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Meiotic DNA Replication

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1. Introduction

Chromosome replication is a basic biological process that is essential for cellular proliferation. Owing to its fundamental nature the process of DNA replication is highly conserved among eukaryotic organisms. In the course of a typical somatic cell cycle, cells are "born" in G1 phase. Throughout this phase the cells grow in size increasing their mass and their protein synthetic capacity. As cells grow during G1 they monitor their size and protein synthetic capacity along with a variety of parameters in the external milieu including nutrient availability (Hartwell, 1974). When the internal and external environments are deemed to be suitable, the cell will commit to a cell division cycle. Initiating a cell cycle is a serious matter for yeast cells in that once they initiate the program they are committed to its completion (Hartwell, 1974). Should the cell have insufficient resources or capacity to successfully complete division the result is arrest and inevitable loss of viability. The decision to initiate a division cycle is taken at START in the budding yeast *Saccharomyces cerevisiae*; this event is referred to as R, the restriction point in mammalian cells (Hartwell, 1974; Pardee, 1974). If conditions are appropriate for proliferation, G1 phase cells will make the transition to S-phase where DNA replication ensues followed by progression through G2 and chromosome segregation at mitosis followed by completion of the cycle at cytokinesis.

Prior to completing the START transition and committing to a round of cell division *S. cerevisiae* can adopt several alternative fates. Haploid cells can conjugate with a partner of the opposite mating type to form diploids (Cross et al., 1988). Alternatively, both haploid and diploid cells are capable of entering a quiescent state in response to deprivation of some nutrients (Gray et al., 2004). Under the appropriate conditions diploid *MAT*a/α cells can exit the mitotic cell cycle and initiate the meiotic differentiation program leading to sporulation (Kupiec et al., 1997). This differentiation program is triggered by starvation for nitrogen and glucose. Upon encountering these conditions diploid *S. cerevisiae* will arrest in G1 phase with unreplicated DNA. The meiotic differentiation process initiates from G1 phase where the starved cells accumulate, and progresses through premeiotic DNA replication followed by extensive homologous recombination. Meiotic recombination is followed by two consecutive rounds of chromosome segregation, the meiosis I (reductional) division, and meiosis II (equational) division. Significantly, these two rounds of chromosome division occur without an intervening S-phase hence the meiotic progeny are haploid and the haploid nuclei are encapsulated in spores that are highly resistant to environmental insult. Hence, with regard to chromosome metabolism, the meiotic program resembles a somatic

cell cycle but many aspects of the process are modified to achieve the desired developmental aim of ploidy reduction, increased genetic diversity, and spore formation.

2. The transition to premeiotic S-phase

In mitotically proliferating *S. cerevisiae* cells, progression past START and entry into S-phase is dependent upon the Cyclin Dependent kinase Cdk1 (formerly known as Cdc28) (Reed, 1992). Cdk1 is activated in G1-phase by the three G1 cyclins Cln1, Cln2, and Cln3 (Richardson et al., 1989). The Cln-Cdk1 complexes serve multiple functions in promoting entry into a mitotic cell cycle (Levine et al., 1995). Starvation for nitrogen and glucose causes diploid cells to arrest in G1 and precludes progression into a mitotic cell division cycle. Under these conditions the *CLN1, CLN2* and *CLN3* genes are repressed (Gallego et al., 1997). The *CLN* cyclins are not required for entry into or progression through meiotic differentiation and indeed enforced expression of *CLN* cyclins under starvation conditions impedes the initiation of meiotic differentiation (Dirick et al., 1998; Colomina et al., 1999). It has been proposed that *CLN* expression acts as the switch that determines whether a cell initiates a mitotic division cycle or enters meiotic differentiation (Colomina et al., 1999).

Nitrogen deprivation, the condition that represses the expression of the *CLN* genes and precludes entry into a mitotic cell division cycle, induces the expression of a cascade of meiosis-specific genes (Kassir et al., 2003). *MAT*a/α diploid cells respond to starvation for nitrogen and glucose by activating expression of *IME1* (Inducer of Meiosis) (Kassir et al., 1988). The induction of *IME1* requires input from a complex signaling pathway that integrates nutritional signals (nitrogen and glucose) and ploidy *MAT*a and *MAT*α mating type genes (Kassir et al., 2003). *MAT*a/α diploids will respond to this condition by initiating the program of meiotic differentiation, whereas haploids, *MAT*a/a or *MAT*α/α diploids will arrest in G1 and enter a quiescent state. *IME1* is a meiosis-specific transcription factor that activates the expression of a large family of early meiotic genes many of which encode proteins that function in DNA replication and recombination (Mitchell, 1994). One of the key targets of Ime1 protein is the *IME2* gene (Smith et al., 1990). Ime2 encodes a meiosis-specific protein kinase with amino acid sequence similarity to Cdks. Indeed it has been proposed that Ime2 may replace the functions of Cln-Cdk1 complexes in meiosis (Dirick et al., 1998). While Ime2 may share some of the roles played by G1 cyclins it is unlikely that Ime2 directly replaces Cdk function in meiosis (Honigberg, 2004). However, Ime2 does play many roles in the meiotic differentiation program including driving the induction of early and middle phase meiosis-specific genes, and promoting inactivation of both APC^{CDH1} (Anaphase Promoting Complex) and the Cdk inhibitor Sic1 (Dirick et al., 1998; Bolte et al., 2002; Sedgwick et al., 2006; Holt et al., 2007). The latter two functions are essential to allow the accumulation of active B-type cyclin-Cdk1 complexes as APC^{CDH1} targets the cyclins for proteolytic degradation and Sic1 binds to the cyclin-Cdk1 complexes and inhibits their catalytic activity.

