1.1 Eukaryotic cell division

Cell division is an essential and fundamental biological process in all organisms, comprising of a series of highly coordinated events (Yu et al., 2006, Kraikivski et al., 2015). During cell division, the duplication of genetic material and organelles is followed by segregation, leading to the formation of the genetically identical daughter cells (Hartwell, 1971; Petronczki et al., 2008). Studies from the evolutionarily divergent organisms have emphasized that cell division mechanisms show a high degree of conservation (Bell and Dutta, 2002; Grewal and Edgar, 2003).

There are two types of cell division: mitosis and meiosis. Mitosis is the fundamental process of vegetative propagation and growth. During mitosis, genomic and functional content of the cell duplicates and divides to form two daughter cells identical to parental cell (Hartwell, 1974; Morgan, 2007). The other type of cell division, meiosis, ensures sexually reproducing organisms produce haploid gametes from the diploid parental cells (Petronczki et al., 2008).

During meiosis, DNA is duplicated in single phase followed by two consecutive rounds of divisions, reducing the chromosome number by half. During the first reductional division (meiosis I), sister chromatids remain attached and homologous chromosomes segregate. Meiosis I is a complex process and thus, for its accurate accomplishment, a series of changes occur in chromosome dynamics during an extended meiotic prophase, which includes pairing, synapsis, and recombination between the homologous chromosomes, resulting in genetic variation (Roeder, 1997; Zickler and Kleckner, 1999; Petronczki et al., 2008; Vaid et al., 2016b). During the second equational division (meiosis II), sister chromatids segregate to opposite poles and divide as in mitosis, thus, producing four, non-identical haploid cells (Roeder, 1997; Marston and Amon, 2004). Defects in the meiotic cell division result in aneuploidy and associated disorders.

1.2 Cell cycle

In eukaryotes, cell division is integrated into a series of programmed cyclic events called cell cycle. The cell cycle consists of four discrete phases: G1, S, G2 and M (Bell and Dutta, 2002; Yu et al., 2006). The cell cycle begins with cell growth phase or Gap1 (G1), followed by DNA synthesis (S-phase), the second growth phase (G2), and subsequent segregation of the
duplicated chromosomes, and cell separation in mitotic phase (M-phase) (Morgan, 2007). M-phase comprises of prophase, metaphase, anaphase and telophase. All eukaryotes, from single-celled yeast to complex multi-cellular vertebrates, pass through the same four phases. In higher eukaryotes like amphibians and mammals, an additional phase called G_0 or quiescent/resting phase is observed which is considered to be an extended G_1 phase where the cell withdraws from the cell cycle under unfavorable growth conditions or due to cell differentiation. Regulation of cell cycle is critical for the normal development of multi cellular organisms, because deregulated proliferation is the hallmark of cancer (Archambault and Glover, 2009; Zitouni et al., 2014).

1.3 Cell cycle kinases

Extensive research has revealed that the progression of eukaryotic cell cycle requires major regulators, called protein kinases. There three major cell cycle kinases namely, polo-like kinases (PLKs), cyclin dependent kinases (CDK), and aurora kinases (AURKs) which are evolutionarily conserved from yeast to humans (Lee and Amon, 2003a). These kinases drive the cell cycle by phosphorylating a distinct array of protein substrates during each phase of cell cycle. While CDKs and PLKs are the master regulators of the cell cycle, aurora kinases control multiple processes during cell division, and coordinate chromatid segregation and cytoskeletal events (Cheng et al., 2016). The concerted action of these regulators plays an important role in maintaining the genome integrity of the organism by orchestrating mitosis and meiosis (Tavernier et al., 2015). The importance of cell cycle kinases is substantiated by the fact that genes encoding either of these kinases are essential for survival in most of the organisms studied (de Cárcer, 2007; Malumbres and Barbacid, 2007).

1.3.1 Polo like kinases (PLKs)

PLKs are a family of serine/threonine kinases that function as prime regulators of cell division (Blagden and Glover, 2003; Barr et al., 2004; Zitouni et al., 2014). Polo, the founding member of this family, was originally identified in the fruit fly, Drosophila melanogaster (Fenton and Glover, 1993), and subsequently in other organisms. PLKs are
highly conserved from yeast to humans, and comprised of catalytic domain towards N-terminus, and a characteristic signature motif called polo-box domain (PBD), consisting of two polo boxes (PB1, PB2) towards C-terminus (Lowery et al., 2005; Zitouni et al., 2014) (Fig. 1.1). While the kinase domain functions as catalytic core of PLKs, the PBD adds diversity to PLK functions by targeting the enzyme to an array of substrates found at different sub-cellular structures for exquisite regulation of cell cycle (Vaid et al., 2016b).

