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The role of RNAi in epigenetic inheritance and cell cycle control in *S.pombe*

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Abstract of the Dissertation

The role of RNAi in epigenetic inheritance and cell cycle control in *S.pombe*

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Heterochromatin is chromosomal material that remains condensed throughout the cell division cycle and silences genes nearby. It is found in almost all eukaryotes, and although discovered (in plants) almost 100 years ago, the mechanism by which heterochromatin is inherited has remained obscure. Heterochromatic silencing and histone H3 lysine-9 methylation (H3K9me2) depend, paradoxically, on heterochromatic transcription and RNA interference (RNAi). Heterochromatin protein 1 in fission yeast (Swi6) is lost via phosphorylation of H3 serine-10 (H3S10) during mitosis, allowing heterochromatic transcripts to transiently accumulate in S phase. Rapid processing of these transcripts into small interfering RNA promotes restoration of H3K9me2 and Swi6 after replication when cohesin is recruited. RNAi in fission yeast is inhibited at high

temperatures, providing a plausible mechanism for epigenetic phenomena that depend on replication and temperature, such as vernalization in plants and position effect variegation in animals. These results explain how "silent" heterochromatin can be transcribed, and lead to a model for epigenetic inheritance during replication. This study also shows that RNAi is important for cell cycle progression. Specifically, Dcr1 is crucial for maintaining genome integrity by acting along with Cds1 and Chk1 to regulate a DNA damage checkpoint. In addition, lack of Dcr1 triggers a Chk1 dependent DNA damage signaling.

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Chapter I: Introduction

1.1 Summary

In this chapter I present a comprehensive overview of heterochromatin. I begin with briefly discussing the concept of epigenetic modifications and their importance for heterochromatin and its propagation through cell divisions. Next, I introduce RNAi as a mechanism for assembly of heterochromatin and specifically concentrate on the role of RNAi at *Schizosaccharomyces pombe* (*S.pombe*) centromeres. This leads to an introduction of recent discoveries that implicate heterochromatic RNAi in maintenance of heterochromatic marks during the cell cycle. I follow with an overview of the correlation between RNAi-dependent silencing and temperature, which has been observed in many organisms. The subsequent sections focus on the broad spectrum of phenomena that are regulated by RNAi. I review the importance of RNAi in regulation of cell cycle, X chromosome inactivation, variegation and transposons. I then briefly introduce each individual chapter of my dissertation and state the rationale behind the work. Sections 1.2-1.10 were published as a review in Trends in Genetics (Kloc and Martienssen 2008).

1.2 Chromatin and its functional properties

Chromosomal material was first designated as euchromatic or heterochromatic by Emil Heitz, working with moss in the 1920s. He showed that heterochromatin remains condensed throughout cell cycle. Indeed, propagation of heterochromatin at the

replication forks presents a challenge during DNA replication. Yet, heterochromatin along with its modifications is restored in the daughter cells, which indicates that a cell has a "memory" of its transcriptional state. For many decades after its initial characterization, heterochromatin was considered to be transcriptionally inert, but newer work indicates that this highly condensed chromosomal material is transcribed, and rapidly silenced, by an orchestrated sequence of events directed by RNA interference (RNAi). Recent studies shed light on the timely assembly and inheritance of heterochromatin within a short period during the cell cycle, thereby providing an explanation for how "silent" heterochromatin can be transcribed during the S phase of the cell cycle.

The unit of chromatin, the nucleosome, comprises a 146 base pair stretch of DNA wrapped around a histone octamer, and is linked to other nucleosomes by 30-40bp DNA and the linker histone H1. Silencing is mediated by covalent modifications of DNA, by the presence of non-histone proteins or by covalent modifications of N-terminal histone tails, which include acetylation, methylation, phosphorylation, sumoylation, ubiquitination and ADP-ribosylation (Jenuwein and Allis 2001). Certain histone modifications are associated with either transcriptional activity or repression. For example, H3K9 methylation is a hallmark of gene silencing, whereas H3K4 methylation is usually associated with transcriptionally active states. Two types of chromatin, heterochromatin and euchromatin, can be distinguished based on its transcriptional properties. Heterochromatin remains condensed during interphase, contrary to euchromatin, which decondenses on exit from mitosis. Heterochromatin can be further classified as facultative or constitutive. Constitutive heterochromatin is associated with

centromeres, telomeres and the silent mating type locus (*mat2/3*) in the fission yeast *S.pombe*, and it was classically considered to be permanently silenced. Facultative heterochromatin, on the other hand, is active in a subset or population of cells; for example, X-chromosome inactivation results in mosaics in which one of the two X chromosomes is active in female mammals. Genome sequencing has revealed that repetitive DNA sequences and low numbers of genes are common features of heterochromatic regions (Smith, Shu et al. 2007). Although heterochromatin is usually gene-poor, examples of genes within heterochromatic regions rich in histone 3 lysine 9 dimethylation (H3K9me2) have been observed in *D.melanogaster* (Yasuhara and Wakimoto 2008).

1.3 *S.pombe* as a model system to study RNA interference and cell cycle

Because heterochromatic regions are highly condensed, and so perhaps less accessible to transcription factors, heterochromatin traditionally has been considered to be transcriptionally inactive. This view has been challenged by studies in *S.pombe*, which revealed that heterochromatin is transcribed and almost immediately silenced by RNAi (Volpe, Kidner et al. 2002).

S.pombe has been traditionally used for studying cell cycle; specifically questions pertaining to progression of cell cycle stages and checkpoints. Fission yeast divides every 2,5 hours, which allows detailed analysis. Fission yeast has recently turned out to be an excellent model system to study RNAi. This single-cell eukaryote has one copy of each of the major genes responsible for RNAi. Heterochromatin is located at three distinct

loci- centromere, mating type locus and telomeres. RNAi guides silencing at the centromere. Combined, *S.pombe* is a great model system to look at timely establishment of heterochromatin during the cell cycle, as well as the role of RNAi in cell cycle regulated processes.

The centromeres of *S.pombe* comprise inverted repeats and are structurally similar to higher vertebrate centromeres (Clarke, Amstutz et al. 1986). The central kinetochore binding site is flanked by innermost repeats, *imr*, which in turn are surrounded by outermost pericentric repeats, *otr*, comprising the heterochromatic *dg* and *dh* sequences (Fig 2-1). RNAi is required for assembly and spreading of heterochromatin at the pericentric *dg* and *dh* repeats. Loss of RNAi results in decreased H3K9me2 at pericentric repeats, but not at the silent mating type loci (*mat2* and *mat3*) (Hall, Shankaranarayana et al. 2002), even though the intervening K region contains a related *CenH* repeat, which itself responds to RNAi. This is because alternative pathways silence *mat2* and *mat3.*

1.4 RNAi in *S.pombe*

Formation of silent heterochromatin at the outer repeats of the centromere is thought to begin when a reverse strand transcript of either *dg* or *dh* is generated by RNA Polymerase II (PolII), and is blocked by mutants in 3 out of its 12 subunits- Rpb1, Rpb2 and Rpb7 (Zaratiegui, Irvine et al. 2007). These transcripts are then processed by RNAi (Fig. 1-2). The presence of a single copy of RNAi proteins makes fission yeast a convenient system to study the RNAi pathway. Argonaute slices the transcript (Irvine, Zaratiegui et al. 2006), which is thought to create entry sites for the RDRC (RNA

dependent RNA polymerase (Rdp) complex), which also contains the polyA polymerase Cid12 and the helicase Hrr1. RDRC generates double stranded RNA, which is simultaneously processed into 21-24 nucleotide small interfering RNAs (siRNAs) by Dicer (Colmenares, Buker et al. 2007). Double stranded siRNAs are first loaded onto the ARC complex, containing Ago1, Arb1 and Arb2 (Buker, Iida et al. 2007). Arb1 and Arb2 presumably convert double stranded siRNAs into single stranded siRNAs (Buker, Iida et al. 2007), which are then bound by the Ago1 PAZ domain, and incorporated into the RITS complex, consisting of Tas3, Chp1 and Ago1 (Verdel, Jia et al. 2004). This interaction is thought to target nascent transcripts for degradation and to recruit RDRC (Verdel and Moazed 2005; Buhler, Verdel et al. 2006).

Through a yet undefined mechanism, the Rik1 complex is recruited during this process. This complex contains the histone H3K9 methyltransferase Clr4 and several other proteins (Huarte, Lan et al. 2007). Swi6, the Heterochromatin Protein-1 (HP-1) homolog, is a chromodomain protein that recognizes dimethylated and trimethylated H3K9 (Yamada, Fischle et al. 2005) (H3K9me2 and H3K9me3, respectively) and silences heterochromatin. Swi6 directly interacts with, and retains cohesin at pericentric repeats (Nonaka, Kitajima et al. 2002), which is crucial for proper alignment of sister chromatids with the mitotic spindle before chromosome segregation. Loss of RNAi leads to partial loss of H3K9me2 from the repeats (Volpe, Kidner et al. 2002), and complete loss from transgenes integrated into them, implicating RNAi in spreading of heterochromatin into the reporter genes (Volpe, Kidner et al. 2002; Sadaie, Iida et al. 2004; Irvine, Zaratiegui et al. 2006). Additional chromatin modification pathways are thought to be responsible for RNAi-independent histone modifications (Shi, Lan et al.

2004; Yamada, Fischle et al. 2005; Huarte, Lan et al. 2007; Lan, Zaratiegui et al. 2007). Clr3, a homolog of mammalian class II histone deacetylase, directs RNAi-independent assembly of pericentric heterochromatin by somehow recruiting Clr4 (Yamada, Fischle et al. 2005).

RNAi also silences endogenous and transgenic heterochromatic sequences in *Caenorhabditis elegans* (Grishok, Sinskey et al. 2005), in the mouse (Girard and Hannon 2008) , in plants (Waterhouse, Graham et al. 1998; Baulcombe 2004) , and in *Drosophila melanogaster* (Pal-Bhadra, Leibovitch et al. 2004) in which PIWI and HP-1 interact reminiscent of the RITS complex in *S.pombe* (Brower-Toland, Findley et al. 2007). The role of heterochromatin and RNAi is not limited to silencing centromeric repeats of *S.pombe*. RNAi components influence cell cycle, checkpoints, and transposons. Heterochromatic structure plays a role in X chromosome inactivation and imprinting.

1.5 Remembering epigenetic marks: cell cycle dependence of RNAi-induced silencing

The involvement of heterochromatin transcription at the pericentric repeats in the propagation of epigenetic states is paradoxical as heterochromatin must be transcribed to remain silent. Although the role of RNAi in heterochromatin assembly at the *S.pombe* centromere is now widely recognized, recent studies indicate that RNAi also participates in the inheritance of histone modifications through cell division, thus accounting for this paradox. Chromosome replication begins in the S phase of the cell cycle and as the replication fork proceeds, modified histones are distributed between daughter chromatids,

while new, not yet fully modified, histones are incorporated alongside (Loyola and Almouzni 2007). Thus, daughter chromatid assembly into fully heterochromatic nucleosomes requires the proper replacement of epigenetic marks during or after histone deposition (Loyola and Almouzni 2007). Cells must "remember" where to appropriately place these marks, via a process sometimes referred to as epigenetic memory.

Recent studies in *S.pombe* have revealed an intriguing mechanism that explains the timely assembly of heterochromatin during the cell cycle and facilitates the propagation of epigenetic marks through cell division (Chen, Zhang et al. 2008; Kloc, Zaratiegui et al. 2008). Heterochromatin in other organisms, and at *S. pombe* telomeres, replicates in late S phase, but the *S.pombe* centromeric repeats are replicated in early S phase (Kim and Huberman 2001), when transcription of *dh* and *dg* repeats begins. RNAi occurs in S phase, after replication of heterochromatic DNA. PolII is recruited to centromeric repeats in S phase (Chen, Zhang et al. 2008) and *dg* and *dh* transcripts appear (Kloc, Zaratiegui et al. 2008); the forward strand transcript of both repeats peaks first and the reverse strand transcript is expressed shortly thereafter. *dg* transcripts appear minutes before *dh* transcripts, perhaps reflecting their proximity to origins of replication (Kloc, Zaratiegui et al. 2008). siRNAs peak at the time when *dg* and *dh* transcripts are expressed, indicating immediate processing of the double stranded transcripts by Dicer (Kloc, Zaratiegui et al. 2008). H3K9me2 levels are lowest during early S phase of the cell cycle, reflecting the distribution of modified nucleosomes onto each daughter chromatid. H3K9me2 levels increase in late S phase and reach their peak in G2.

Heterochromatin silencing is dependent on the "phospho-methyl switch": histone H3 serine-10 (H3S10) phosphorylation prevents Swi6– H3K9me2 binding (Fischle,

Wang et al. 2003; Yamada, Fischle et al. 2005). H3S10 phosphorylation levels increase at the onset of mitosis, and during S phase, leading to decreased Swi6–H3K9me2 binding (Chen, Zhang et al. 2008; Kloc, Zaratiegui et al. 2008), thereby allowing heterochromatin transcription (Volpe, Kidner et al. 2002). Indeed, H3K4me2, a mark of transcription, is associated with *dh* repeats when Swi6 is lost (Volpe, Kidner et al. 2002; Kloc, Zaratiegui et al. 2008). Swi6 is very briefly associated with heterochromatin at the G1/S boundary, accompanied by a decrease of H3K4me2, but Swi6 is only fully recruited back to H3K9me2 when H3S10 phosphorylation is lost in early G2 (Kloc, Zaratiegui et al. 2008), when Swi6 facilitates retention of cohesin (Nonaka, Kitajima et al. 2002). Therefore, heterochromatin formation is a dynamic process that occurs each time a cell enters S phase and results in heterochromatin reassembly and the reestablishment of epigenetic marks.

Recent studies have also shed light on cohesin recruitment to euchromatin. Convergent genes generate overlapping readthrough transcripts in G1 (Gullerova and Proudfoot 2008). These transcripts form double stranded RNAs and give rise to a transient heterochromatic structure. H3K9me2 recruits Swi6 and cohesin to these sites in G1, preceding replication. Cohesin remains bound and prevents the formation of readthrough transcripts in G2 (Gullerova and Proudfoot 2008). The loss of cohesin at the onset of mitosis promotes the regeneration of readthrough transcripts in the subsequent G1.

RNAi is required to guide CENP-A deposition to newly introduced centromeric DNA, and cohesin might also be involved (Folco, Pidoux et al. 2008). Interestingly, CENP-A chromatin and kinetochore proteins can be reassembled at existing centromeres

in the absence of heterochromatin and RNAi (Folco, Pidoux et al. 2008), so that progression through S phase might be sufficient to create an epigenetic memory of its assembly.