Ime1 and Ime2 are key regulators of the meiotic differentiation program. Loss of Ime1 function causes diploid cells to arrest in G1 phase in response to starvation, and precludes initiation of the meiotic program (Kassir et al., 1988; Smith et al., 1990). Mutations that inactivate Ime2 result in diploid cells undergoing a prolonged G1-phase arrest in response to starvation followed by delayed and defective DNA replication that leads to loss of viability (Foiani et al., 1996).

3. Initiation of premeiotic DNA replication

The initiation of DNA replication is a tightly regulated process that begins with the stepwise assembly of a protein complex that ultimately recruits a DNA helicase and the DNA polymerases required for replication of the chromosomal DNA (Remus & Diffley, 2009). Although meiotic differentiation differs from mitotic proliferation, in some cases drastically, the fundamental aspects of the premeiotic DNA replication mirror the processes that occur during mitotic proliferation.

DNA replication initiates from specific chromosomal locations referred to as origins of replication (Ori). These sequences are distributed along each of the chromosomes so that DNA replication can initiate from multiple sites. The relatively compact Oris of *S. cerevisiae* have been well defined and have an essential core sequence 5'-TTTTATGTTTA-3' and a set of three less well conserved accessory sequences that stimulate the efficiency of Ori activation (Marahrens & Stillman, 1992; Marahrens & Stillman, 1994). Data from numerous investigations support the contention that origins of replication are activated with variable efficiency in mitotically proliferating cells. Additionally, not every Ori is activated in every S-phase (McCune et al., 2008; Patel et al., 2008). Thus, Oris that are activated more efficiently have a higher probability of being activated at higher frequency.

Origin of DNA replication usage has been less rigorously investigated during premeiotic DNA replication. Analysis of origin activation by two-dimensional gel electrophoresis suggested that in *S. cerevisiae* the same origins that are activated during mitotic proliferation are also utilized during premeiotic DNA replication (Collins & Newlon, 1994). This study did not investigate the efficiency or frequency of origin activation. A more recent investigation of origin usage in the fission yeast *Schizosaccharomyces pombe* using microarray technology revealed that the same origins of DNA replication are used during mitosis and meiosis in this yeast (Heichinger et al., 2006). These authors found an overall reduction in the efficiency of origin utilization in premeiotic DNA replication relative to proliferation and they speculated that this might at least in part explain why premeiotic DNA replication takes so much longer than mitotic DNA replication (2 hours for premeiotic S-phase vs. 20 minutes for mitotic S-phase in *S. cerevisiae)* (Cha et al., 2000; Heichinger et al., 2006). Transcription initiating near or proceeding through Ori sequences can influence the efficiency of origin utilization (Donato et al., 2006; Mori & Shirahige, 2007). Additionally, the state of histone modification, in particular acetylation, has been correlated with the efficiency of Ori assembly and activation (Vogelauer et al., 2002; Weber et al., 2008; Unnikrishnan et al., 2010). Some of these conclusions were reached by using chemical inhibition of histone deacetylases (HDACs) to alter the modification state of the chromatin. These conclusions are controversial since altering HDAC activity can affect many aspects of gene regulation and one effect of HDAC inhibition is to reduce the supply of pyrimidine nucleotide precursors for DNA replication resulting in reduced fork speed and the recruitment of latent origins of DNA replication: (Gay et al., 2010).

Since transcription patterns and the landscape of protein binding to DNA changes dramatically when cells transition from mitotic proliferation to meiotic differentiation it is not surprising that there would be a change in the usage of origins. It is unclear why there is an overall reduction in origin firing in meiotic cells but one possibility is that that some factor required for origin firing is limited during meiosis. Owing to the fact that meiotic differentiation initiates under starvation conditions where there may be no exogenous source of amino acids or nitrogen and that the total number of ribosomes decreases by 50%

or more (Hopper et al., 1974; Magee & Hopper, 1974) it is likely that the overall rate of protein synthesis is reduced in cells undergoing meiosis (Esposito et al., 1969). Under these conditions labile factors such as Dbf4 may become limiting to origin activation (Patel et al., 2008). During mitotic proliferation Ori activation is temporally regulated with a subset of origins being activated early and another subset being activated later in S-phase (Raghuraman & Brewer, 2010). Activation of these origins displays distinct properties, early firing origins are activated even in the presence of hydroxyurea which inhibits or reduces activation of late origins (Santocanale et al., 1999). S-phase initiation requires Cdk1, and the S-phase form of *S. cerevisiae* Cdk1 is activated by the cyclins Clb5 and Clb6. Early origins are activated in the absence of the S-phase cyclin Clb5 whereas late activated origins depend upon Clb5 (Donaldson et al., 1998). This particular characteristic has been attributed to the fact that Clb6-Cdk1 complexes are present early in S-phase but Clb6 is rapidly degraded as cells progress through S-phase (Jackson et al., 2006). The role of cyclin Cdk complexes in triggering DNA replication will be considered in section 5 of this chapter. The mechanisms governing this temporal pattern of origin activation have been speculated upon but have yet to be clarified. Recent investigations suggest that earlier firing origins are more efficient and have a higher probability of firing in any given cell cycle. It has not yet been determined whether temporal regulation is imposed upon Ori activation during premeiotic DNA replication.