![Figure 1.1: Schematic representation of the domain organization of Cdc5, PLK in S. cerevisiae.](image)

Cdc5 protein consists of 702 amino acids. The kinase domain (KD) spans 82-337 amino acids and harbors nuclear localization signal (NLS) between 171-174 amino acids. The polo box domain (PBD) consists of polo box1 (PB1) and polo box2 (PB2) spanning 520-587 amino acids, and 619-692 amino acids, respectively, along with the linker spanning 588-618 amino acids, comprise the polo box domain (PBD). Each number denotes the position of initial and last amino acid residue of each domain (adapted and modified from Lee et al., 2005; Dai, 2005).

Till date, different PLKs have been identified in many organisms showcasing their wide range of functions (Vaid et al., 2016b), including budding yeast Saccharomyces cerevisiae (Cdc5) (Kitada et al.,1993); fission yeast Schizosaccharomyces pombe (plo1) (Ohkura et al., 1995); frog Xenopus laevis (Plx1, Plx2, Plx3) (Kumagai et al., 1996); pathogenic yeast Candida albicans (CaCdc5) (Bachewich et al., 2003); the fungus Aspergillus nidulans (PlkA) (Mogilevsky et al., 2012); worm Caenorhabditis elegans (plk1, plk2, plk3, ZYG-1) (Archambault and Glover, 2009); humans Homo sapiens (Plk1, Plk2/hSNK, Plk3/PRK, Plk4/SAK, Plk5) (Ohkura et al., 1995; Park et al. 2010; Zitouni et al., 2014). PLKs are not identified so far in plants and believed to have been lost during evolution (Zitouni et al., 2014; Vaid et al., 2016b).

The members of PLK family share a high degree of structural as well as functional homology, for e.g., the kinase domain of Cdc5 and human Plk1 exhibit 49 % identity, while
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PBDs show 33-40 % of identity, respectively (Lee et al., 2005; Fig. 1.2). Accordingly, defects in cdc5 mutants of yeast can be complemented by human Plk1, which indicates that cell cycle functions of PLKs are conserved across evolution from yeast to humans (Lee and Erikson, 1997).

Figure 1.2: Schematic representation and comparison of PLKs from *S. cerevisiae* (Cdc5) and *H. sapiens* (Plk1). Both sequence identities and similarities (percentages in parenthesis) and position of amino acid residues are indicated. The KD of two PLKs show 49 % identity and 69 % similarity, while the two polo-boxes show 33 % and 40 % identity, (57 % and 53% similarity), respectively (adapted and modified from Lee et al., 2005).

1.3.2 Functions of PLKs

PLKs play a vital role in cell division in all the organisms studied so far (Archambault and Glover, 2009). They regulate crucial events of cell division and cell cycle beginning from centriole (spindle pole bodies in yeast) biogenesis and duplication, activation of DNA damage checkpoint, DNA replication during S-phase, maturation of centrioles, mitotic onset, progression through mitosis, golgi fragmentation and assembly, chromosome segregation, exit from mitosis and cytokinesis (Archambault et al., 2015). Consistent with the indispensable role of PLKs during cell cycle, Plk1 is over expressed in human tumors and has prognostic potential in cancer, indicating its involvement in carcinogenesis, and it’s potential as a therapeutic target (Strebhardt et al., 2006; Gutteridge et al., 2016). Some potential
inhibitors discovered for Plk1 are BI-2536 and volasertib which are revolutionizing the cancer treatments (Valianou et al., 2015; Gjertsen and Schöffski, 2015). Plk1 represents an ideal protein-kinase target for cancer drug development because, in addition to its kinase domain, which is related to members of the protein-kinase superfamily, it also encompasses the unique PBD.

The functions of PLKs have been well characterized in S. cerevisiae. Cdc5 is primarily a late kinase, which mediates G2 to M phase transition, metaphase to anaphase transition during mitosis, exit from mitosis through MEN (Mitotic Exit Network) pathways and cytokinesis through the FEAR (Cdc Fourteen Early Anaphase Release) pathway (Lee et al., 2005; Fig. 1.3). Cdc5 also plays an important role during meiosis, including resolution of the recombination intermediates called joint molecules (JMs), chiasmata formation, pachytene exit, mono-orientation of sister kinetochores, cohesin removal, accurate chromosome segregation and establishment of meiotic divisions (Clyne et al., 2003; Sourirajan and Lichten, 2008; Marston, 2014; Duro and Marston, 2015; Fig. 1.3).
Figure 1.3: Schematic diagram representing an overview of mitotic and meiotic functions of *S. cerevisiae* PLK, Cdc5. The functions have been summarized from the existing literature on functions of Cdc5.

1.4 Substrates of PLKs

Being a kinase, PLKs mediate their effect by phosphorylation of their substrate proteins during cell division. PLKs lack a well defined consensus sequence for their phosphorylation pattern, which has limited identification of its substrates. Multiple substrates for PLKs have been identified in animals and budding yeast (Lowery et al., 2005; Lee et al., 2005).

