-ED) samples of ASd1. The corresponding band was not detected in ASd2 cells expressing inactive kinase (cdc5-N209A-TAP-IN), indicating that phosphorylation is due to kinase activity of Cdc5. Thus, Southern and Western analysis validate that Cdc5-TAP is expressed from pGAL1 upon addition of ED and is functionally active.

After functional validation of the experimental system, large scale preparation of the meiotic yeast cells of ASd1 and ASd2 strains (2 liter meiotic culture of each strain) in the presence (1 μM ED) and absence of ED (-ED) was achieved. The induced samples were collected at 9 h of meiosis (2 h post induction), by the time when Cdc5-TAP expression was maximum. The induced expression of Cdc5-TAP and cdc5-N209A-TAP in large scale was confirmed by Western analysis using anti-TAP antibodies.

The successful outcome of the TAP pull-down relies on the presence of intact protein in the whole cell protein extracts (WCEs) without proteolysis. Therefore, the methodology for the preparation of whole cell protein extracts (WCEs) of the meiotic samples was optimized using FastPrep method of cell lysis. The protein extracts were confirmed for the intactness of Cdc5-TAP and cdc5-N209A-TAP fusion proteins by Western blotting using anti-TAP antibodies showing specific bands corresponding to ~ 101 kDa in ASd1 and ASd2, respectively.
Towards the identification of the substrates, binding to Cdc5 kinase or cdc5-N209A kinase, large scale TAP affinity purification from the meiotic whole cell protein extracts containing Cdc5-TAP or cdc5-N209A-TAP was carried out. TAP-purification was carried out from whole cell protein extracts, both of uninduced and induced meiotic cells of ASd1 and ASd2. For TAP purification, WCEs (~ 50 mg total proteins) were subjected to binding with IgG-sepharose beads, followed by the elution of Cdc5-TAP and cdc5-N209A-TAP, and the associated proteins with the TEV protease. The eluate was subsequently bound with Calmodulin sepharose beads and eluted with EGTA. The eluate fractions from TAP purification columns were analyzed by silver staining and Western blotting with anti-TAP antibodies, which confirmed the presence of Cdc5-TAP and cdc5-N209A-TAP in the eluates.

To identify the proteins and potential substrates, pulled down by Cdc5-TAP and cdc5-N209A-TAP, the TAP purification eluates were resolved on 10 % SDS-PAGE, and gel pieces were excised, followed by tryptic digestion and Liquid Chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The analysis of the peptide peaks from MS by MASCOT using 3 different databases (Yeast Proteome Database, Swiss-Prot and TrEMBL) revealed the presence of several proteins. Non-specific protein hits were filtered and eliminated, and proteins with at least one peptide hit were selected from the dataset. Peptide hits obtained for the uninduced (-ED) datasets of Cdc5-TAP and cdc5-N209A-TAP pull-down were filtered out from the respective induced (+ED) datasets; the housekeeping enzymes of metabolic pathways, and translation factors were eliminated. The hits were further refined on the basis of their relevant function in cell cycle and cell division. The substrates identified from Yeast Protein database included Bck1 (MAPKKK), and Ira2 (GTPase activating protein) as hits for ASd1 (Cdc5-TAP) pull-down. Ira2 (GAP), Hof1 (contractile ring protein Imp2), Mdn1 (cytoskeletal protein Midasin), Bni1, Bnr1 [Cytokinesis protein Sepa (fh1/2)], and Rad16 (DNA repair protein) as hit for ASd2 (cdc5-N209A-TAP) pull-down. GAP was a common hit that was retrieve in pull-downs of both ASd1 and ASd2, whereas Dmc1 (RecA), a DNA repair protein, was unique in ASd1 (Cdc5-TAP) pull-down searched against Swiss-Prot database.

Proteomic analysis of the Cdc5-TAP and cdc5-N209A-TAP pull-downs for ASd1 and ASd2 primarily provided an increasing list of interacting proteins. Therefore, an In silico approach was used to screen the potential substrates for consensus sequences reported for kinase
binding [(D/E/N) X (S/T)] and polo-box domain binding [S(pS/pT)(P/X)] of Cdc5. The highest number of consensus sites for kinase domain was present in Mdn1 protein, whereas, the largest number of the polo-box domain consensus sites was found in Ira2 protein. Amongst the known meiotic substrates of Cdc5, maximum numbers of the kinase phosphorylation sites as well as sites for polo-box domain were revealed in Rec8 protein. Filtering down the data further, we selected Dmc1, Ira2 and Rad16 from Saccharomyces Genome Database (SGD), after reviewing all the eight substrates for physical and genetic interaction with Cdc5 listed in Saccharomyces Genome Database. The three substrates were analyzed for binding to kinase domain of Cdc5 by docking analysis. The homology model of kinase domain of Cdc5 was generated and the three substrates were docked. Cdc5-Dmc1 and Cdc5-Ira2 enzyme-substrate interactions showed minimum binding energy with $E_{total}$ value of zero, while Cdc5-Rad16 showed the $E_{total}$ value of -233.58 kcal/mol. The three substrates were also docked with kinase domain of human Plk1. While Rad16 docked with Plk1 with an $E_{total}$ value of -400.13 kcal/mol, Plk1-Dmc1 and Plk1-Ira2 interaction values of binding energies were not significant.

Therefore, docking analysis projected Rad16 to be the most potential substrate of Cdc5 phosphorylated during pachytene exit. Rad16 also showed the maximum enzyme-substrate interaction with human Plk1, which can be explored more in the direction of cancer therapeutics in the future. Thus, the present study forms for the first report of potential meiotic substrates for the PLK, Cdc5 during pachytene exit, which have been not been identified earlier as substrates of Cdc5 in mitosis and meiosis. In future, the substrates can be validated by mutational analysis in vivo and serve as potential targets for cancer therapeutics.

**Key words:** Ser/Thr kinases, PLK, Cdc5, Meiosis, *Saccharomyces cerevisiae*, Kinase-inactive, Estrogen inducible, Estradiol, Gal4, Pachytene, Meiotic recombination, Ndt80, Joint molecules (JMs), Crossovers (COs), Synaptonemal complex, Tandem Affinity Purification, Substrates/Targets, Phosphorylation, P-Thr, Plk1, Cancer