4. Assembling the prereplication complex

DNA replication is initiated from specific origins of replication by assembling complex multiprotein machines on origins of replication and then activating those complexes. PreReplication Complex (PreRC) assembly at origins of DNA replication has been extensively investigated in mitotically proliferating cells (Diffley, 2001). Contributions from several laboratories have revealed that assembly of the complex proceeds in a step-wise fashion (Diffley et al., 1995). In yeast the Ori sequence is constitutively bound by the Origin Recognition Complex (Orc) composed or the proteins Orc1 – Orc6 (Rowley et al., 1995). In contrast the Orc proteins assemble on the origins in G1-phase in metazoans and Orc1 is destabilized as the cells pass through S-phase (DePamphilis, 2005). The Orc complex marks the chromosomal origins of DNA replication, and serves as a nucleation site for the assembly of a functional PreRC (Diffley et al., 1995; Rowley et al., 1995).

A key cell cycle regulated step in promoting further assembly of the PreRC is the accumulation of Cdc6 and Cdt1 in late G1 phase. Transcription of the Cdc6 gene is induced in late G1 phase by the MBF transcription factor (Zhou & Jong, 1990). In mitotically proliferating cells the accumulating Cdc6 in conjunction with Cdt1 binds to Orc complexes tethered to Ori DNA (Devault et al., 2002). The ATPase activity associated with Cdc6 is then required to promote loading of the Mcm (Mini Chromosome Maintenance) complex on to the ORC complex (Stillman, 2005). The loading of Mcm is a key step in assembling the origin complex. Failure of this loading or loss of Cdc6 function precludes further assembly of PreRCs and blocks the initiation of DNA replication (Piatti et al., 1995). Surprisingly, loss of Cdc6 function does not arrest chromosome metabolism. Rather, cells depleted of Cdc6 simply skip over S-phase and progress to a reductional chromosome division leading to loss of viability (Piatti et al., 1995).

The Cdc6 gene is subject to regulated transcription during meiotic differentiation where it accumulates during G1 phase (Ofir et al., 2004). Cdc6 is essential for progression through

premeiotic S-phase and meiotic recombination in both yeast and higher eukaryotes (Lemaitre et al., 2002; Lemaitre et al., 2004; Ofir et al., 2004). Loss of Cdc6 in meiotic differentiation precludes the initiation of premeiotic DNA replication but allows an attempted chromosome division without having replicated the DNA or forming a synaptonemal complex, leading to loss of viability (Hochwagen et al., 2005). Current thinking ascribes progression to meiotic chromosome divisions in the absence of S-phase to the idea that progression through G1 – S – M phases is restrained by the S-phase checkpoint which detects single stranded DNA or some aspect of DNA replication fork structure. A failure to initiate DNA replication results in no signal that could be detected by the checkpoint and so the checkpoint is not activated. Under these conditions the transcriptional program that drives meiotic differentiation proceeds and the cells progress to attempt the segregation of unreplicated chromosomes (Stuart & Wittenberg, 1998).

The primary role of Cdc6 is to promote the association of the hexameric Mcm complex with DNA bound Orc complexes (Tanaka et al., 1997). The Mcm proteins Mcm 2 - 7 form sub-complexes that can subsequently be loaded on to the Orc complex as a dimer of hexamers (Stillman, 2005). The Mcm complex functions as an ATP-dependent DNA helicase (Ishimi, 1997). By loading a dimer of Mcm hexamers on to Orc complexes the hexamers can drive unwinding of the duplex DNA leading to bidirectional DNA replication. In addition to their role in DNA unwinding, the Mcm helicase also provides binding sites for the recruitment of other factors that form the replication forks such as DNA polymerases (Forsburg, 2004).

Components of the Mcm complex accumulate cooperatively in the nucleus during G1-phase and are required for activation not only of S-phase but also of the S-phase checkpoint (Labib et al., 2001). Surprisingly, it has been reported that Mcm proteins are not required for premeiotic DNA replication in yeast (Forsburg & Hodson, 2000). This study made use of a set of yeast strains harboring temperature sensitive mutations in a subset of the *MCM* genes. The opposite conclusion was reached by investigators using temperature sensitive degron mutants that eliminated or profoundly reduced the abundance of the Mcm proteins upon activation of the degron (Lindner et al., 2002). The difference between these two investigations may simply have been the degree to which the Mcm proteins could be depleted from the cells.

Subsequent to the loading of the hexameric Mcm complex in late G1-phase, assembly of the PreRC continues with the recruitment of several additional factors Cdc45, Dpb11, Sld2, Sld3, and the GINS complex (Remus & Diffley, 2009; Tanaka & Araki, 2010). Cdc45 has no identified catalytic activity but it makes specific contacts with the Mcm complex and is essential for mitotic DNA replication (Hopwood & Dalton, 1996). Cdc45 is essential for premeiotic DNA replication and meiosis in plants (Stevens et al., 2004), and it is assumed to be essential for premeiotic DNA synthesis in yeast although this has never been formally reported.

Cdc45 binding to the Mcm complex at the preRC is linked to binding of Dpb11, Sld2, and Sld3. Dpb11 is thought to act as a scaffold to allow association of Sld2, Sld3 and the replicative DNA polymerases Pol1 (DNA polymerase alpha) and Pol2 (DNA polymerase epsilon) with the preRC (Masumoto et al., 2000; Tanaka et al., 2007; Zegerman & Diffley, 2007). Sld2, Sld3, and Dpb11 are all essential for the initiation of DNA replication in proliferating cells (Araki et al., 1995; Kamimura et al., 1998; Kamimura et al., 2001). Stable assembly of Sld2 and Sld3 with Dpb11 and the preRC is dependent upon Cdk activity. Phosphorylation of Sld2 and Sld3 on multiple Cdk sites promotes their stable binding to Dpb11 and the preRC (Tanaka et al., 2007; Zegerman & Diffley, 2007). Temperature sensitive

sld2, and mutants display a profound defect in premeiotic DNA replication implying that they serve the same essential role in meiotic cells that they play in proliferating cells (Figure 1).