Dicer, in addition to its role in heterochromatin silencing, has been implicated in other epigenetic phenomena. Recently, a connection between X chromosome inactivation (XCI) and RNAi has emerged (Ogawa, Sun et al. 2008), with Dicer being involved in "xiRNA" generation. In addition, maintenance of DNA hypermethylation in human cancer cells is thought to require Dicer (Ting, Suzuki et al. 2008). However, Dicer has also been implicated in regulating de novo DNA methylation (Sinkkonen, Hugenschmidt et al. 2008) and telomere length (Benetti, Gonzalo et al. 2008) indirectly in mouse ES cells, through the microRNA cluster miR-290. Dicer knockout cells lack miR-290, which leads to elevated target messages including retinoblastoma-like 2 protein (Rbl2) which represses the expression of DNA methyltransferases (Dnmt) (Benetti, Gonzalo et al. 2008; Sinkkonen, Hugenschmidt et al. 2008). Thus caution needs to be applied in concluding the mechanism for Dicer requirement in these cases.

1.6 Heterochromatin and X chromosome inactivation

In the 1960s Mary Lyon suggested that one copy of the X chromosome, coming from either parent, is silenced in each cell (Lyon 2002), in order to explain variegated expression observed in female mice heterozygous for coat color genes. Indeed, to ensure balance in the amount of transcript between mammalian female (XX) and male (XY) cells, one X chromosome in a female cell becomes inactive (Xi) in process referred to as

X chromosome inactivation (XCI). Further research showed that Xi becomes silenced by packing it into a heterochromatic structure. The "decision" to inactivate one of the X chromosomes involves X-X chromosome pairing (Xu, Tsai et al. 2006), by which the inactivating centers of the two X chromosomes, known to mediate inactivation, physically interact at the onset of XCI (Augui, Filion et al. 2007). Inactivation of a single X chromosome in a cell is regulated by the antiparallel non-coding transcripts Xist and Tsix, and deletion of either of these two genes results in lack of XCI or else in inactivation of both Xs (Xu, Tsai et al. 2006). In mouse embryonic fibroblasts (MEFs), the inactive X chromosome (Xi) localizes to perinucleolar sites in mid and late S phase (Zhang, Huynh et al. 2007), a process that requires Xist. Therefore, it is thought that this cell cycle specific event maintains silencing of the Xi. The many similarities between Xist mediated silencing and RNAi have led to speculation that RNAi might be involved in XCI, and recently a connection has been reported (Ogawa, Sun et al. 2008). In mouse ES cells (mESC) Xist and Tsix form duplexes, which are turned into 25-40 nt "xiRNAs", perhaps by Dicer, as diminished xiRNA levels were observed in its absence (Ogawa, Sun et al. 2008). Interestingly, xiRNAs are absent from mature MEFs and mESCs when XCI has already occurred. Dicer can also influence silencing indirectly via miRNA (Benetti, Gonzalo et al. 2008; Sinkkonen, Hugenschmidt et al. 2008), and the role of miRNA in XCI, if any, has not yet been explored.

1.7 RNAi, heterochromatin and cell cycle

RNAi guided heterochromatin affects not only silencing, but also the cell cycle, differentiation, development and stability of repeated DNA sequences. In fission yeast, components of the RNAi pathway influence cell cycle checkpoints and progression: *ago1*$^{-}$ and *dcr1*$^{-}$ mutants undergo altered heterochromatic modifications, which leads to chromosome segregation defects during mitosis, a high number of lagging chromosomes during anaphase (Volpe, Schramke et al. 2003), delayed cytokinesis and mating defects (Carmichael, Provost et al. 2004). Both fission yeast Ago1 and human Ago2 N-termini bind the conserved family of 14-3-3 proteins, which are important for cell cycle regulation (Stoica, Carmichael et al. 2006) and participate in numerous cellular signaling cascades. The S-phase replication checkpoint and S-M cell cycle checkpoints are affected in *ago1*$^{-}$ and *dcr1*$^{-}$ mutants (Carmichael, Provost et al. 2004), delaying progression. Both *dcr1*$^{+}$ and *ago1*$^{+}$ are required for Cdc2 hyperphosphorylation, and hence for the replication checkpoint (Carmichael, Provost et al. 2004). Ago1 overexpression prevents Cdc25 nuclear localization (Stoica, Carmichael et al. 2006) by decreasing the ability of Cdc2 to phosphorylate Cdc25. RNA-dependent RNA polymerase, Rdp1, does not seem to play a role in these cell cycle events although it is indispensable for RNAi in *S.pombe*. Interestingly, *D. melanogaster* Ago2, a homolog of *S.pombe* Ago1, is thought to affect the mitotic spindle, which leads to chromosome displacement (Deshpande, Calhoun et al. 2005). Dicer knockouts result in cell death and premature chromosome segregation in vertebrate cells (Fukagawa, Nogami et al. 2004).

Histone modifications also play a role in ensuring proper cell cycle progression. The citron kinase protein, encoded by *sticky*, is conserved from invertebrates to humans and mediates cell cycle progression (Sweeney, Campbell et al. 2008). Recent work showed that citron kinase is required for H3K9 methylation, heterochromatin silencing and proper HP1 localization (Sweeney, Campbell et al. 2008). On the other hand, Clr6–HDAC dependent H4 deacetylation is thought to regulate Cds1 kinase, which participates in DNA checkpoint control and mitosis induction (Kunoh, Habu et al. 2008). Once Cds1 kinase is activated in a *clr6*$^{-}$, it cannot be inactivated presumably because H4-deacetylation by Clr6 might be required to remove Cds1 from chromatin.

RNAi also influences meiotic progression. Ego1, a *C.elegans* RNA-Dependent RNA Polymerase, is required for H3K9me2 deposition on unpaired chromosomes during meiosis (Maine, Hauth et al. 2005). This process is independent of Dcr1, and therefore differs from pericentric heterochromatin assembly in *S.pombe*. RNAi mediates meiotic silencing in *Neurospora crassa*. Sad-1 (Shiu, Raju et al. 2001) (*S.pombe* Rdp1 homolog), Sms-2 (Lee, Pratt et al. 2003) (Argonaute-like) and Sms-3 and DCL-1 (Alexander, Raju et al. 2008) (Dicer-like) are involved in silencing the expression of unpaired DNA during meiosis, a process known as Meiotic Silencing by Unpaired DNA (MSUD) (Kelly and Aramayo 2007). *N.crassa* RNAi components are also required for fertility, suggesting a role in sexual development (Kelly and Aramayo 2007). The RNAi pathway has been suggested to be involved in MSUD in the mouse: the MAEL protein associates with unsynapsed chromosomes and interacts with the Argonaute related proteins, MILI and MIWI, in the germ cell specific structure, characterized by accumulation of dense fibrous material, known as the nuage (Costa, Speed et al. 2006).

RNAi and H3K9me2 have been involved in stabilization of repeated DNA sequences (Peng and Karpen 2008). Recent work in *D. melanogaster* has revealed the importance of many RNAi pathway components and the *Su(var)3-9* H3K9 methyltransferase in rDNA and nucleolar organization. Mutants in *Su(var)3-9* and *HP1/Su(var)2-5* disrupt both the number and volume of nucleoli; they also have dispersed rDNA foci (Peng and Karpen 2007). *Su(var)3-9* mutants have decreased H3K9me2 levels, and this is thought to promote extrachromosomal circular DNA (ecc) formation (Peng and Karpen 2007). DNA Ligase 4, which participates in the non-homologous end joining (NHEJ) pathway, partially suppresses the *Su(var)3-9* multiple nucleolus phenotype. This finding suggests a role for DNA repair in ecc DNA formation (Peng and Karpen 2007).

1.8 RNAi is temperature sensitive

RNAi, in addition to its role in heterochromatin assembly and silencing, is also a conserved eukaryotic mechanism for host defense against viruses and parasites (Buchon and Vaury 2006). RNAi-mediated host protection is temperature sensitive in plants. For example, viral infection-induced siRNA production in *Nicotiana benthamiana* protoplasts is temperature dependent (Szittya, Silhavy et al. 2003). Virus-derived siRNAs are prevalent at 27°C, but almost completely absent at 15°C (Szittya, Silhavy et al. 2003), suggesting that low temperatures inhibit RNA silencing. Indeed, plants tend to recover from viral infection at higher temperatures, a phenomenon known as “heat masking”. In both *Arabidopsis thaliana* and potato, accumulation of transgene-derived siRNAs

influenced by temperature (Szittya, Silhavy et al. 2003). The amounts of siRNA are low at 15°C and high at 24°C. Interestingly, the accumulation of miRNAs, which regulate many developmental process in *A.thaliana*, is not temperature dependent (Szittya, Silhavy et al. 2003). This may be because siRNAs in *A.thaliana* are processed by DCL III (DICER-LIKE RNAse III enzymes) while miRNAs arise via DCL I (Ramachandran and Chen 2008), and different DICER-LIKE proteins might have different susceptibility to temperature. Because miRNAs have important developmental functions it might be evolutionary advantageous to maintain miRNA levels at various temperatures.

Epigenetic phenomena control flowering time in *Arabidopsis.* In a process referred to as vernalization, long exposure to cold periods during winter is required to induce flowering in spring. FLOWERING LOCUS C (FLC), which prevents flowering, is epigenetically repressed during cold periods. Silencing depends on both RNAi and H3K9 methylation. FLC repression is associated with stable chromatin changes that include histone modifications characteristic of inactive chromatin structure, such as H3K9me2 and H3K27me2 (Bastow, Mylne et al. 2004; Sung and Amasino 2004; Sung and Amasino 2005). The maintenance of repressed *FLC* is accomplished by H3K27me3. This process is thought to require DNA replication (Finnegan and Dennis 2007), as mature mitotically inactive *A. thaliana* cells cannot maintain H3K27me3 at *FLC* after vernalization (Finnegan and Dennis 2007).

Temperature also influences RNAi-dependent centromere silencing in *S.pombe.* RNAi is inhibited at high temperatures (Allshire, Javerzat et al. 1994; Kloc, Zaratiegui et al. 2008); therefore, pericentric *dg* and *dh* transcripts in a wild type fission yeast population are elevated at high temperatures, whereas the amount of siRNAs decreases

(Kloc, Zaratiegui et al. 2008). Conversely, the transcript levels decrease at lower temperatures and siRNAs increase. In *cdc25-22* mutant cells, which arrest in G2 when shifted to 36°C, siRNA amounts are reduced and only increase gradually after releasing the culture to 26°C, the permissive temperature (Kloc, Zaratiegui et al. 2008). In contrast the *nda3-KM311* mutant strain is cold sensitive and arrests in mitosis at 20°C. A culture shifted to 20°C has elevated levels of siRNAs that decrease gradually once the cells are incubated at 33°C, the permissive temperature. Thus in *S.pombe* silencing of transgenes inserted into heterochromatic repeats is alleviated as temperature increases (Allshire, Javerzat et al. 1994). In *Drosophila*, heterochromatic silencing of transgenes also decreases as temperature is raised from 18°C to 29°C (Lloyd, Dyment et al. 2003), as does post-transcriptional gene silencing mediated by inverted repeats (Fortier and Belote 2000).

It is interesting that sRNA guided silencing in *A.thaliana* is enhanced at high temperatures, whereas in *S.pombe* and *D.melanogaster* silencing is most effective at lower temperatures. These differences might reflect the rapid evolution of viral resistance in plants; however, it should be noted that *A.thaliana* was not tested at temperatures higher than 24°C, while *S.pombe* cells were not grown below 20°C, when sRNAs in plants are still abundant. Perhaps, then, sRNA guided silencing is alleviated at both high and low temperatures. One idea is that low and high temperatures might result in aberrant pairing of sRNAs, which could alter the efficiency of silencing. Thus, temperature sensitivity of RNAi silencing might reflect the temperature that is optimal for a given organism.

1.9 Variegation and the cell cycle.

When placed in the vicinity of heterochromatin, eukaryotic genes tend to be partially silenced, displaying a variegated phenotype. This phenomenon is known as position effect variegation (PEV) in *D. melanogaster* when chromosomal rearrangements result in the juxtaposition of euchromatic genes with heterochromatin (Henikoff 1990; Henikoff 2000) (Fig. 1-1). Examples of PEV can also be found in *S. pombe* and mammals. Transcriptional repression owing to placement near heterochromatin usually affects only a subset of cells, resulting in mosaic expression (Schotta, Ebert et al. 2003). This repression can be stably transmitted through mitotic divisions. In *S.pombe,* fusion of a heterochromatin promoting repeat to a reporter gene leads to silencing. For example the *cenH* region found at the silent mating type locus can silence a linked *ade6+* reporter gene leading to a variegated phenotype (Ayoub, Goldshmidt et al. 2000). The silent state can be visualized by red colonies, whereas white color corresponds to loss of silencing. Silencing depends on RNAi, and occurs in variegated or sectored colonies indicating it may be lost during cell divisions (Fig. 1-1).

Improper expression of siRNAs during S phase or failure to assemble heterochromatin during DNA replication could lead to variegated phenotypes. The dependence of siRNAs on S phase has implications for other non-coding RNAs (ncRNAs) which regulate other variegated epigenetic phenomena, such as XCI, paramutation, and transposon silencing (Zaratiegui, Irvine et al. 2007; Costa 2008). Recently, a correlation between RNAi and XCI has been found, and localization of the inactive X chromosome (Xi) within the nucleus is thought to be S phase specific (Zhang,

Huynh et al. 2007), which suggests that this stage of the cell cycle may ensure the epigenetic memory of the inactive state.

1.10 Transposon regulation and RNAi

Transposable elements (TE) are involved in many epigenetic phenomena, including PEV (Schotta, Ebert et al. 2003), XCI (Cohen, Davidow et al. 2007) and imprinting (Youngson, Kocialkowski et al. 2005). Although TEs play an important role in evolution and speciation, active transposition can result in mutations and in misexpression if regulatory sequences are disrupted. As a result, most TEs are silenced, and RNAi is often involved in this process (Slotkin and Martienssen 2007). Transposon regulation also depends on the cell cycle (Mc 1951) and on temperature; for example, in classical studies, *Activator (Ac)* element transposition in maize occurs just after DNA replication in S phase (Chen, Greenblatt et al. 1992).

Argonaute protein and bound siRNA are involved in regulation of retrotransposons (DNA sequences generated by reverse RNA transcription) in *Trypanosoma brucei*. *Ingi* and RIME retroposons, often inserted into *RHS* pseudogenes are the most prevalent families of transposable elements in *T.brucei* (Bringaud, Biteau et al. 2002; Bringaud, Biteau et al. 2004). siRNA-like molecules were detected from both *ingi* and SLACS (Djikeng, Shi et al. 2001) and the *T.brucei* Argonaute protein, *TbAGO1*, is involved in regulation of *ingi*, RIME and the *RHS* pseudogenes (Durand-Dubief, Absalon et al. 2007). The amounts of sense and antisense transcripts from both *ingi* and *RHS* increased in the absence of *TbAGO1* as did RIME retroposon transcripts; this defect

could be reversed by the expression of GFP::AGO1 (Durand-Dubief, Absalon et al. 2007). Interestingly, none of the retroposons transposed upon the loss of *TbAGO1*.