In spite of multiple known functions of the PLK, Cdc5, in yeast, very few substrates have been identified for Cdc5. There include the cohesin Scc1, phosphatase, Cdc14 (Nasmyth et al., 2000; Amon, 2001; Alexandru et al., 2001; Sumara et al., 2002; Clyne et al., 2003; Lee and Amon, 2003b). Cyclin B, and Swe1 kinase (Bartholomew et al., 2001; McMillan et al., 2002; Park et al., 2003); Hsl1 and septins in the bud-neck ring; Cdc11,12 during G2/M transitions (Song and Lee, 2001; Sakchaisri et al., 2004; Lee et al., 2005). Cdc5 also phosphorylates components of spindle pole body, Nud1, Bbp1, Bfa1, Bub2, Spc72, Stu2 and Slk19, which participate in microtubular dynamics and help in establishing mitotic exit (Gruneberg et al., 2000; Hu et al., 2001; Ho et al., 2002; Park et al., 2004). Based on the analysis of substrates of Cdc5, potential consensus for Cdc5 kinase domain [(D/E/N) X (S/T)] and PBD [S(pS/pT)(P/X)] were predicted (Snead et al., 2007).

Few substrates of Cdc5 have been identified during meiosis, which include Ime2 (a meiosis-specific CDK-like kinase), Ndt80 (a meiosis specific transcription factor), Mam1 and Lrs4 (monopolin components), and Rec8, the meiosis-specific kleisin subunit of cohesin (Clyne et al. 2003; Lee and Amon 2003a; Matos et al, 2008).

1.5 Role of Cdc5 in pachytene exit of meiosis-I

Pachytene exit during prophase of meiosis I is a crucial transition during meiosis, after which cells are committed to enter meiotic divisions. Pachytene exit is a crucial event as it is accompanied by the joint molecules (JMs) resolution as crossovers (COs), noncrossovers (NCOs), and synaptonemal complex (SC) disassembly and directs the cell towards final
meiotic divisions (Xu et al., 1995; Sourirajan and Lichten, 2008). In S. cerevisiae, exit from pachytene stage and the associated events is triggered by a meiosis-specific transcription factor called Ndt80, which activates transcription of genes required for pachytene exit, meiotic divisions and spore formation (Xu et al., 1995; Allers and Lichten, 2000). Accordingly, yeast cells lacking Ndt80 (ndt80Δ) exhibit pachytene arrest with unresolved JMs, and intact SC, and failure to undergo meiotic division (Xu et al., 1995; Allers and Lichten, 2000). Ndt80 activates the transcription of more than 200 genes during meiosis known as middle sporulation genes, which includes cell cycle kinases CDK (cyclin-B subunits) and PLK (Cdc5) (Chu et al., 1998; Chu and Herskowitz, 1998; Sourirajan and Lichten, 2008). Interestingly, loss of Cdc5 function during meiosis results in defect in pachytene exit, similar to the ndt80Δ mutants (Clyne et al., 2003; Lee and Amon, 2003b). Subsequent studies have shown that the PLK, Cdc5 is the only member of the huge Ndt80 transcriptome that is necessary and sufficient for crucial events of pachytene exit, including the dissolution of SC assemblage, and resolution of recombination intermediates between the homologous chromosomes as crossover recombinants (Sourirajan and Lichten, 2008).

How does Cdc5 regulate events of pachytene exit? Since Cdc5 is a kinase, the most obvious mechanism is that Cdc5 phosphorylates one or more of its substrate proteins, which get activated, and in turn, promote pachytene exit by activating JM resolution, and SC breakdown. In favor of this possibility is the fact that kinase inactive mutants of Cdc5 (cdc5-N209A) fail to promote JM resolution (Sourirajan and Lichten, 2008). Consequently, the present study was undertaken to identify the molecular targets of budding yeast PLK during meiosis, specifically during exit from pachytene. To identify the substrates of the PLK, Cdc5, a high-throughput proteomic approach was adopted. To limit the screen to the substrates of Cdc5 involved in pachytene exit, ndt80Δ yeast cells (these cells arrest in pachytene) containing the estrogen-inducible CDC5 allele (CDC5-IN) was used. To isolate the substrates bound to Cdc5 kinase, a TAP-tagged (Tandem Affinity purification) version of CDC5-IN allele (pGAL1-CDC5-TAP), and the kinase-inactive CDC5 allele, cdc5-N209A-IN (pGAL1-cdc5-N209A-TAP) were used. The proteins bound to Cdc5-TAP and cdc5-N209A were pulled-down using TAP-tag, and subjected to LC-MS-MS (tandem mass spectrometry) for identification.

1.6 Importance of the study
The present study aimed to identify the substrates of Cdc5 during pachytene exit, which in turn will shed light into the molecular players involved in JM resolution and SC breakdown, both of which are unknown yet. This would also generate an atlas of the meiotic kinome of Cdc5, the sole PLK in budding yeast.

1.7 Objectives of the present study

The present study was undertaken with the following objectives:

- Generation and validation of *CDC5-IN* and *cdc5-N209A-IN* (kinase inactive) diploid yeast strains
- Preparation of whole cell protein extracts and TAP-tag pull down of Cdc5 and cdc5-N209A and their associated substrates during meiosis
- Identification of substrates of Cdc5 and cdc5-N209A during meiosis and their validation by *in silico* analysis