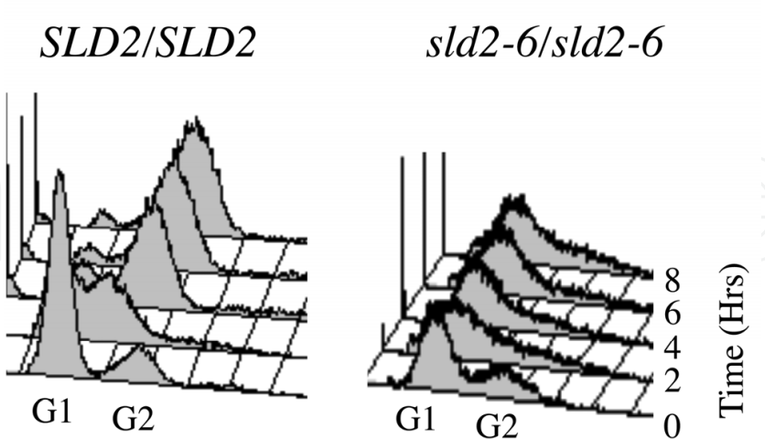

Fig. 1. Flow cytometry profiles of *SLD2/SLD2* and *sld2-6/sld2-6* mutant cells that have been induced to initiate meiotic differentiation at the non-permissive temperature of 36°C. The *sld2-6* mutant arrests in G1-phase – early S-phase and does not complete S-phase. The *sld2-6* temperature sensitive allele was provided by Dr. H. Araki and was introduced into the *S. cerevisiae* SK1 genetic background by gene replacement.

The GINS complex is a tetrameric protein assembly whose name derives from Go-Ichi-Ni-San; the Japanese numbers 5,1,2,3, in reference to Sld5, Psf1, Psf2, Psf3, the components of the complex (Takayama et al., 2003). GINS associates with Sld2, Dpb11, and Pol2 in a sub-complex that is loaded on to the preRC (Muramatsu et al., 2010). The GINS complex is essential for the initiation of DNA replication and replication fork progression in proliferating cells (Takayama et al., 2003). The function of GINS in premeiotic S-phase has not been directly investigated, however the finding that *psf2* mutants display meiotic chromosome segregation defects in *S. pombe* suggests that Psf2 and GINS also play a role in meiotic DNA replication (Gómez et al., 2005).

The assembly of the correct preRC architecture is important for regulating the initiation of DNA replication and the ultimate function of this complex is to recruit and position the replicative DNA polymerases to the Ori where the chromosomal DNA will be unwound by the Mcm complex and bidirectional DNA synthesis will initiate. Pol1, the catalytic subunit of DNA polymerase alpha-primase is essential for meiotic DNA replication and likely serves the purpose of synthesizing the lagging strand as it does in proliferating cells (Budd et al., 1989). Not unexpectedly, subunits of the DNA primase, Pri1 and Pri2, are also required for the synthesis of the lagging strand (Longhese et al., 1993). The leading strand DNA polymerase Pol3 (DNA polymerase delta) plays a role in meiotic DNA replication and meiotic homologous DNA recombination (Schild & Byers, 1978; Maloisel et al., 2008). Pol2 (DNA polymerase epsilon) is also found at the replication fork and has a role as leading strand DNA polymerase in proliferating cells (Calzada et al., 2005). Surprisingly the catalytic activity of Pol2 is not essential in mitotically proliferating cells likely owing to redundancy with Pol3 activity (Kesti et al., 1999; Ohya et al., 2002). Both of the leading strand polymerases are expected to be required for premeiotic DNA replication but this has not been formally demonstrated.

Thus, while preRCs are composed of primarily the same components during the initiation of S-phase in meiotic and proliferating cells, it should be noted that the composition of meiotic preRC has not been formally investigated and may have components remaining to be identified that impart meiosis-specific regulation to the activation of premeiotic DNA synthesis.

5. Activating premeiotic DNA synthesis: the role of Cyclin-Cdk and Dbf4-Cdc7

In proliferating cells the activation of DNA synthesis is dependent upon B-type cyclin-Cdk1 and Dbf4-Cdc7. *S. cerevisiae* has a single cell cycle regulating Cdk, referred to as Cdk1. Cdk1 is essential for all the major cell cycle transitions in *S. cerevisiae* (Nasmyth, 1993). Cdk1 can bind to and is activated by nine different cyclins. *CLN1, CLN2*, and *CLN3* are expressed in proliferating G1 phase cells. Six B-type cyclins *CLB1 – CLB6* are expressed in S-phase, G2 and mitosis (Lew & Reed, 1992). Cyclin binding to Cdk1 serves the purposes of activating the catalytic function of the kinase through promoting a change in the conformation of Cdk1 and they direct the Cdk1 kinase activity against specific substrates (Morgan, 1995; Loog & Morgan, 2005). Cdk1 is essential for activating S-phase in proliferating cells and during meiotic development (Reed, 1980; Benjamin et al., 2003). Cdk1 in complex with the cyclins Clb5 and Clb6 is the primary Cdk1 complex that drives the initiation of DNA replication. Clb5 is the dominant cyclin of the Clb5, Clb6 pair as it is more stable and accumulates to greater abundance (Jackson et al., 2006). Although other B-type cyclins can activate Cdk1 to promote S-phase in proliferating cells none do so as efficiently as Clb5 (Donaldson et al., 1998; Cross et al., 1999; Hu et al., 2008). This is in part due to the consideration that Clb5 and Clb6 accumulate in late G1 whereas the other B-type cyclins Clbs1 – 4, normally accumulate later in G2 and M-phase (Fitch et al., 1992; Richardson et al., 1992; Schwob & Nasmyth, 1993). However, even when other cyclins are ectopically expressed in G1 they can induce DNA replication but with a significant delay relative Clb5 (Donaldson, 2000; Hu & Aparicio, 2005). It is likely that this is due to substrate specificity. Clb5 and Clb6 have an amino acid motif (MRAIL) that has affinity for the substrate sequence RxL that is present in proteins like Sld2 and Sld3 that are high affinity substrates for Clb5 (Cross & Jacobson, 2000; Ubersax et al., 2003; Loog & Morgan, 2005). Hence Clb5 and Clb6 are referred to as S-phase cyclins.