The importance of the cell cycle in mobile element activation is further illustrated by LINE-1 (L1) retrotransposons, which constitute a large part of the mammalian genome. In primary human fibroblasts, cell cycle arrest at G0, G1, S, G2 or M phase represses L1 retrotransposition (Shi, Seluanov et al. 2007). A similar effect is observed in HeLa cells arrested in S phase or in the G2/M transition (Shi, Seluanov et al. 2007), suggesting that L1 retrotransposons require cell division for activity. Accordingly, L1 retrotransposition is abolished in cells undergoing replicative senescence and L1 transcript levels are decreased in non-dividing cells (Shi, Seluanov et al. 2007). Interestingly, the majority of somatic cells are arrested at G0 or at the G0/G1 transition,which could be a form of defense against retrotransposition induced genome instability. In summary, these results emphasize the importance of S phase, and in particular, chromosomal replication, for transposition.

1.11 Summary of dissertation

During my studies I focused on three topics: RNAi and epigenetic inheritance, RNAi and cell cycle and RNAi and propagation of variegation. When I began my work, RNAi of *S.pombe* had been already well established as a model system for heterochromatin assembly at the centromere. Therefore, I initially concentrated on how epigenetic modifications associated with heterochromatin are propagated through cell divisions. I also explored a potential involvement of RNAi in non-random segregation.

Towards the end of my work, my interests focused on involvement of RNAi in cell cycle regulation.

Chapter II represents a detailed study of epigenetic inheritance based on RNAi-dependent heterochromatin assembly in *S.pombe*. Inheritance of epigenetic modifications has been an unsolved mystery for some time. I initially started looking at cell cycle regulation of RNAi to determine if epigenetic marks associated with heterochromatin were steady or "active" with respect to the cell cycle. This work was particularly suitable in *S.pombe* because of its rapid division and well characterized cell cycle. It was possible to achieve good synchronization and examine accumulation of RNAi-dependent centromeric transcripts, small interfering RNAs and histone modifications associated with silencing. I found that epigenetic modifications and RNAi itself are cell cycle regulated, which lead to a model of epigenetic inheritance during S phase and revealed that the process is dependent on the phospho-methyl switch. I briefly explored the influence of RNAi on temperature, as it was crucial for cell cycle analysis methodology. This work was published in Current Biology (Kloc, Zaratiegui et al. 2008).

In Chapter III, I discuss the potential involvement of RNAi in cell cycle control of *S.pombe*. My interest in this topic was originally sparked by the discovery of cell cycle regulation of RNAi-dependent heterochromatin assembly (discussed in detail in Chapter II). The occurrence of RNAi in S phase of the cell cycle could imply involvement in processes characteristic of this cell cycle stage. Using a *cdc25-22* temperature sensitive mutant, I showed that Dcr1 is involved in genome integrity during S phase of the cell cycle, presumably through regulating replication fork stability. I also showed that Dcr1 is involved in Cdc25-dependent DNA damage signaling. Although the exact mechanism of

this phenomenon is still to be explored in detail, my genetic analysis reveals that RNAi participates in cell cycle regulation and genome stability.

In Chapter IV I explore the instability of RNAi induced silencing, manifested by variegation using a series of lineage analysis experiments. I asked whether tethering of strand specific transcripts could be a mechanism for variegation and preferential segregation of DNA templates. I used two constructs with *ade6+* reporter gene to assess if loss of silencing was dependent on lineage, and then performed a statistical analysis of loss of silencing in mother and daughter cells. This work has implications for the Immortal Strand Hypothesis, which has been one of the most controversial topics in stem cell biology. The analysis of multiple lineages showed that loss of silencing could be dependent in part on inheritance of a specific DNA strand by a daughter cell.

Figure 1-1

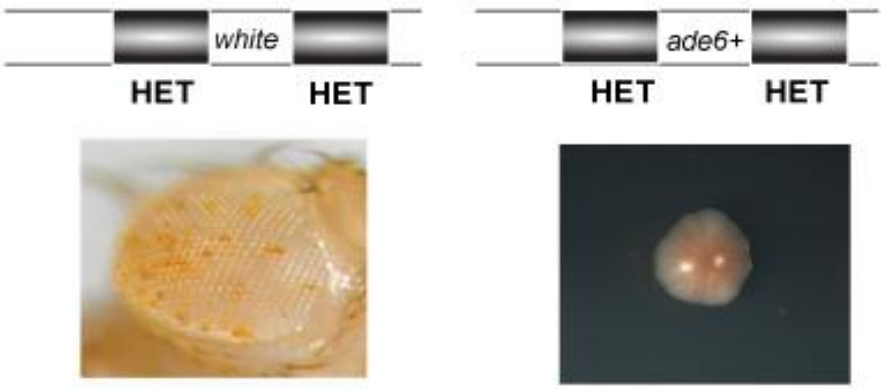


Fig. 1-1 Position Effect Variegation
A. Juxtaposition of a gene with a heterochromatic domain (HET) leads to variegated phenotypes in many organisms, and is known as position effect variegation (PEV). *D. melanogaster* eye shows variegated expression of a *white* transgene inserted in pericentric heterochromatin. If *white* is properly transcribed, the eye is red [reproduced with permission from Reference (Pal-Bhadra, Leibovitch et al. 2004)]
B. Variegated expression of the *ade6+* reporter gene in fission yeast. A part of the K region from the silent mating type region, *cenH*, which contains heterochromatic *dg* and *dh* repeats, was fused with the *ade6+* reporter gene and inserted at the *ura4* locus (Ayoub, Goldshmidt et al. 2000). This resulted in variegated expression, which was dependent on RNAi (Hall, Shankaranarayana et al. 2002).

Figure 1-2

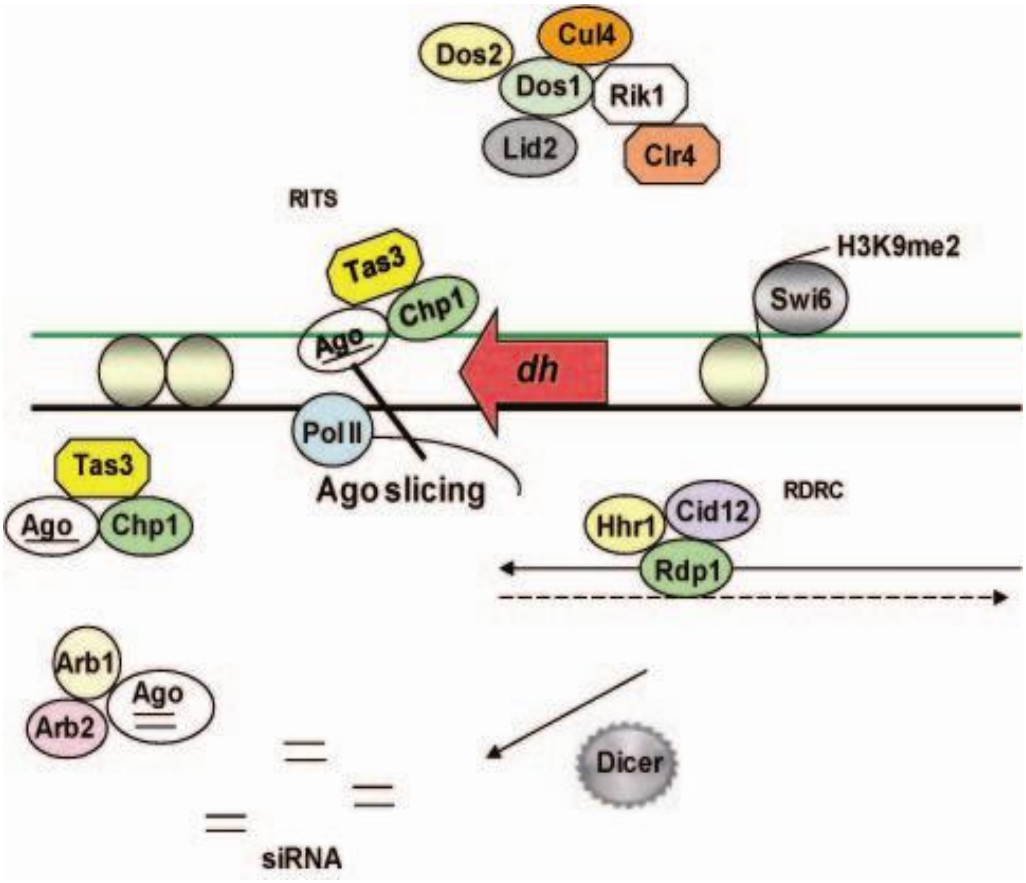


Fig. 1-2 RNAi induced silencing of *dh* pericentric transcripts
Reverse strand *dh* transcript generated by Pol II is sliced by Ago1 to create entry points for RDRC complex. A double stranded transcript is generated by RDRC and processed by Dcr1 into siRNAs. These are first incorporated into Arb1/Arb2/Ago complex and then in RITS complex, where they are bound by Ago1. Rik1 complex is recruited and H3K9 is methylated by Clr4. Swi6 binds directly to H3K9me2, which results in recruitment of cohesin.

Chapter II: RNA interference guides histone modification during the S phase of chromosomal replication

2.1 Introduction

Epigenetic changes are heritable changes of either DNA or histones modifications that are transmittable through mitosis and meiosis but do not alter the DNA sequence. Unlike DNA sequence changes, epigenetic marks are potentially reversible at high frequency. Propagation of these modifications is challenged during S phase when replication forks pass through DNA. Different mechanisms have evolved to ensure inheritance of epigenetic marks.

In plants, mammals and fungi DNA can be methylated on cytosine residues (Gautier, Bunemann et al. 1977; Gruenbaum, Naveh-Many et al. 1981; Saze 2008). In *Arabidopsis,* DNA methyltransferase 1, homologous to mammalian DNMT1, regulates methylation of CpG residues (Ronemus, Galbiati et al. 1996) during DNA replication in S phase (Bostick, Kim et al. 2007; Sharif, Muto et al. 2007). Components of the RNAi machinery are involved in *de novo* methylation, which is directed by DRM1 and DRM2 methyltransferases related to Dnmt3 (Matzke, Kanno et al. 2007). The semiconservative nature of DNA methylation replication ensures that a newly synthesized, unmethylated strand of DNA will attain the same methylation pattern as the "mother" strand, facilitating inheritance throughout generations (Saze 2008). Maintenance of CpG and non-CpG methylation of repetitive sequences requires the chromatin remodeling factor, DDM1 (Vongs, Kakutani et al. 1993).

Contrary to DNA methylation pattern propagation, the mechanism of epigenetic inheritance of histone modifications has not been well understood. Histone based inheritance of epigenetic marks faces challenges after DNA replication, as nucleosomes have to be assembled *de novo* and distributed to two daughter strands (Jackson 1988; Saze 2008). Studies by Jackson *etal* have shown that H3 and H4 are deposited first on new DNA strands and H2A and H2B are deposited afterwards (Jackson 1988; Groth, Rocha et al. 2007). Currently, there are two models that explain how histone modification patterns are passed on to the next generation. A "conservative" nucleosome replication model proposes random distribution of parental histone octamers to DNA strands (Jackson, Granner et al. 1975). The epigenetic information associated with parental histone octamers has to be copied to newly assembled nucleosomes on the other DNA strand. A "semi-conservative" model of epigenetic inheritance of histone modifications proposes a non-random distribution of histones that is directly tied to DNA replication. According to this model, two H3/H4 dimers, which come from $(H3/H4)_2$ tetramers, are each distributed to one DNA strand and guide reassembly of nucleosome structure and reestablishment of "old" epigenetic marks (Tagami, Ray-Gallet et al. 2004). Specifically, histone variant H3.3 exist as a dimeric H3.3/H4 and H3.1/H4 unit and a histone chaperone complex split the $(H3/H4)_2$ tetramer (Tagami, Ray-Gallet et al. 2004). However, the exact mode of nucleosome assembly after DNA replication is yet to be determined.

Many epigenetic phenomena, such as gene and transposon silencing, X chromosome inactivation (XCI) and imprinting, to name but a few, are dependent on proper heterochromatin formation. Because of its broad applications for gene silencing,

reestablishment of epigenetic modifications associated with heterochromatic modifications has been an area of active research. Compactness and limited accessibility of either facultative or constitute heterochromatin adds an additional level of complexity to inheritance of epigenetic marks. However, heterochromatin, along with its modifications, is restored each time a cell divides. A newly formed daughter cell has the same pattern of epigenetic marks, which indicates that a cell has a "memory" of its transcriptional state. RNA interference has been shown to regulate assembly and spreading of heterochromatin at the pericentric *dg* and *dh* repeats in *S. pombe* (Volpe, Kidner et al. 2002; Sadaie, Iida et al. 2004; Irvine, Zaratiegui et al. 2006) and lately, the role of RNAi has been potentially extended to XCI (Ogawa, Sun et al. 2008). In this chapter I show that assembly of heterochromatin and reestablishment of epigenetic modifications occurs during a specific time during cell cycle and is regulated by the "phospho-methyl switch" (Chen, Zhang et al. 2008; Kloc, Zaratiegui et al. 2008), thereby providing an explanation for how "silent" heterochromatin can be transcribed during this time.

In *S. pombe* heterochromatic centromeric outer repeats are replicated early, just before the beginning of S phase via origins of replication (Smith, Caddle et al. 1995; Kim and Huberman 2001) that lie between the *dh* and *dg* transcription units (Fig. 2-1). In order to measure heterochromatin transcription and modification during the cell cycle I synchronized wild type cells. This can be achieved by first arresting them in the cell cycle, and then releasing to progress through each phase at more or less the same time. Cells can be arrested by using drugs or by using temperature sensitive mutants in cell

cycle progression. I found that RNAi occurs specifically in the S phase of the cell cycle, but is inhibited at high temperatures such as those used to arrest cell cycle mutants.

2.2 Results

2.2.1 RNAi occurs in S phase

I synchronized cells by incubation in hydroxyurea (HU), which stalls replication forks, preventing cells from completing DNA synthesis in S phase. Cells were released when septation was complete. Measurement of the septation index and expression of 4 different cell cycle specific genes (Ace2, Hhf2, Psu1 and Cig2) indicated the cells went through one full cycle and one partial cycle of synchronous division (Fig. 2-2). Fractions were collected every 10 minutes and analyzed for expression of strand specific *dh* and *dg* repeats, small interfering RNAs, and histone H3 modification.

Transcripts from both strands of the *dg* and *dh* repeats accumulated predominantly in S phase, with the forward strand transcript appearing first, but at much lower levels than the reverse strand (Volpe, Kidner et al. 2002; Djupedal, Portoso et al. 2005) (Fig. 2-3). The forward transcripts initiate closer to the origins of replication (Fig. 2-1). *dh* and *dg* reverse strand heterochromatic transcripts are processed co-transcriptionally into 21-24 nucleotide long siRNA via RdRP and Dicer (Kato, Goto et al. 2005; Buhler, Verdel et al. 2006; Irvine, Zaratiegui et al. 2006), and I found that these siRNAs accumulate in S phase, detected on Northern blots in the first and second cell cycles (Fig. 2-3). Both forward and reverse strands of siRNAs appear simultaneously.

Origins of replication fire under hydroxyurea arrest (Patel, Arcangioli et al. 2006), but replication forks fail to elongate, so that siRNA accumulation follows origin firing.

2.2.2 Analysis of histone modifications

The levels of modified histones associated with *dh* heterochromatic repeats throughout the cell cycle were investigated by Chromatin Immunoprecipitation (ChIP) (Fig. 2-5). Synchronized WT cells were fixed in formaldehyde and incubated with various antibodies. Quantification was achieved using input DNA from whole cell extract (WCE) and control primers, via quantitative PCR. H3K9me2 levels associated with heterochromatic repeats decreased during the cell cycle, reaching a minimum in early S phase, precisely when the repeats have replicated (Fig. 2-5). This may reflect distribution of parental modified histones on each of the two daughter chromatids immediately after replication (Dodd, Micheelsen et al. 2007). Histone H3 serine-10 (H3S10) phosphorylation peaked in mitosis, as expected (Dormann, Tseng et al. 2006). H3S10ph prevents binding of the heterochromatin protein-1 (HP-1) homolog Swi6 to H3K9me2 via the "phospho-methyl switch" (Yamada, Fischle et al. 2005) (Fig. 2-4 and 2-5). As H3S10ph increased, levels of Swi6 fell, first to a local minimum in M phase, and then to an absolute minimum in S phase. Each minimum corresponded to a peak of H3S10ph, and Swi6 levels only returned to normal in G2, once H3S10ph was lost (Fig. 2-5). Swi6 levels were thus inversely related to H3S10ph, and the septation index.

Swi6 is required after S phase for retention of cohesin in heterochromatin and ensures proper chromosome segregation (Bannister, Zegerman et al. 2001). It is also

required for transcriptional silencing (Volpe, Kidner et al. 2002) so that loss during the cell cycle might allow accumulation of transcripts from *dh* and *dg* heterochromatic repeats. In agreement with this model, forward transcripts accumulated weakly during M phase, and much more strongly during S phase. The more abundant reverse transcripts (Volpe, Kidner et al. 2002) followed the same pattern, and accumulated predominantly in S phase. H3K4me2 is a robust mark of active transcription and was associated with *dh* repeats when Swi6 was lost (Fig. 2-5). This pattern of transcripts accounts for S phase specific accumulation of siRNA (Fig. 2-3), as dsRNA is generated from reverse transcripts by Rdp1 and processed into siRNAs (Volpe, Kidner et al. 2002; Djupedal, Portoso et al. 2005). It also neatly accounts for the paradoxical transcription of heterochromatic repeats, as heterochromatic marks are reduced transiently during replication.

Dcr1^{+} , *rdp1*$^{+}$ and *ago1*$^{+}$ themselves are not cell cycle regulated (Rustici, Mata et al. 2004), although lack of *dcr1*$^{+}$ and *ago1*$^{+}$ has been linked to cytokinesis defects (Carmichael, Provost et al. 2004). siRNA, H3K9me2 and Swi6 are lost or reduced in RNAi mutants, while centromeric transcripts accumulate to high levels (Volpe, Kidner et al. 2002). We performed cell cycle analysis of centromeric transcripts in *dcr1*$^{-}$ mutants (Fig. 2-6). Septation was delayed in *dcr1*$^{-}$ as previously reported (Carmichael, Provost et al. 2004). *dh* forward transcripts were elevated in G2 (Fig. 2-6), consistent with loss of transcriptional silencing, and in agreement with expression levels in *clr4*$^{-}$ cells (Chen, Zhang et al. 2008). However, reverse transcripts still peaked in S phase, at much higher levels than in WT, consistent with loss of post-transcriptional silencing as well (Volpe, Kidner et al. 2002).

2.2.3 Swi6 and the phospho-methyl switch

siRNA accumulation guides modification of H3K9me2 (Volpe, Kidner et al. 2002), which increases steadily during S phase and peaks in G2, closely following the G2 cell cycle marker *psu1*$^{+}$ (Fig. 2-5). One prediction is that mutants in Histone H3S10 phosphorylation should lose H3K9me2 after each round of replication. Substitution of alanine for serine-10 in histone H3 is viable in *S.pombe* and prevents phosphorylation (Mellone, Ball et al. 2003). Consistent with our model, H3K9me2 accumulation is lost in these strains along with centromeric silencing (Mellone, Ball et al. 2003). However, alanine substitution and/or loss of H3K9me2 also reduce binding of Swi6 (Fig. 2-7) (Mellone, Ball et al. 2003). I therefore reduced levels of serine-10 phosphorylation transiently, so as not to lose H3K9me2. Histone H3S10 is phosphorylated by aurora kinase (Ark1), which has many other substrates and is essential for growth. I used an *nmt1::ark1*$^{+}$ "shut off" plasmid to reduce Ark1 levels in an Δ*ark1* deletion strain, which undergoes cell cycle arrest (Petersen and Hagan 2003). H3S10ph levels decreased upon repression of *nmt1::ark1+* (Fig. 2-8) while levels of Swi6 were elevated in arrested cells, without loss or gain of H3K9me2. In contrast, *swi6*$^{-}$ mutants gain H3K9me2 (Yamada, Fischle et al. 2005). Taken together, these genetic experiments strongly support our model.

2.2.4 RNAi is inhibited at elevated temperatures

In order to investigate RNAi during the cell cycle I arrested mutant cells at different points in the cell cycle using temperature sensitive mutants, namely *cdc25-22*

(G2 arrest), *cdc10-129* (G1/S phase arrest) and *nda3-KM311* (M phase arrest). Synchronization was followed using cell cycle marker genes expressed in G2, M and S phase, and was most complete in *cdc25-22*. In a *cdc25-22* strain, small RNA accumulated at low levels at the restrictive temperature (36ºC), and gradually increased through the M, S, and G2 phases reaching a peak at the end of the second S phase (Fig. 2-10). The same pattern could be observed in a *cdc10-129* strain, which can be synchronized at 36ºC. It is obvious that the pattern of siRNA accumulation is not cell cycle regulated, but rather increases gradually after release to the permissive temperature (26ºC). For example, levels of siRNA were much higher from 180-210 minutes than from 0-30 minutes, although both were in G2, the predominant phase in unsynchronized cells. When *nda3-KM311* mutant cells were arrested in mitosis at cold temperature (20ºC), I observed the opposite pattern, with a steady decline through S, G2 and subsequent S phases. We therefore suspected that RNA interference in *S.pombe* is suppressed at high temperatures, but enhanced at low temperatures, confounding any cell cycle regulation. In unsynchronized wild type cells, I found that centromeric transcripts were sharply elevated at 36˚C (Fig. 2-9) consistent with the loss of centromeric silencing at high temperatures previously reported (Allshire, Javerzat et al. 1994). Therefore, alternative means of synchronizing cells were employed to study RNAi.

2.3 Discussion

Heterochromatin is defined by its inheritance from interphase to interphase (Lippman and Martienssen 2004) and represents an intriguing example of propagation of epigenetic material. Our results suggest a mechanism for epigenetic inheritance of

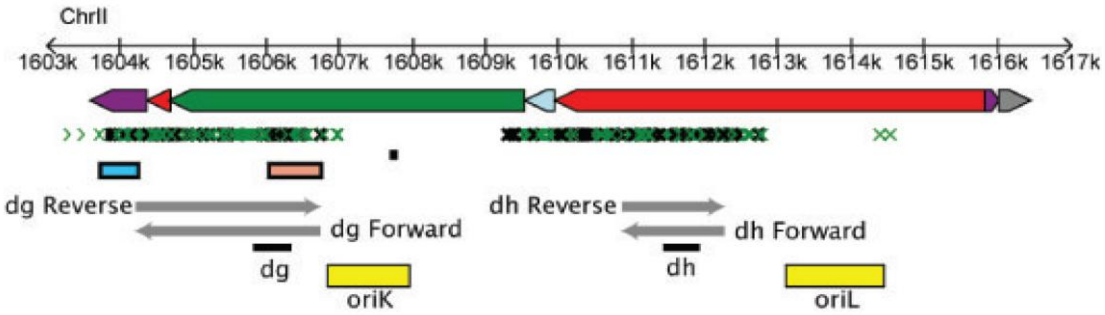
modified histones during replication (Fig. 2-11). Replication results in transmission of genetic information (DNA) into each daughter cell, but nucleosomes are disrupted as the replication fork proceeds (Gruss, Wu et al. 1993), resulting in the loss of epigenetic marks as fresh histones are deposited onto replicated DNA (Santoro and De Lucia 2005). H3S10 phosphorylation during mitosis leads to loss of Swi6 and transcription of heterochromatic repeats. Double stranded RNA is quickly processed into siRNA in S phase leading to restoration of H3K9me2 levels. H3K9me2 may even "spread" across the replication fork from unreplicated to replicated DNA, via transcription of the reverse strand and processing by RNAi (Irvine, Zaratiegui et al. 2006). Thus, we can view epigenetic inheritance as a cyclic event that is based on inheritance of modified histones during replication, guided by RNAi. Interestingly, heterochromatic silencing in budding yeast also requires passage through S phase, though neither DNA replication nor RNAi are thought to be involved (Kirchmaier and Rine 2001). Thus fission and budding yeasts seem to have evolved distinct mechanisms to propagate silent chromatin after replication.

In multi-cellular organisms, if heterochromatic siRNA only arise in S phase, then they would only accumulate in dividing cells or in cells undergoing endoreduplication. Indeed, genes involved in replication are also required for RNAi-mediated transgene silencing in Arabidopsis and in fission yeast (Vaughn, Tanurdzic et al. 2005). S phase specificity has major implications for epigenetic phenomena that depend on RNAi, such as transposon silencing, and for phenomena that depend on non-coding RNA, such as X inactivation and imprinting. Occasional failure to maintain heterochromatic siRNA during replication could contribute to variegated patterns of expression, which are hallmarks of these epigenetic phenomena. Further, paramutation in maize and position

effect variegation in *Drosophila*, exhibit temperature sensitivity and are regulated, at least in part, by RNAi (Chandler 2007; Zaratiegui, Irvine et al. 2007). Our results suggest that siRNA in each case should accumulate in dividing cells. They also provide an attractive explanation for vernalization in Arabidopsis: The process by which *FLOWERING LOCUS C (FLC)* is silenced during long periods of cold, resulting in a cellular memory of winter and flowering the following Spring (Amasino 2005). Permanent silencing only occurs if cold periods are experienced by dividing cells (Finnegan and Dennis 2007) and requires, among other things, histone methylation (Bastow, Mylne et al. 2004) and heterochromatic RNAi (Swiezewski, Crevillen et al. 2007). Thus replication-dependent and temperature-sensitive RNA-mediated silencing may contribute to environmental response, as well as to the epigenetic inheritance of heterochromatin.

Figure 2-1

A.



B.

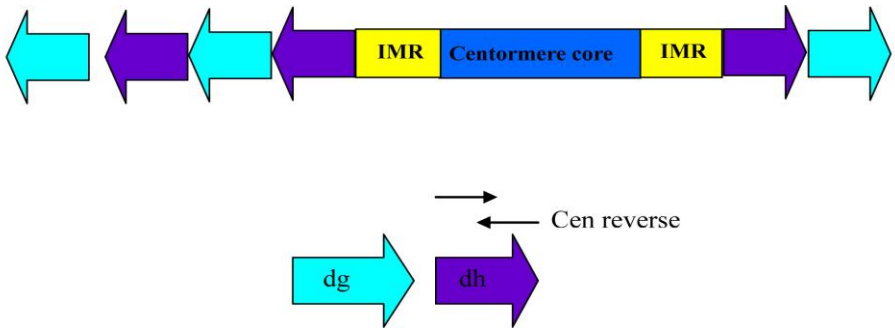


Fig. 2-1 Heterochromatic repeats and replication origins near centromere 2
A. Repeats are oriented with telomere to the left and centromere to the right. Non coding features are depicted as *dg*, *dh* and *imr* repeats (green, red and purple rectangles, respectively) along with chromosomal coordinates. Small RNA appear as small green (Cam, Sugiyama et al. 2005) and black (Reinhart and Bartel 2002) arrows. Centromeric promoters (Djupedal, Portoso et al. 2005) are represented by blue (reverse) and sepia (forward) rectangles while transcripts from *dg* (Djupedal, Portoso et al. 2005) and *dh* (Volpe, Kidner et al. 2002) are shown as grey arrows. Origins of replication (Smith, Caddle et al. 1995) are depicted by yellow rectangles: oriK is the strongest and contains an ars-binding protein (Abp1) binding site consensus sequence (black). Black rectangles under *dh* and *dg* transcripts indicate regions used for PCR amplification (p30 and p33 probes, respectively).
B. Centromeric repeats of *S.pombe* centromere II (cen2). Directions of forward and reverse transcripts of *dh* outer repeat are shown below. The reverse transcript initiates in *dh* and towards *dg* repeat.

Figure 2-2

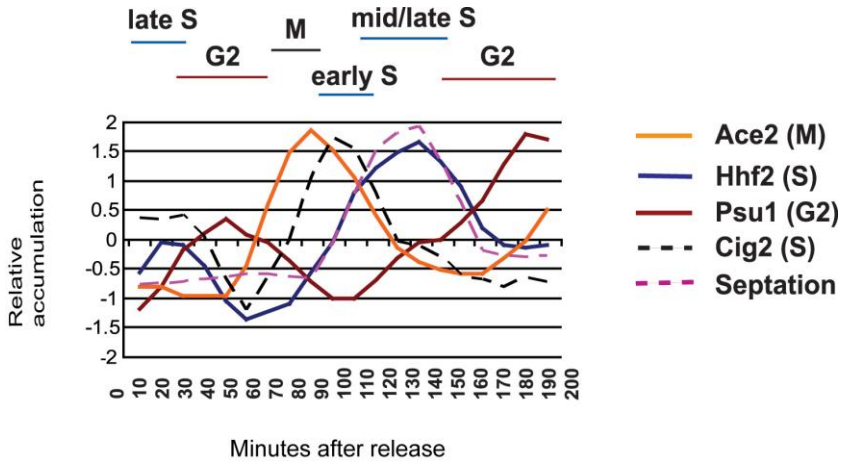


Fig. 2-2 Synchronization profile of a wild type population of *S.pombe*
Synchronization of wild type *S.pombe* cells with hydroxyurea (HU) was followed by quantitative RT-PCR of cell cycle genes; blue- *hhf2* (S phase), dashed black-*cig2* (early S phase), brown- *psu1* (G2), orange- *ace2* (M) and by measuring the septation index, which peaks during S phase (dashed pink).