The key and possibly only essential role for Cdk1 in activating DNA replication is to phosphorylate Sld2 and Sld3 to allow them to bind Dpb11 and the preRC complex or stabilize their binding through creation of high affinity sites (Tanaka et al., 2007; Zegerman & Diffley, 2007). There may be additional roles in supporting DNA polymerase binding since the DNA polymerase epsilon subunit Dpb2 is also Cdk substrate (Kesti et al., 1999).

Cdk1 activity is required for several aspects of chromosome metabolism in the early stages of meiotic differentiation. Cdk1 is strictly required for premeiotic DNA replication as demonstrated by the meiotic G1 arrest when mutants expressing an analog sensitive version of Cdk1 are treated with the inhibitor 1-NM-PP1 (Benjamin et al., 2003). Cdk1 is also required for the initiation of meiotic DNA recombination and synaptonemal complex formation (Henderson et al., 2006; Zhu et al., 2010). Both of these processes are dependent upon successful completion of premeiotic DNA replication.

Temperature sensitive alleles of Cdk1 cannot be inactivated sufficiently to block premeiotic DNA replication, which led to the idea that Cdk1 activity was not required for progression through meiotic differentiation (Shuster & Byers, 1989). It is unclear why proliferating cells

need more Cdk1 activity to initiate S-phase than do meiotically differentiating cells. One possibility is that in proliferating cells Cdk1 is required to activate the transcription factors MBF (MluI binding factor) and SBF (SCB binding factor) (deBruin et al., 2004). These transcription factors induce the expression of the genes whose products function to promote S-phase and DNA replication *CLB5, POL1, RNR1* etc. (Koch & Nasmyth, 1994). In contrast, many of the genes whose products are required for premeiotic S-phase are regulated by the meiosis-specific transcription factor Ime1 (Mitchell, 1994). SBF is not active in meiotic cells; however, MBF is active during meiotic G1 and promotes G1-specific transcription; however, inactivation of MBF does not eliminate expression of the genes required for DNA replication and does not cause any defect in premeiotic DNA replication (Raithatha & Stuart, 2005). Additionally, there is evidence that a subset of MBF regulated genes are differentially regulated during mitotic and meiotic G1-phase (Raithatha & Stuart, 2005).

In mitotically proliferating *S. cerevisiae* the S-phase cyclins *CLB5* and *CLB6* are not essential for S-phase activation, indeed cells will only arrest in G1 when all six of the B-type cyclins are inactivated (Schwob et al., 1994). In contrast *CLB5* and *CLB6* are strictly required for the initiation of premeiotic S-phase (Dirick et al., 1998; Stuart & Wittenberg, 1998). During the course of unperturbed meiotic differentiation Clb5 and Clb6 are the first cyclins to accumulate and these trigger DNA replication whereas the other B-type cyclin genes are regulated by Ndt80 and only accumulate during the middle phase of sporulation during pachytene (Chu et al., 1998). Even if Clb1 or Clb3 is expressed early in meiosis under the regulation of a *CLB5* promoter these cyclins fail to trigger premeiotic S-phase in a *clb5 clb6* mutant (J. DeCesare & D. Stuart unpublished observation). Suggesting that Clb5 and Clb6 have some particular property that allows them to activate DNA synthesis during meiosis. Possibilities include subcellular localization, recruitment to specific substrates or substrate specificity. Clb5 accumulates in the nucleus both in proliferating and sporulating cells as expected since it effectively phosphorylates components of the preRC. The fact that any B-type cyclin can induce DNA replication in proliferating cells implies that all cyclin-Cdk1 complexes can gain access to the appropriate substrates at least in proliferating cells. However, it is unlikely that simple subcellular localization gives Clb5 its specific ability to activate premeiotic DNA replication because Clb3 has been localized to the nucleus in both proliferating and sporulating cells so in principle it should have access to the same suite of substrates as Clb5-Cdk1 complexes. The S-phase cyclins Clb5 and Clb6 display homology with other B-type cyclins in their Cdk1 binding domain however the amino-terminal portions of each cyclin are highly diverged from each other as well as other B-type cyclins and it is not clear what sequences direct these cyclins to their proper nuclear location.

Even though all cyclin-Cdk1 complexes can enter the nucleus they may not all have access to the same substrates. The potential remains for compartmentalization within the nucleus or sequestration of cyclin-Cdk1 complexes to locations within the nucleus where they may not have access to the critical substrates required to initiate premeiotic DNA replication. It is very likely that the architectural organization of the nucleus differs between mitosis and meiosis owing to the unique demands for synaptonemal complex formation, extensive homologous recombination, and the reductional meiosis I chromosome division. Within this altered nuclear landscape Clb5 and Clb6-Cdk1 complexes may have a unique ability to interact efficiently with the substrate proteins required to assemble and fire origins of DNA replication.