Figure 2-3

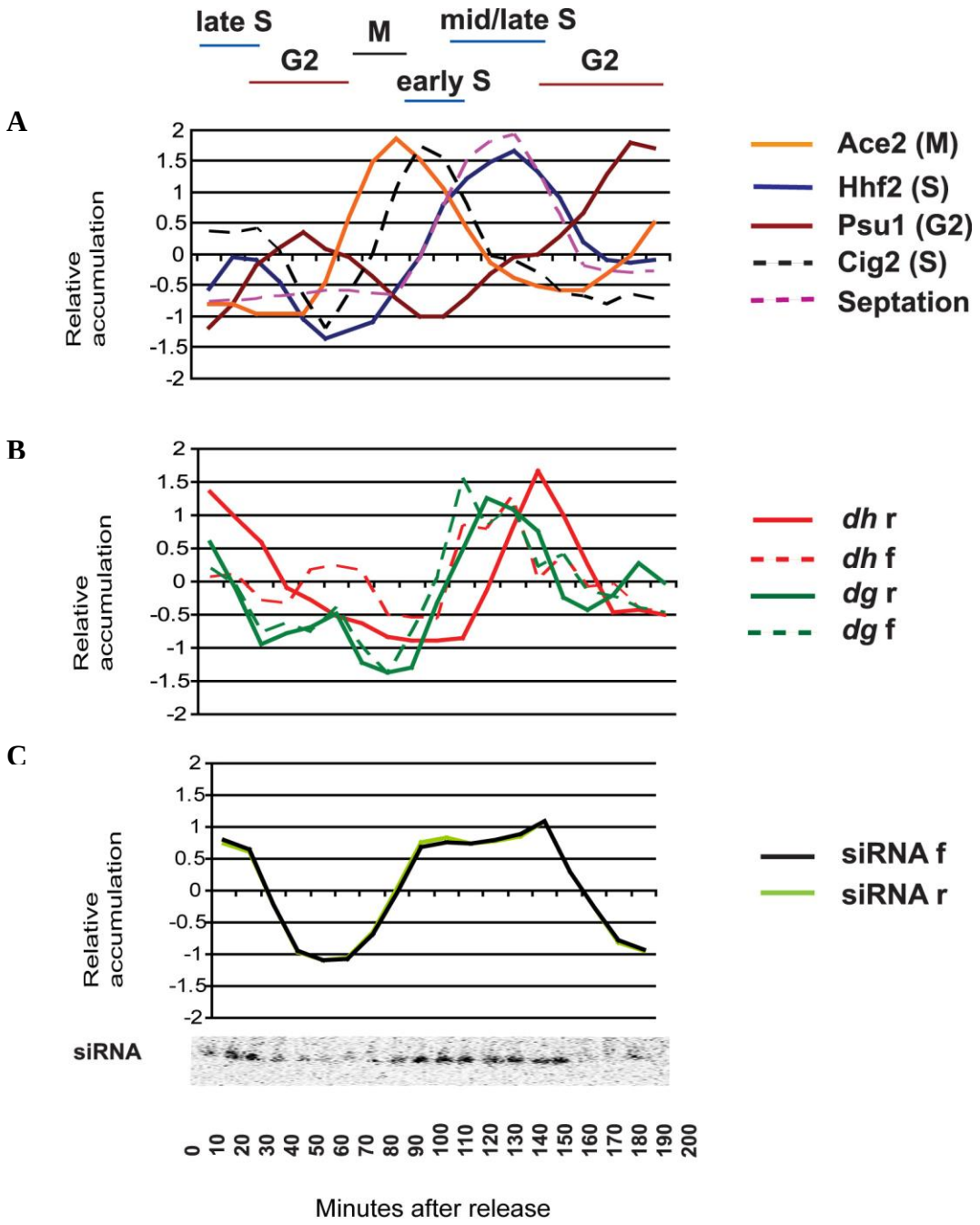

late S
M
mid/late S
G2
early S
G2
A
Relative accumulation
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
Ace2 (M)
Hhf2 (S)
Psu1 (G2)
Cig2 (S)
Septation
B
Relative accumulation
dh r
dh f
dg r
dg f
C
Relative accumulation
siRNA f
siRNA r
siRNA
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200
Minutes after release

Fig. 2-3 Cell cycle analysis of centromeric transcripts and small RNA in a synchronized population of wild type cells

A. Cell cycle profile of synchronized samples.

B. Quantitative PCR analysis of strand specific *dh* (red) and *dg* (green) transcripts. In both cell cycles, both *dh* and *dg* forward transcripts (dashed lines) appear before reverse transcripts (solid lines). Reverse transcripts are much more abundant than forward transcripts, but have been normalized for comparison (Materials and Methods).

C. Forward (black) and reverse (lime) strands of siRNAs appear throughout S phase, as shown on the small RNA Northern Blot (inset). The two strands accumulate in parallel with each other.

Figure 2-4

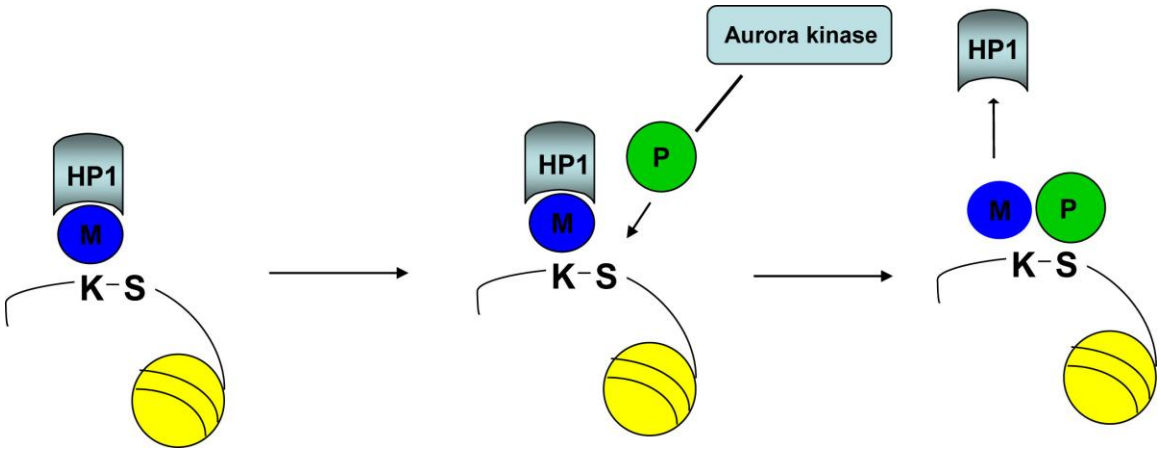


Fig. 2-4 Phospho-methyl switch
In G2 stage of the cell cycle HP1, a homolog of Swi6, is bound to H3K9me2. Phosphorylation of H3S10 (green) by Aurora kinase (Ark1 homolog) during mitosis leads to ejection of HP1 from its binding site.

Figure 2-5

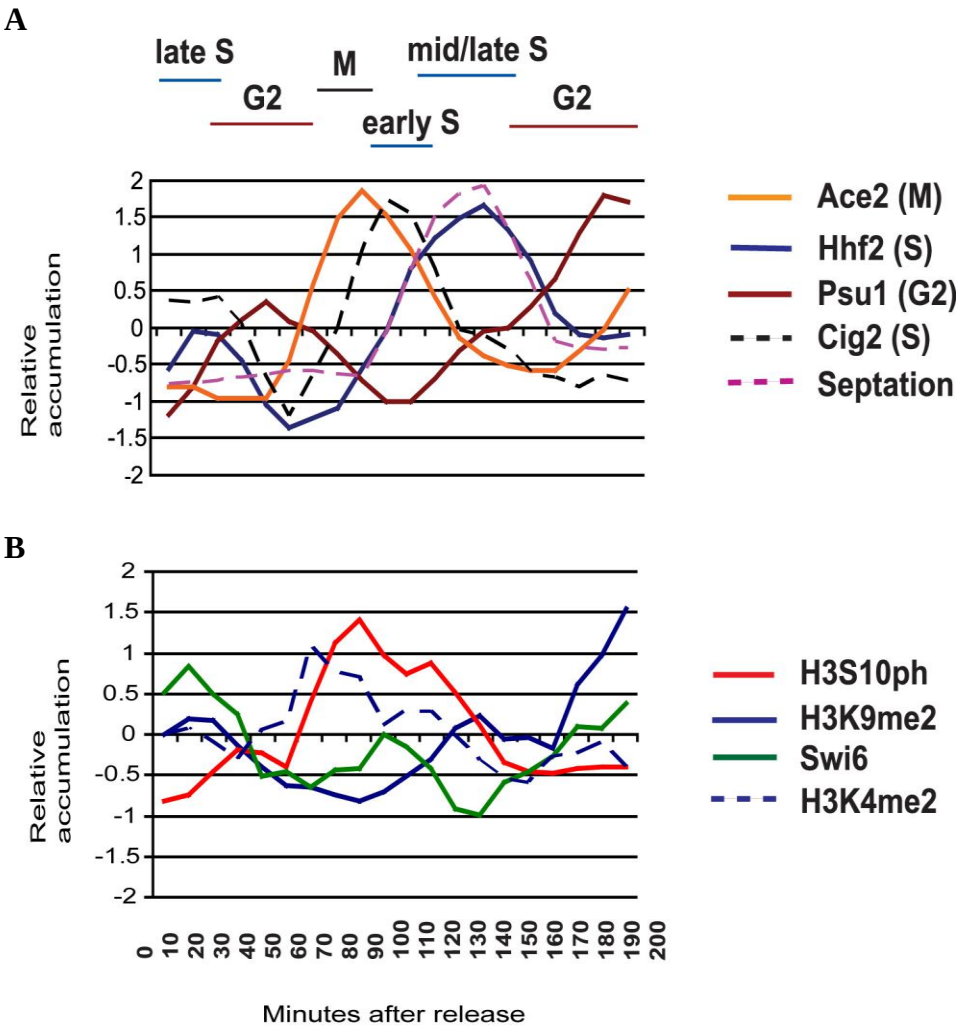


Fig. 2-5 Chromatin precipitation of histone modifications associated with *dh* pericentric repeats

A. Synchronized fractions of wild type cells.

B. ChIP of H3S10ph (red), H3K9me2 (blue), Swi6 (green) and H3K4me2 (dashed blue) associated with the *dh* repeat was performed on samples taken between 0 and 210 minutes following HU release. H3K9me2 is highest in G2. Swi6 (green) is lost in M and again in S phase when H3S10 is phosphorylated (red). Swi6 levels return to normal in G2.

Figure 2-6

Fig. 2-6 Analysis of *dh* transcripts in a *dcr1*$^{-}$ mutant
Quantitative RT-PCR of *dh* centromeric transcripts (violet) during the cell cycle in *dcr1*$^{-}$ mutant cells. The reverse strand transcript (solid) still only increases in S phase and remains high. The forward strand transcript (dashed) is ectopically expressed throughout cell cycle. Septation index (dashed teal line) indicates S phase in *dcr1*$^{-}$ is delayed relative to WT.

Figure 2-7

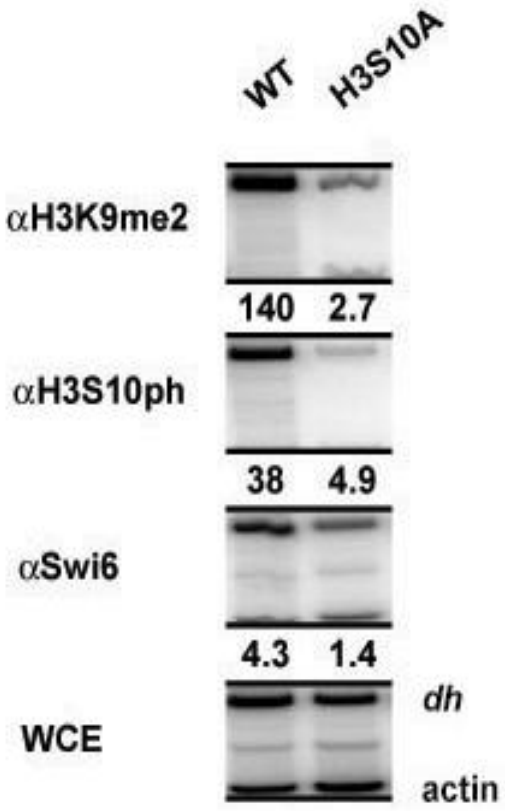


Fig. 2-7 Chromatin immunoprecipitation of the *dh* pericentromeric repeat in H3S10A strain

In a substitution strain in which H3S10 has been replaced by alanine (Mellone, Ball et al. 2003), and cannot be phosphorylated, H3S10ph and H3K9me2 are lost while levels of Swi6 are decreased substantially (about 3 fold).

Figure 2-8

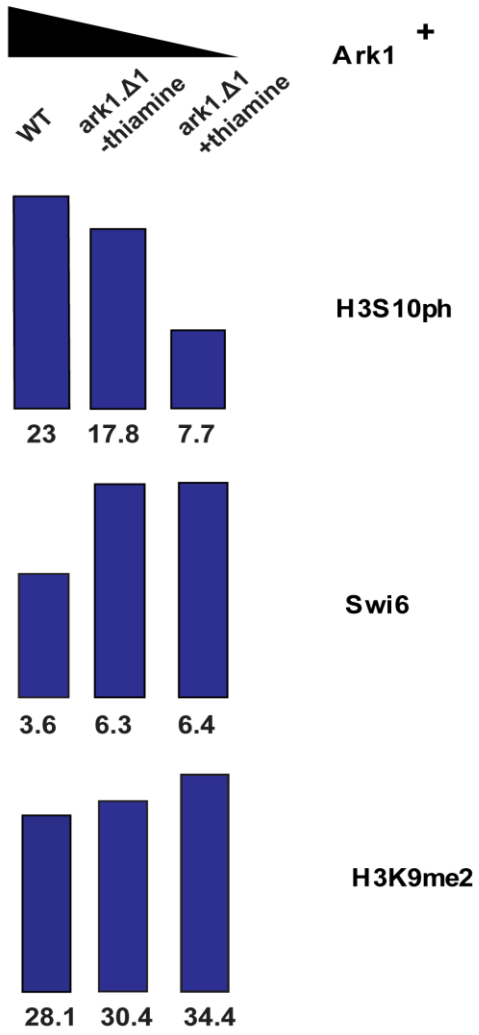


Fig. 2-8 Histone modification in serine-10 kinase mutants
ark1.Δ1 cells are deficient for the H3S10 Aurora kinase Ark1 and undergo cell cycle arrest, but can be rescued in part by a *nmt1::ark1+* gene fusion on a complementing plasmid (*ark1.Δ1* - thiamine). When grown in thiamine (*ark1.Δ1* + thiamine), *nmt1::ark1+* is shut off, and the cells undergo cell cycle arrest . In *ark1.Δ1* cells, H3K9me2 and Swi6 were increased, while H3S10ph decreased relative to strains with intact *ark1+* (wild type, WT).

Figure 2-9

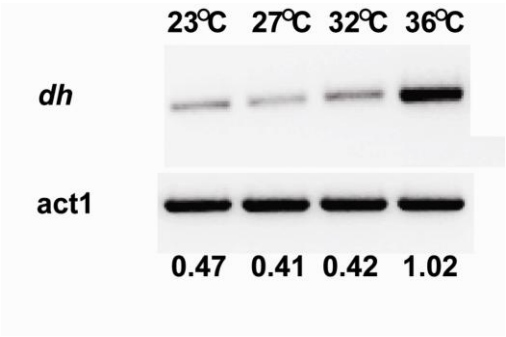


Fig. 2-9 Temperature effect on *dh* pericentric transcripts
Unsynchronized wild type *S.pombe* cells were grown at four different temperatures; 23˚C, 27˚C, 32˚C and 36˚C. Total RNA from cells grown at each temperature was analyzed by RT-PCR using primers from the *dh* heterochromatic repeat. The signal was quantified using actin (act1). RNAi mediated silencing is lost at 36˚C, confounding the use of temperature sensitive cell cycle mutants to study RNAi.

Figure 2-10

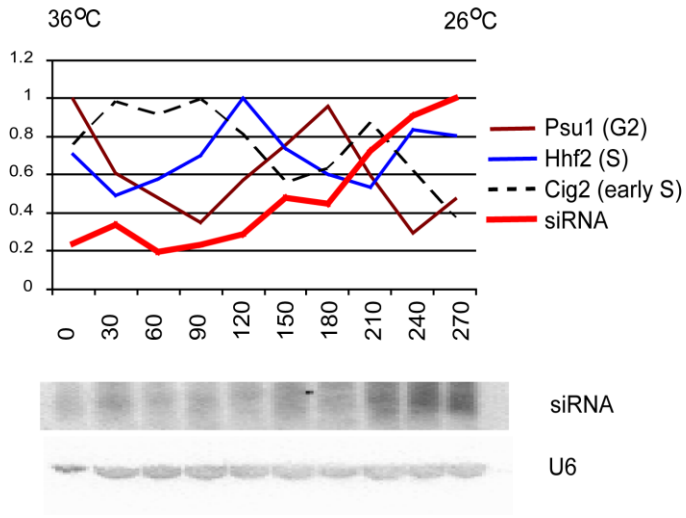


Fig. 2-10 Temperature effect on siRNA
Synchronization of *cdc25-22* temperature sensitive mutant cells was achieved by incubation at 36˚C, release and growth at 26˚C. Synchronization was analyzed with cell cycle specific genes; dashed black-*cig2* (early S), blue-*hhf2* (S) and brown-*psu1* (G2). siRNA levels, shown below, increase as the cells are released and grown at permissive temperature (26˚C).

Figure 2-11

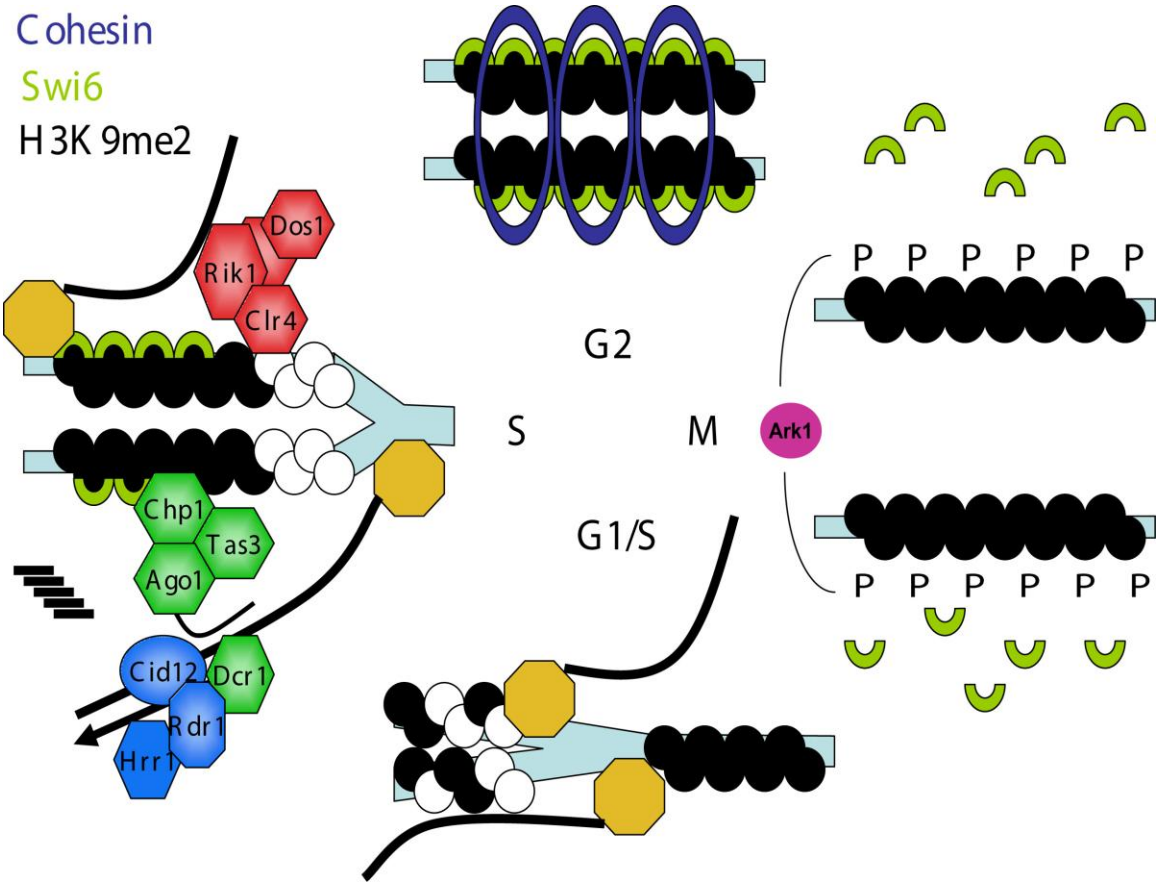


Fig. 2-11 RNAi guided heterochromatin assembly during cell cycle
The Ark1 aurora kinase (purple) mediates phosphorylation of H3S10 during mitosis which leads to Swi6 (light green) ejection via the "phospho-methyl switch". Pericentric *dh* and *dg* repeat transcripts appear in S phase, following replication, and are immediately processed by RNA dependent RNA polymerase and Dicer into siRNAs. These siRNAs are then incorporated into the RITS complex. Clr4 methylates H3K9 in the G2 phase, and Swi6 is recruited back to chromatin via binding to H3K9me2. Cohesin is recruited back to pericentric repeats through its direct interactions with Swi6 in G2.

2.4 Materials and Methods

2.4.1 Fission yeast strains

ago1^{-}, *dcr1*$^{-}$, and *rdp1*$^{-}$ null alleles with G418 resistant cassettes were generated as described previously (Volpe, Kidner et al. 2002). *cdc25-22*, *nda3-KM11* and *cdc10-129* temperature sensitive mutants were obtained from M. Yanagida. The mutants were grown at permissive temperatures (26˚C for *cdc25*-22 and *cdc10-129*, 33˚C for *nda3-KM11*). *Ark1*$^{+}$ function was ablated by growth in thiamine as described (Petersen and Hagan 2003).

2.4.2 Hydroxyurea synchronization and analysis of *dh* centromeric transcripts

Log phase wild type *S.pombe* cells were treated with 15mM hydroxyurea (Sigma) for 4.5 hours. The cells were washed twice and grown for 5 hours. Total RNA was prepared using hot phenol (Irvine, Zaratiegui et al. 2006). Synchronization efficiency was estimated by septation index and PCR analysis of cell cycle specific genes; Hhf2 (S phase), Ace2 (M) and Psu1 (late G2) of cDNA samples taken at 10 minute intervals.

2.4.3 Strand specific analysis of *dh* and *dg* centromeric transcripts

Following a hot phenol RNA extraction, each sample was treated with DNAseI and reverse transcribed with either forward or reverse *dh* (p30) or *dg* (p33) primers along with either forward or reverse actin (act1) primers to obtain strand specific cDNA (SuperScript III Reverse Transcriptase; Invitrogen). The strand specific samples were amplified using SYBR Green qPCR Universal Kit (Invitrogen) with either *dh* (p30) or *dg* (p33) primers

and control actin (act1) primers. The samples were analyzed with Opticon Program. In order to standardize all three technical replicates, the mean of all values for a given profile was subtracted from each individual value for that profile. The difference was divided by the standard deviation of the entire profile, giving a value similar to a Z score. A moving average was calculated for every 3 consecutive samples and plotted.

2.4.4 *Cdc25-22* temperature sensitive mutant analysis

The *cdc25-22* cells were grown at 26˚C and shifted to 36˚C for 4 hours to induce arrest. The cells were then released back to permissive temperature (26˚C) and grown for 4,5 hours. The small RNA blots were probed with *dh* (p30) forward and reverse probe, as well as U6. The blots were quantified by dividing *dh* probe signal by U6 signal.

2.4.5 Analysis of temperature effect on levels of *dh* centromeric transcripts

Unsynchronized wild type *S.pombe* cells were grown at four different temperatures; 23˚C, 27˚C, 32˚C and 36˚C. RNA was isolated and RT-PCR was performed using *dh* (p30) and actin (act1) primers. Gel scans were used for quantification. The enrichment was calculated by dividing *dh* signal by actin signal.

2.4.6 Small RNA quantification

Small interfering RNAs were isolated from total RNA using Polyethylene glycol precipitation. Samples were resuspended in formamide and run on 15% urea denaturing polyacrylamide gel. The blots were transferred onto a nitrocellulose membrane and incubated at 42˚C with both sense and antisense α^{32}P-UTP labeled riboprobe specific for

dh centromeric repeats (p30), which was generated using T3 and T7 in vitro transcription Maxiscript (Ambion) and hydrolyzed with sodium bicarbonate. The blot was probed with a U6 snRNA control end labeled with γ^{32}P-ATP and Polynucleotide Kinase (NEB). Washing was with 2xSSC 2% SDS at 50˚C. Blots were exposed onto phosphoimager screens for 1-3 days and quantified. Enrichment was calculated by dividing siRNA signal by U6 control signal.

2.4.7 Chromatin Immunoprecipitation (ChIP)

ChIP was performed as previously described (Li, Goto et al. 2005). HU synchronized fractions of wild type *S.pombe* cells were fixed in 1% formaldehyde and the chromatin was purified, sonicated and incubated overnight with either of the antibodies: anti-H3K9me2 (Upstate), anti- H3K4me2 (Upstate), anti-phospho-H3 Ser10 (Upstate) or rabbit polyclonal sera against Swi6 (Abcam). DNA was extracted using phenol and Ethanol-precipitated. For quantification, ChIP samples were amplified using multiplex PCR in the presence of α-32P-dCTP, run on 5% polyacrylamide gel, dried and exposed to a phosphoimager screen. Quantification was obtained using actin as a measurement of background (with the exception of H3S10ph, which was quantified without normalization to actin, because actin association with H3S10ph is also cell-cycle regulated) and comparing the intensity of the test and actin bands in the ChIP sample with those in DNA purified from Whole Cell Extract (WCE). The enrichment was calculated as (target band IP/normalization band IP)/ (target band WCE/normalization band WCE). ChIP results were analyzed by quantitative PCR in 3 replicates. The following formula was used: 2^-(input *dh*-input actin)/2^-(wce *dh*-wce actin), except for H3S10ph, for which the formula

2^-(input *dh*)/2^-(wce *dh*), was used. This is because the euchromatic actin gene also associates with H3S10ph. The profiles were then standardized as described above and the moving average of all three replicates was plotted on the graph.

2.4.8 Analysis of *ark1* mutant

Chromatin IP on *ark1*.Δ*1* cells deficient for the H3S10 Aurora kinase (Ark1) was used to analyze H3K9me2 and Swi6 levels. *ark1*.Δ*1* undergo cell cycle arrest, but can be rescued in part by a *nmt1::ark1+* gene fusion on a complementing plasmid (*ark1*.Δ*1* - thiamine). When grown in thiamine (*ark1*.Δ*1* + thiamine), *nmt1::ark1+* is shut off, and the cells undergo cell cycle arrest . The levels of H3K9me2, Swi6 and H3S10ph were measured in cells grown in both +thiamine and –thiamine medium. The enrichment was calculated as (target band IP/normalization band IP)/ (target band WCE/normalization band WCE).

Chapter III: Dicer1 regulates genome integrity during the cell cycle through Cds1 and Chk1 dependent signaling

3.1 Introduction

Proper functioning of a cell is dependent on its genomic stability. Replication errors or DNA damage lead to chromosomal breakage, DNA mutations, and contribute to cancer and other diseases. Checkpoint mechanisms are employed to detect any abnormalities in a timely manner and prevent their transmission to the next generations. Here we report that RNA interference acts along with Cdc25, a crucial cell cycle mediator, to maintain genome integrity. Lack of Dcr1 causes a Chk1 dependent DNA damage response upon inactivation of Cdc25 and leads to cell death. We also show that Dcr1 is involved in DNA replication initiation during S phase through the Cds1 dependent checkpoint. These results suggest a novel function for RNAi in cell cycle control and genome stability.

3.1.1 Cell cycle and genome stability

Exposure to genotoxic agents, such as UV and gamma radiation, chemical mutagens, or oxidative stress, as well as stalled or collapsed replication forks can cause double stranded breaks or nucleotide mutations (Hakem 2008). A healthy cell responds to DNA damage by slowing down or arresting the cell cycle. This ensures that an affected cell has time to repair the damage, or, if the damage is irreversible, induce apoptosis.

Timing of the arrest is crucial for recovery- for instance, if DNA is damaged cells should arrest before chromosomes separate during mitosis, so that the damage is not propagated to daughter cells. Therefore, the arrest usually occurs at three critical time points during cell cycle: G1 (prevents commitment to a new cycle), S (slows down replication allowing time for repair) and G2/M (does not allow progression through mitosis and chromosome segregation). The elaborate cellular mechanisms involved in DNA damage signaling help prevent genomic instability.

Damage that occurs during DNA replication often results in G2 arrest and it is therefore referred to as DNA replication dependent G2 arrest. In *S.pombe* the G2 checkpoint is crucial for viability (Furnari, Rhind et al. 1997). G2 arrest is coordinated by a large number of proteins and some of them are also involved in DNA repair (Furnari, Rhind et al. 1997). These proteins include Rad3 kinase (Bentley, Holtzman et al. 1996), Chk1 and Cdc2. The G2 stage of the cell cycle is characterized by rapid cell elongation, so that cells that have defects during the G2/M transition can become unusually long. Similarly, DNA damage, or DNA replication checkpoints that cause arrest in G2 often results in elongated cells.