The molecular mechanisms that confer substrate specificity to cyclins have yet to be fully resolved (Miller & Cross, 2001). It is clear that Clb5 has a preference for a specific collection

of protein substrates that can interact with the cyclin MRAIL motif. Indeed Clb5 may harbor additional substrate interaction motifs that confer increased affinity to one or more meiosis-specific substrates. The cyclins that are shown to have specific functions in S-phase: *S. cerevisae* Clb5, and Clb6 and *C. albicans* Clb4, have a conserved motif of charged amino acids at their carboxyl-terminus that is not present in other cyclins (Figure 2). This sequence may have a role in the S-phase functions of these cyclins but no analysis of mutations in this motif have been reported. Premeiotic S-phase is influenced by proteins that are only expressed during meiotic differentiation (see next section) and it may well be that Clb5-Cdk1 can specifically phosphorylate or interact with those substrates. While it is not surprising that there would be cyclins with specialized roles in meiotic differentiation the mechanism by which that specificity is conferred remain to be elucidated.

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Sc. Clb5: 374 IHDEAFQSLCIDLVKDIASSKTHLDSLILKYKKPRYGS
Sc. Clb6: 330 ESDPAFKDFISELVEDIAVPDTNLDSLRLKYKKPKHGM
Kl. Clb : 340 DNEIELKELSQILINDIAKPTTQLNALIHKYKKIGTWN
Ca. Clb4: 433 YTEKQLQPLADVLLENCRHAEINHKAIFEKYKERRYRK
                 +  +  +   L+           +  KYK
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Fig. 2. A conserved cluster of charged amino acids near the carboxyl-terminus of the *S. cerevisae* S. phase cyclins Clb5 and Clb6. A similar cluster is present in the *C. albicans* Clb4 (an S-phase cyclin) and a cyclin from *K. lactis* (r_klactIV3887) whose function has not been confirmed. This charged cluster is not found in other *S. cerevisiae* cyclins.

In addition to cyclin-Cdk1, activation of DNA replication requires the kinase activity associated with the Dbf4-Cdc7 protein kinase (Masai et al., 1999). Cdc7 is the catalytic subunit of the protein kinase whereas Dbf4 is an activating and substrate targeting subunit similar to a cyclin (Jackson et al., 1993). The enzyme complex is referred to as DDK (Dbf4 Dependent Kinase). In *S. cerevisiae* Dbf4 accumulates in late G1 phase and binds Cdc7, which is constitutively expressed. The complex is then recruited to preRCs through interactions with both Mcm and Orc proteins (Dowell et al., 1994). The catalytic activity of Cdc7 is required to activate DNA replication in proliferating cells as inactivation of *cdc7* temperature sensitive mutants results in a strict G1-S-phase arrest (Newlon & Fangman, 1975). This phenotype is replicated by the analog sensitive alleles of Cdc7 that can be specifically inhibited by the small molecule PP1 [4-amino-1-tert-butyl-3-(p-methylyphenyl)pyrazolo [3,4-d]pyrimidine] (Wan et al., 2006). Recruitment of Dbf4 to the PreRC is essential for the activation of DNA replication, mutations that allow Dbf4 to bind Cdc7 but not the preRC cause a failure in DNA replication (Duncker et al., 2002).

The role of Cdc7 in premeiotic DNA replication has been controversial since both *cdc7* temperature sensitive mutants and analog sensitive mutants arrest post S-phase prior to the onset of meiotic recombination (Schild & Byers, 1978; Buck et al., 1991; Wan et al., 2006). In contrast the activating subunit Dbf4 is essential for premeiotic DNA replication (Valentin et al., 2006). Dbf4 is a highly unstable protein that can be rapidly depleted from cells unlike Cdc7, which is very stable (Ferreira et al., 2000). It may be the case that like Cdk, there is a

lower threshold of Cdc7 kinase activity required in meiosis. Nonetheless the requirement for Dbf4 argues that Dbf4-Cdc7 activity is required for premeiotic DNA replication.

The essential substrates of Cdc7 have been intensively sought after. Genetic analysis revealed that a mutation in Mcm5 (Mcm5-$P^{83}L$) could bypass the need for Cdc7 or Dbf4 both during mitotic proliferation and for premeiotic DNA replication (Hardy et al., 1997; Matos et al., 2008). Interestingly DDK does not appear to phosphorylate Mcm5. Rather it seems likely that phosphorylation of other components of the MCM complex Mcm2, Mcm4 and Mcm6 by DDK leads to a conformational change in the complex allowing its activation (Hoang et al., 2007). The $P^{83}L$ mutation in Mcm5 may result in a similar change that allows activation of the complex in the absence of DDK phosphorylation (Hoang et al., 2007). Currently the best candidates for the essential substrates of Dbf4-Cdc7 are Mcm2, Mcm4 and Mcm6. DDK can phosphorylate these Mcm proteins both in vitro and in vivo (Lei et al., 1997; Masai et al., 2006; Francis et al., 2009). Indeed Cdc7 preferentially phosphorylates the Mcm proteins within the context of the PreRC and chromatin bound Mcms are much better Cdc7 substrates than the free Mcm proteins (Francis et al., 2009). The role of Mcm phosphorylation is not entirely clear but there is evidence that it stabilizes the binding of Cdc45 and perhaps other components of the preRC (Zou & Stillman, 2000). Additionally, it has been posed that phosphorylation of the Mcm complex may lead to a conformational change that relieves autoinhibition of the helicase activity (Sheu & Stillman, 2010).

DDK has additional roles in meiotic chromosome metabolism. Strains that harbor *cdc7* mutations are defective in the formation of DNA double strand breaks (DSBs) that initiate meiotic homologous recombination (Wan et al., 2006). DDK phosphorylates several residues on Mer2 protein allowing it to stably associate with the chromatin and trigger double stand breaks (Sasanuma et al., 2008; Wan et al., 2008). Phosphorylation of Mer2 by DDK is dependent upon prior phosphorylation of Mer2 by Cdk1 (Sasanuma et al., 2008; Wan et al., 2008). Thus, the initiation of DSB formation and homologous recombination requires integration of inputs from both Cdk1 and DDK kinases.