3.1.2 RNAi and DNA damage response

RNAi mutants in *S.pombe* have chromosome segregation defects that result in lagging chromosomes (Volpe, Schramke et al. 2003). HP1/Swi6 directly binds cohesin Psc3 subunit and recruits it to centromeric heterochromatin (Bernard, Maure et al. 2001; Nonaka, Kitajima et al. 2002), which could provide a functional link between

missegregation and RNAi. Recent work also suggests that cohesin function is related to DNA double strand break (DSB) repair (Strom, Karlsson et al. 2007; Strom and Sjogren 2007), which could potentially imply a role of RNAi in the process. In both human and yeast cells cohesin localizes to DNA damage sites in S and G2 phases of the cell cycle (Kim, Krasieva et al. 2002; Strom, Lindroos et al. 2004; Unal, Arbel-Eden et al. 2004) and remains there after the repair is completed (Strom and Sjogren 2005).

The involvement of RNAi in directing inheritance of histone modifications (Kloc, Zaratiegui et al. 2008), in addition to its less well established role in cell cycle progression (Carmichael, Provost et al. 2004; Stoica, Carmichael et al. 2006), suggests perhaps a more specific role of RNAi in maintaining genome stability. Here I show that Dcr1 is involved in maintaining genome integrity in S phase. Its role during the cell cycle is tied to Cdc25 phosphatase and it provides a functional link between the DNA replication checkpoint in S and DNA induced damage response in G2. Lack of functional Cdc25 and Dcr1 during S phase leads to irreversible DNA damage and elicits a Chk1-mediated DNA damage response. Dcr1 is also involved in regulation of S phase progression along with Cds1 kinase.

3.1.3 Bringing the cell cycle to a halt: chk1, cdc25 and cdc2

Chk1, a serine-threonie kinase, is involved in maintaining genome integrity by performing a variety of functions during the G1/S transition. Chk1 knockouts have incomplete S phase, presumably due to aberrant transcription of genes required for DNA replication (Naruyama, Shimada et al. 2008). *S.pombe* mutants defective in DNA

replication initiation require Chk1 for viability (Yin, Locovei et al. 2008), which indicates that DNA damage can result from DNA initiation defects. Perhaps the most important function of Chk1 involves coupling of the DNA damage signal to cell cycle arrest in mitosis (referred to as G2 DNA damage induced checkpoint) (Fig. 3-1). Six genes: *rad1*$^+$, *rad3*$^+$, *rad9*$^+$, *rad17*$^+$, *rad26*$^+$ and *hus1*$^+$ (al-Khodairy and Carr 1992; Rhind and Russell 1998) first signal the damage. Rad3 activates Chk1 immediately after DNA damage by phosphorylating its C terminus Serine 345 (Lopez-Girona, Tanaka et al. 2001), which results in timely arrest before mitosis (Furnari, Rhind et al. 1997). The phosphorylated form of Chk1 interacts with 14-3-3 proteins (Chen, Liu et al. 1999).

Cdc25 phosphatase is necessary for maintaining genome integrity by functionally linking the DNA replication checkpoint and the G2 cell cycle stage. *S.pombe cdc25* knockouts are not viable. Similarly *cdc25A* null mice die during pre-implantation, implying a role in embryonic development (Ray, Terao et al. 2007). The Cdc25 checkpoint pathway has been also shown to function as a tumor suppressive barrier (Bartkova, Horejsi et al. 2005). Cdc25 phosphatase is a target of Chk1 mediated phosphorylation during DNA damage response (Furnari, Rhind et al. 1997). Phosphorylated Cdc25 interacts with 14-3-3 proteins (Conklin, Galaktionov et al. 1995), which is thought to provoke export of Cdc25 out of the nucleus (Lopez-Girona, Furnari et al. 1999), and therefore facilitate mitotic arrest (Walworth 2001). Cdc25 and 14-3-3 protein interaction is not affected by DNA damage (Chen, Liu et al. 1999).

Cdc2 is the target of DNA damage arrest. Cdc2 phosphorylation is carried out by Wee1 and Mik1 protein kinases (Russell and Nurse 1987; Featherstone and Russell 1991; Lee, Enoch et al. 1994). In a normal cell, dephosphorylation of Cdc2 tyrosine 15 (Y15)

by Cdc25 is crucial for Cdc2 activation and mitotic entry. This mechanism, conserved in fission yeast (Gould and Nurse 1989), *Xenopus* (Gautier, Matsukawa et al. 1989) and mammalian cells (Morla, Draetta et al. 1989), brings about changes in the cytoskeleton, chromosome condensation and in vertebrate cells, nuclear envelope breakdown (Nurse 1990). Cdc2 is also necessary for the function of Cdc10/Sct1 transcription complex, which controls transcriptional activity in G1 (Connolly, Caligiuri et al. 1997). However, when DNA is damaged, Cdc25 cannot dephosphorylate Cdc2, which renders arrest before cells enter mitosis (Rhind, Furnari et al. 1997; Rhind and Russell 1998).

Both Cdc25 inhibition and Cdc2 phosphorylation are also thought to be required for slowing down replication during S phase (Kumar and Huberman 2004), which is referred to as the "intra-S phase checkpoint", although this subject is still a matter of dispute (Kommajosyula and Rhind 2006). Recent work by Kumar and Huberman reveals that MMS treatment, which triggers the DNA damage checkpoint response, delays firing of the early-replicating origins found in *dg* centromere repeats (Kumar and Huberman 2009). Interestingly, mutations in histone deacetylase genes, *clr3* and *clr6*, also result in delayed firing of early-replicating origins (unpublished data cited in (Kumar and Huberman 2009)).

3.1.4 Cds1 and its role in DNA replication checkpoint

Cds1, a mammalian Chk2 homolog, is an important effector of the S phase of the cell cycle. It is activated and phosphorylated during the S phase and mediates the replication checkpoint (Lindsay, Griffiths et al. 1998; Rhind and Russell 2000). Similar

to Chk1, Cds1 is activated by *rad* genes. Cds1 has been reported to stabilize stalled replication forks through regulation of three proteins involved in fork stability: Mus81-Eme1, Rqh1 (suppressing aberrant recombination) and Rad60 (required for recombinational repair) (Kai and Wang 2003). The Cds1 kinase phosphorylates Mik1, which along with Wee1, deactivate Cdc2 by an inhibitory phosphorylation. Therefore, stalled replication forks result in G2 arrest. Cds1 is also involved in phosphorylating Cdc25 (Fig. 3-1).

In summary, *rad* genes mediate activation of both Chk1 and Cds1, which can then signal DNA damage and induce arrest, and therefore maintain the integrity of the genome. If the DNA damage is repaired, the cells recover from the arrest and resume cycling, although the mechanism of the recovery is not well understood.

3.2 Results

3.2.1 RNAi is involved in Cdc25 dependent regulation of cell cycle control

S.pombe cdc25-22 harbors a point mutation, which allows temporary inactivation of Cdc25 and results in G2 arrest upon incubation at 36˚C. Cells arrested at 36˚C have an elongated phenotype, but once a culture of G2 arrested cells is shifted back to 26˚C- the permissive temperature- Cdc25 phosphatase activity is restored and cells reenter the cell cycle. I have found that RNAi mutants crossed to *cdc25-22* strain are unusually long when grown at the permissive temperature; even though RNAi and *cdc25-22* single

mutants do not have any morphological changes (Fig. 3-2), suggesting that RNAi is involved in regulation of cell cycle control.

3.2.2 RNAi and hydroxyurea induced arrest in S phase

While the elongated phenotype is observed in all *cdc25-22 dcr1⁻*, *cdc25-22 ago1⁻* and *cdc25-22 rdp1⁻* mutants, *cdc25-22 dcr1⁻* has by far the most severe phenotype, which includes a significantly delayed growth rate, sensitivity to temperature changes and defects at the G2/M cell cycle stage. This observation is supported by an analysis of cell cycle progression after exposure to hydroxyurea (HU). All single mutants can arrest in S phase after a three hours exposure to HU, which results in shifting of the DNA content peak on a FACS profile (Fig. 3-3). FACS analysis of the three double mutants shows a broad peak of DNA content, characteristic of cell cycle defects (Fig. 3-3). Unlike single *cdc25-22* mutant, *cdc25-22 dcr1⁻* is unable to enter S phase after exposure to the drug, demonstrated by the lack of a 1C DNA content peak. *Cdc25-22 rdp1⁻* and *cdc25-22 ago1⁻* have a milder defect when treated with HU. *Cdc25-22 ago1⁻* becomes fully synchronized in S phase upon treatment (Fig. 3-3) and is able to re-enter the cell cycle after removal of the drug (data not shown). *Cdc25-22 rdp1⁻* has an intermediate defect, and although it never fully enters S phase, there is significant shift after HU exposure (Fig. 3-3).

3.2.3 "Double arrest"- Dcr1 plays a role in Cdc25 dependent response to DNA damage

The *cdc25-22* phosphatase is a key component connecting DNA damage occurring during S phase to cell cycle arrest in G2. Phosphorylated Cdc25 cannot dephosphorylate Cdc2, which leads to its inactivation and arrest in the G2 stage of the cell cycle. Therefore, we suspected that the severely elongated phenotype and growth defect of *cdc25-22 dcr1*$^{-}$ mutant may indicate the presence of DNA damage during S phase.

Cdc25-22 dcr1^{-} double mutant has significant defects in both S and G2 stages of the cell cycle. Contrary to *cdc25-22* single mutants, *cdc25-22 dcr1*$^{-}$ double mutants never properly arrest in G2 when shifted to 36˚C. Instead, the arrest results in two separate populations of cells - G2 (2C DNA content) and S (1C-2C DNA content) (Fig. 3-4, Fig. 3-5). This is because cells that successfully completed S phase upon arrest at 36˚C could enter G2 due to inactivation of Cdc25. However, cells that already passed G2 upon the time of arrest were not able to complete S phase, which resulted in a "double arrest" phenotype. An unsynchronized population of *S.pombe* cells has only around 10-15% of G1 and S phase cells; therefore, a 1C peak is hardly present. *Cdc25-22 dcr1*$^{-}$ have around 35% of S phase cells that do not enter G2 (Fig. 3-4), which may be due to aberrant replication fork structures or to DNA damage. Releasing *cdc25-22 dcr1*$^{-}$ mutants back to the permissive temperature did not rescue the phenotype and caused cell death instead. Contrary to *cdc25-22* single mutants, which began cycling once transferred to the permissive temperature, *cdc25-22 dcr1*$^{-}$ attempted to resume DNA replication

without division. Only the G2 peak shifted towards right resulting in DNA content greater than 2C. The S phase peak stayed unchanged, indicating that the damage that occurred during S phase in the absence of functional Cdc25 and Dcr1, was irreversible. Common defects observed in the double mutants upon release included loss of DNA integrity, as shown by smeary DAPI staining (Fig. 3-4B). Multiple septa observed in a single cell indicated loss of coupling between DNA replication and growth checkpoint, as the cell attempted to divide without fully completing S phase. In sharp contrast with the *cdc25-22 dcr1*$^{-}$ mutant, *cdc25-22 ago1*$^{-}$ and *cdc25-22 rdp1*$^{-}$ had only a slight cell cycle delay at the G1/S transition and they could complete S phase (Fig. 3-6).

3.2.4 Chk1 mediates DNA damage response that occurs in *cdc25-22 dcr1*$^{-}$ mutants

In organisms ranging from yeast to humans, DNA damage response is mediated by Chk1, which is also involved in enforcing the DNA replication checkpoint (Boddy, Furnari et al. 1998). In order to investigate the involvement of RNAi in Cdc25 dependent response to DNA damage, *cdc25-22 dcr1*$^{-}$ was crossed to a *chk1Δ* null strain. *Cdc25-22 dcr1*$^{-}$ *chk1*$^{-}$ triple mutant cells were not significantly elongated at 26˚C (Fig. 3-4C). A shift to restrictive temperature rendered proper synchrony in G2 and the arrested cells resumed cycling when shifted back to permissive temperature (Fig. 3-4A). *Cdc25-22 dcr1*$^{-}$ *chk1*$^{-}$ triple mutant cells grew much faster than both *cdc25-22 dcr1*$^{-}$ and *dcr1*$^{-}$ mutants (Fig. 3-4D). *Cdc25-22 dcr1*$^{-}$ takes over 5 hours to complete a full cell cycle, whereas the triple mutant completes one in 3 hours.

Chk1 mediates mitotic arrest. This function of the Chk1 kinase is accomplished by phosphorylation of the serine 345 (Lopez-Girona, Tanaka et al. 2001). In order to test if the rescue of *cdc25-22 dcr1*$^{-}$ mutant in *chk1*$^{-}$ background was dependent on lack of DNA damage signaling we took advantage of a Chk1-S345A allele, in which serine 345 is substituted with alanine. Chk1-S345A does not respond to DNA damage and therefore does not arrest at the G2/M transition. The triple mutant, cdc25-22 *dcr1*$^{-}$ *chk1-S345A* fully rescued the *cdc25-22 dcr1*$^{-}$ phenotype. When shifted to 36˚C, the triple mutants arrested in G2 and began cycling as soon as the cell culture was shifted to permissive temperature (Fig. 3-4A). The triple mutant was less elongated and had a faster growth rate compared to the *cdc25-22 dcr1*$^{-}$ double mutant. Chk1 also rescued the *dcr1*$^{-}$ growth defect in a *cdc25*$^{+}$ background (Fig. 3-4D). Interestingly, wild type cells expressing *chk1-S345A* become elongated and have cell cycle progression defects (Lopez-Girona, Tanaka et al. 2001), resembling a *cdc25-22 dcr1*$^{-}$ mutant. The rescue of the *cdc25-22 dcr1*$^{-}$ mutant upon introducing a *chk1*$^{-}$ mutation indicates that lack of Dcr1 causes DNA damage in the absence of functional Cdc25. Interestingly, a *cdc25-22 dcr1*$^{-}$ *rad3*$^{-}$ strain does not fully rescue the double arrest phenotype, which suggests that other damage response could also be involved.