6. Does DNA replication differ between meiosis and mitosis?

The process of initiating DNA replication in proliferating cells and cells undergoing meiotic differentiation are fundamentally the same. All of the proteins required to initiate S-phase in mitotically proliferating cells seem to also be required for premeiotic S-phase although not all have been rigorously tested in meiotic cells (Simchen, 1974). Premeiotic S-phase displays a reduced requirement for Cdk and DDK activity but these enzymes are still required. Based upon genetic and molecular studies it appears that the preRC assembled in cells initiating premeiotic S-phase is similar to or the same as that assembled in proliferating cells. However, a variety of investigations have revealed significant differences between mitotic and premeiotic S-phase. One fundamental physiological difference between the two processes is that proliferating cells display a specific cell size requirement before they can transition from G1 to S-phase (Cook & Tyers, 2007). Premeiotic DNA replication and indeed the entire meiotic differentiation process in *S. cerevisiae* is independent of cell size control (Stuart, 2008). This issue is somewhat controversial as some investigators have observed cell size regulation of meiotic differentiation but the differences may be related to the genetic background of the strains used in the experiments (Calvert & Dawes, 1984; Day et al., 2004).

Genetic studies have revealed several genes whose products are required for meiotic but not mitotic S-phase: *SPO7, SPO9, MUM2*, and *CDC55* (Esposito & Klapholtz, 1981; Davis et al.,

2001; Nolt et al., 2011). *SPO7* and *CDC55* encode subunits of protein phosphatases and may be candidates for meiosis-specific regulators of DNA replication. *MUM2* displays genetic interactions with both the replicative DNA polymerase *POL1* and *ORC2* suggesting that this protein may participate in activating premeiotic origins of DNA replication; however, the basis for its requirement has yet to be reported (Davis et al., 2001). The function of *SPO9* remains to be characterized. Currently the molecular basis for the meiosis-specific requirement for these proteins remains unknown.

Premeiotic S-phase is also dependent upon Ime2, a meiosis-specific kinase whose amino acid sequence is similar to Cdks (Foiani et al., 1996). Loss of Ime2 function causes a profound delay in premeiotic S-phase. Ime2 may not directly regulate the initiation of premeiotic DNA replication because *ime2* mutants display a plethora of defects including reduced recombination, reduction in early and middle meiotic gene expression (Mitchell et al., 1990; Benjamin et al., 2003). Ime2 is implicated in the inactivation of the APC^{CDH1} an event necessary to allow accumulation of B-type cyclins (Bolte et al., 2002; Holt et al., 2007). Ime2 is also implicated in destabilizing Sic1 although this effect may not be direct (Dirick et al., 1998; Sedgwick et al., 2006).

One of the most easily observable differences between premeiotic S-phase and S-phase in proliferating cells is the prolonged time frame for premeiotic DNA replication. S-phase lasts for nearly 2 hours in meiotic cells whereas the process lasts a mere 20 minutes in proliferating cells (Cha et al., 2000). Since the same origins of replication are utilized in meiotic and mitotic S-phase the most likely explanation for the slow progress of DNA replication is that origins are activated with lower efficiency as observed in fission yeast (Heichinger et al., 2006). Alternatively, the rate of fork movement may be slower in meiotic cells. Experiments using metabolic labeling and DNA fiber autoradiography suggested that the rate of replication fork movement is the same in meiotic and mitotic cells (Johnston et al., 1982). However, during premeiotic DNA replication the replication forks must contend with the assembling Spo11 DSB complexes on the chromatin. Replication forks have been shown to pause when encountering stable protein-DNA complexes (Azvolinsky et al., 2009). It is possible that replication fork pausing caused by the DSB complexes slows the progression of premeiotic DNA replication. Indeed deletion of *SPO11* shortens premeiotic S-phase supporting this contention (Cha et al., 2000).

7. Premeiotic DNA replication and meiotic recombination: What's the connection?

The ultimate goal of the meiotic differentiation process is to produce the haploid progeny required for sexual reproduction. One of the hallmarks of the meiotic process is elevated rates of recombination that effectively "shuffle the genome" allowing for relatively unbiased assortment of the parental genetic material. The process of generating haploid progeny yields at least two significant advantages to *S. cerevisiae*. First the genetic assortment that occurs increases the likelihood of generating at least a subset of progeny that are in possession of increased fitness relative to the parent. Second by passing through a haploid stage any deleterious recessive mutations that have accumulated in the diploid parent may be eliminated since a haploid harboring this allele will be inviable, a type of genetic "quality control' mechanism.

Meiotic recombination has been extensively studied in *S. cerevisiae* owing to the ease with which meiotic progeny can be separated and tracked. Meiotic DNA recombination is

generally predicated upon the successful completion of premeiotic DNA replication. The DNA double strand breaks (DSBs) that initiate meiotic recombination only form after the DNA has been replicated. This order of events is clearly important since the formation of DSBs prior to DNA replication would be catastrophic for genome integrity. This dependent relationship was elegantly demonstrated by a study that removed origins of DNA replication from the left arm of chromosome III, effectively delaying the completion of the replication of that arm of the chromosome. While DNA replication and DNA double strand break formation occurred with the expected normal timing on the right arm of chromosome III, DNA replication and DSB formation was profoundly delayed on the left arm (Borde et al., 2000).

A variety of possible mechanisms have been put forth to explain this relationship such as altered chromatin structure of the replicated DNA allowing DSB formation or access to the DSB forming complex (Berchowitz et al., 2009). Another potential explanation is that the DNA replication machinery may interact with and "license" or activate the protein complex that produces the meiotic DNA double strand breaks.

DNA DSBs are formed by a protein complex that binds to the chromatin in meiotic G1-phase. The complex is minimally composed of Spo11, Rec102, Rec104, Ski8 Mer2, Me14, Rec114, Mre11, and Rad50 (Keeney, 2001). Some of the integral components of the DSB formation complex are substrates for the S-phase protein kinases Clb5-Cdk1 and Dbf4-Cdc7. Mer2 is phosphorylated by Clb5-Cdk1 and this modification acts as a priming phosphorylation to trigger phosphorylation by Dbf4-Cdc7 (Henderson et al., 2006; Sasanuma et al., 2008; Wan et al., 2008). These phosphorylation events are essential for meiotic DSB formation and recombination. A mutated Mer2 lacking Cdk phosphorylation sites is defective in interacting with other components of the DSB forming complex (Henderson et al., 2006). Could Cdk1 and DDK be the connection between premeiotic DNA replication and meiotic recombination?

A parsimonious model to explain the temporal regulation of DSB formation and the involvement of Clb5-Cdk1 and Dbf4-Cdc7 could be that when Clb5-Cdk1 accumulates in premeiotic G1 phase it phosphorylates proteins required to assembly the PreRC (Sld2 and Sld3) as well as the DSB initiation complex (Mer2). Dbf4 accumulating in premeiotic G1-phase binds to Cdc7 and recruits the active Cdc7 to the PreRC. Upon the initiation of DNA replication the Mcm complex and other residents of the replication forks move along the chromosomes and Dbf4-Cdc7 moves along with the fork. As replication forks pass and possibly pause at sites of DSB initiation complexes Dbf4-Cdc7 phosphorylates members of the complex allowing for complete assembly or activation of the complex such that DSB formation can ensue following the passage of the replication fork.

This model is consistent with most published data however *cdc6* mutants that fail to assemble PreRCs form DSBs despite the absence of any detectable DNA replication (Hochwagen et al., 2005). Although this mutation decouples replication and recombination it is not inconsistent with the proposed model. The Dbf4-Cdc7 complex normally becomes tethered to the PreRC following loading of the Mcm complex by Cdc6. In the absence of Cdc6 the Mcm complex fails to load on to the DNA and Dbf4-Cdc7 complexes are thus not sequestered and can proceed to phosphorylate target proteins such as the DSB initiation complex factors hence allowing DSB formation. The localization and activity of Dbf4-Cdc7 has not been determined in *cdc6* mutants and so this model currently remains a matter of conjecture. Indeed it is also possible that the DNA replication checkpoint regulates the DSB formation complex and impedes the formation of DSBs until DNA replication is complete (Tonami et al., 2005).

8. Conclusion

DNA replication is an integral component of the meiotic differentiation program and is essential to the integrity of chromosome cohesion, meiotic recombination, and chromosome segregation. Defects in meiotic DNA replication have consequences that range from infertility and death of the progeny gametes to genomic instability and aneuploidy that can manifest as genetic diseases, birth defects and cancer. Despite our growing understanding of the mechanisms that govern DNA replication in mitotically proliferating cells, the regulation of premeiotic DNA replication remains largely uncharacterized. Several specific topics hold great promise for new discovery in the future, in particular the role of cyclin-Cdk1 in triggering premeiotic DNA synthesis, the regulation of preRC assembly in meiotic cells, and the relationship between DNA replication and homologous recombination.

Premeiotic DNA replication is subject to several distinct forms of regulation involving meiosis-specific regulatory proteins and meiosis-specific requirements for cyclin-Cdk and DDK. Future investigations will likely focus on clarification of substrate specificity, subcellular localization, and protein-protein interactions involving cyclin-Cdk1 and DDK. Additionally, premeiotic DNA replication requires some proteins that are meiosis-specific. Mum2 has no close orthologs in other organisms and as yet has no clearly defined role but that it is required for premeiotic DNA replication in *S. cerevisiae*. PreRC complexes have not yet been closely examined in cells undergoing meiotic differentiation. Biochemical characterization of these complexes through proteomic investigation may yield a wealth of new information about the components of meiotic replication complex and how premeiotic DNA replication is regulated. The biochemical relationship between the DNA replication machinery and the recombination machinery remains to be characterized and such investigations will certainly lead to an understanding of how DNA DSB formation is restrained until the DNA replication has been replicated. The molecular mechanisms governing the distinct regulation of premeiotic DNA replication remain to be elucidated but these will undoubtedly be geared to accomplishing the specific developmental aims of meiotic differentiation, namely the production of haploid gametes.

9. References

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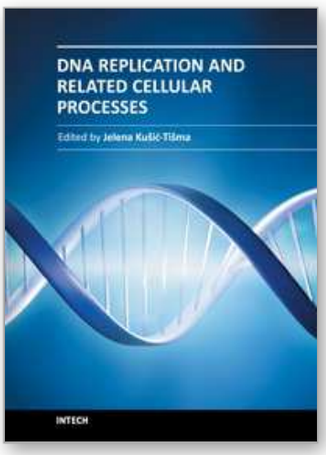

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Since the discovery of the DNA structure researchers have been highly interested in the molecular basis of genome inheritance. This book covers a wide range of aspects and issues related to the field of DNA replication. The association between genome replication, repair and recombination is also addressed, as well as summaries of recent work of the replication cycles of prokaryotic and eukaryotic viruses. The reader will gain an overview of our current understanding of DNA replication and related cellular processes, and useful resources for further reading.

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