3.2.5 Camptothecin treatment induces severe growth delay in *cdc25-22 dcr1*$^{-}$ mutants

DNA damage can be induced by camptothecin (CPT), which specifically causes inhibition of DNA replication during S and is mediated by ATR/Chk1 pathway. It is

thought that the damaging effects of the drug are caused by formation of DNA double stranded breaks when the DNA replication fork encounters CPT-topoisomerase I complex (Wan, Capasso et al. 1999). Therefore, only actively dividing cells respond to the drug (Wan, Capasso et al. 1999). Depending on dosage and duration, exposure to CPT causes Chk1 phosphorylation, slow growth or complete arrest in S phase. A dilution assay was used to verify that phenotypic defects of *cdc25-22 dcr1*$^{-}$ mutant are a result of activation of Chk1 mediated DNA damage response pathway. Compared to wild type and *chk1*$^{-}$ cells, *cdc25-22 dcr1*$^{-}$ showed a slower growth and a lower survival rate after 3 days of growth at 26˚C on 5μM CTP plates (Fig. 3-7). *Dcr1*$^{-}$ single mutant had a slightly slower growth rate than wild type cells, whereas *cdc25-22* and *cdc25-22 chk1*$^{-}$ single mutant did not have any noticeable growth defect. These results are in agreement with FACS data and strongly suggest that *cdc25-22 dcr1*$^{-}$ mutant triggers a Chk1 mediated DNA damage response.

3.2.6 DNA damage in *cdc25-22 dcr1*$^{-}$ mutant leads to cell death and over-accumulation of mitochondrial DNA

I used a cell viability fluorescence assay to assess cell death in *cdc25-22 dcr1*$^{-}$ mutants (Fig. 3-8). Over 25% of double mutant cells were found to be dying in growing cultures compared to only 3.5% of *cdc25-22* and 6.7% of *dcr1*$^{-}$ cells. However, only 6.5% of *cdc25-22 dcr1*$^{-}$ *chk1*$^{-}$ mutant die, which confirms that *cdc25-22 dcr1*$^{-}$ mutant is rescued in a *chk1*$^{-}$ background. *Cdc25-22 ago1*$^{-}$ and *cdc25-22 rdp1*$^{-}$ double mutants

also undergo some cell death, although the percentage of dying cells is much lower compared to *cdc25-22 dcr1*$^{-}$ (Fig. 3-8).

Analysis of DNA cesium chloride preparations of *cdc25-22* grown at both permissive and restrictive temperatures reveals two distinct DNA bands: chromosomal and mitochondrial (Fig. 3-9). The mitochondrial DNA band is not normally visible in an *S.pombe* cesium chloride DNA preparation; however, since Cdc25 is essential for survival of an organism, its transient inactivation at 36˚C results in inhibition of chromosomal DNA replication and hence enrichment of mitochondrial DNA. Interestingly, enrichment of mitochondrial DNA can be also seen in *cdc25-22 dcr1*$^{-}$ mutant cells at permissive temperature, which indicates that a *cdc25-22* defect is enhanced even at permissive temperature in the absence of Dcr1, consistent with the slow growing phenotype of these elongated cells (Fig. 3-4). These results are also consistent with FACS data and provide further evidence for a loss of genomic integrity in the double mutant. Accumulation of mitochondrial DNA can be also observed in *cdc25-22 chk1*$^{-}$ *dcr1*$^{-}$ and *cdc25-22 chk1*$^{-}$ mutants and it is comparable to *cdc25-22* mutant at permissive temperature.

3.2.7 Dcr1 is involved in proper S phase completion

DNA damage signaling and the replication checkpoint in S are not solely dependent on Chk1. Cds1 kinase is involved in stabilizing replication forks (Noguchi, Noguchi et al. 2003), as well acting along with Chk1 to delay mitosis (Boddy, Furnari et al. 1998; Tanaka and Russell 2004). Cds1 phosphorylates Cdc25, rendering it inactive

and therefore facilitating mitotic arrest. Mrc1 specifically interacts with Cds1 in S phase through binding to its FHA domain, which is required for Rad3-Rad6 mediated Cds1 phosphorylation (Tanaka and Russell 2004). *Cds1*$^{-}$ mutants treated with HU show accumulation of stalled and/or regressed replication forks (Lopes, Cotta-Ramusino et al. 2001). I found that *cdc25-22 dcr1*$^{-}$ mutants accumulate cell death in a *cds1Δ* null background. Arrest and release analysis shows that these triple mutants accumulate dead cells even at permissive temperature. *Cdc25-22 dcr1*$^{-}$ *rad3*$^{-}$ mutant behaves similarly to *cdc25-22 dcr1*$^{-}$ *cds1*$^{-}$ (Fig. 3-10). On the other hand, *ago1*$^{-}$ and *rdp1*$^{-}$ strains crossed to *cdc25-22 cds1*$^{-}$ have less severe defects at both permissive and restrictive temperatures. Cds1 is involved in stabilizing stalled replication forks and enforcing proper initiation of DNA replication. The defects observed in *cdc25-22 dcr1*$^{-}$ *cds1*$^{-}$ mutant reveal a novel role of Dcr1 in genomic stability during S phase, presumably through stabilizing replication forks.

3.3 Discussion

3.3.1 Dcr1 plays a role in Cdc25 dependent DNA damage response

Analysis of *cdc25-22* mutant in RNAi deficient background reveals a novel role for RNAi in maintaining genome stability. Although all RNAi mutants display misregulation of cell cycle in a *cdc25-22* background, Dcr1 appears to be a key component of Cdc25 dependent regulation during S phase (Fig. 3-11). The absence of functional product of Cdc25 protein combined with the loss of Dcr1 leads to severe

genome instability, aberrant S phase and ultimately cell death. RNAi has been recently shown to be directly tied to the S phase of the cell cycle (Chen, Zhang et al. 2008; Kloc, Zaratiegui et al. 2008). Therefore, RNAi could be involved in processes that occur at this particular stage. A functional link between Cdc25 and Dcr1 is of particular significance, since Cdc25 plays a pivotal role in cell cycle progression, DNA replication and recovery from checkpoint, and its misregulation has been associated with human cancer (Ray and Kiyokawa 2008).

3.3.2 Dcr1 is involved in regulation of the S phase of the cell cycle

The outcome of *cdc25-22 cds1*$^{-}$ analysis in RNAi mutants sheds light on involvement of Dcr1 during S phase. Cds1 is involved in DNA replication checkpoint, as well as stabilizing the replication fork. On the other hand, Chk1 is predominantly involved in response to damage that occurs during S phase and involves a G2 arrest.

According to our results, the lack of Dcr1 may cause DNA damage during S phase, which induces a Cds1 replication checkpoint. The lack of Cds1 does not allow proper response to the damage. As the *cdc25-22 dcr1*$^{-}$ mutant struggles to complete the cell cycle, which is displayed by slow growth (Fig. 3-4), Chk1 senses the damage and elicits a DNA damage signal. The lack of Chk1 in *cdc25-22 dcr1*$^{-}$ *chk1*$^{-}$ mutant allows "bypassing" the damage induced during S phase and results in faster growth (Fig. 3-4). Because both Cds1 and Chk1 are involved in DNA damage checkpoint during S phase, the lack of Cds1 could also trigger an increased Chk1 DNA damage response in the *cdc25-22 dcr1*$^{-}$ *cds1*$^{-}$ mutant, which could result in cell death. *Cdc25-22 dcr1*$^{-}$ *rad3*$^{-}$

mutant analysis supports that both Cds1 and Chk1 checkpoints are involved in Dcr1 induced damage. Arrest of *cdc25-22 dcr1*$^{-}$ *rad3*$^{-}$ mutant at the restrictive temperature enhances cell death. The triple mutants can survive at the permissive temperature despite accumulating damage, but inactivation of Cdc25 (hence imposing more damage) at 36°C results in further damage and increases the number of dead cells. Because Cds1 is involved in stabilizing DNA replication forks, the phenotypes of *cdc25-22 dcr1*$^{-}$ and *cdc25-22 dcr1*$^{-}$ *rad3*$^{-}$ could indicate the involvement of Dcr1 in replication fork stability. Additional experiments are needed to uncover the mechanism.

Dcr1 processes long, ds RNA transcripts into siRNAs (Introduction 1-4). In the absence of Dcr1 these transcripts could remain tethered to the DNA template, potentially forming short stretches of DNA-RNA. The DNA-RNA hybrids have been shown to form co-transcriptionally in *S.cerevisiae hpr1* mutant during mRNA elongation, which affects transcription, genomic stability and promotes transcription associated recombination (Huertas and Aguilera 2003). Generation of DNA-RNA hybrids in *cdc25-22 dcr1*$^{-}$ mutant could lead to formation of aberrant structures at the replication fork, or limit accessibility of replication factors, which could impair the progression of the fork and trigger a Cds1 replication checkpoint. The presence of DNA-RNA hybrids would likely lead to a halt at the replication fork or its collapse, which could result in formation of recombinogenic DNA breaks. In fact, it has been shown that DNA-RNA hybrids are involved in class switching recombination of Ig immunoglobulin (Mizuta, Iwai et al. 2003). Dcr1 could be involved in the cleaving of potential DNA-RNA hybrids, which would ensure proper progression of replication forks in S phase.

The role of Dcr1 in potential regulation of S phase is further supported by the analysis of *Drosophila* rasiRNA (repeat associated small interfering RNA). Mutations in *armi* and *aub* genes, which are required for production of rasiRNAs are suppressed by mutants in the ATR/Chk2 pathway (Klattenhoff, Bratu et al. 2007). Embryonic axis specification phenotypes associated with rasiRNA pathway mutants are specifically suppressed by *mei-44* and *mnk*, which encode ATR and Chk2 homologs. In addition, because rasiRNA pathway mutants accumulate γ-H2Av loci associated with double stranded DNA breaks, it is inferred that rasiRNA pathway specifically suppresses DNA damage signaling in *Drosophila* germline (Klattenhoff, Bratu et al. 2007). Our analysis of *cdc25-22 dcr1*$^{-}$ shows that Dcr1 is specifically involved in both Cds1 and Chk1 mediated checkpoints during S phase, perhaps through stabilization of the replication forks. Lack of Dcr1 triggers a DNA damage response, which is consistent with the proposed role of rasiRNA pathway in *Drosophila*.

Analysis of *D.melanogaster Su(var)3-9* and *dcr-2* mutants shows increased DNA damage of heterochromatin (Peng and Karpen 2009). Loss of *Su(var)3-9* function results in increased number of ds DNA breaks, defective sister chromatin cohesion and high level of heterochromatin recombination. Interestingly, *dcr-2* mutants accumulate γ H2Av foci, indicative of ds DNA breaks, specifically during S phase (Peng and Karpen 2009). This could perhaps extend S phase, which would allow more time to repair the damage. My observations in *cdc25-22 dcr1*$^{-}$ mutant also indicate DNA damage occurs in the absence of Dcr1, which then causes extension of S phase and triggers a DNA damage checkpoint response.

In addition to Dcr1, Rdp1 and Ago1 null mutants also have a phenotype in the *cdc25-22* background. Rdp1, a component of RDRC complex, is needed for proper formation of siRNAs. *Cdc25-22 rdp1*$^{-}$ mutants are elongated and have cell cycle defects as shown by HU treatment (Fig. 3-3). On the other hand, *ago1*$^{-}$ mutants have only a minor defect in a *cdc25-22* background, which suggests that its role in maintaining genome integrity could be different from Dcr1 and Rdp1, and perhaps independent of siRNA biogenesis. Ago1 could also be a substrate of Chk1, which would explain a mild phenotype of *cdc25-22 ago1*$^{-}$ mutant. A detailed analysis of RNAi pathway mutants is needed to reveal the role of RNAi in maintaining genome integrity.

Figure 3-1

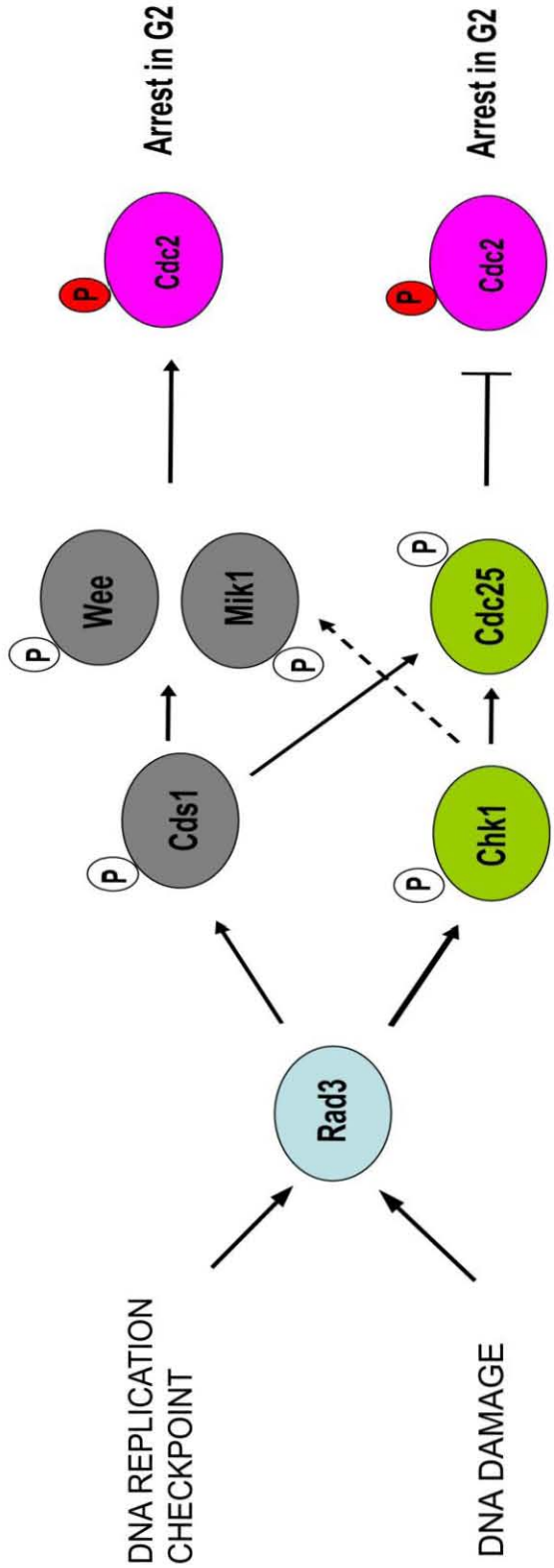

Arrest in G2
Arrest in G2
Cdc2
Cdc2
P
P
Wee
Mik1
Cdc25
P
P
P
Cds1
Chk1
P
P
Rad3
DNA REPLICATION CHECKPOINT
DNA DAMAGE

Fig. 3-1 Chk1 and Cds1 are effectors of DNA replication checkpoint, DNA damage checkpoint and mediate G2 arrest

Mitosis is initiated by dephosphorylation of tyrosine 15 of Cdc2. The timing of this event is regulated by Cdc25 phosphatase and Wee1 and Mik1 tyrosine kinases. When DNA damage or DNA replication checkpoint occurs, cells arrest in G2 to delay or inhibit mitosis. When DNA is damaged, Rad3 phosphorylates Chk1 on Serine 345 (Ser-345), which leads to its activation. Chk1 then phosphorylates Cdc25 kinase, which results in decreased nuclear localization of Cdc25. Phosphorylated Cdc25 cannot dephosphorylate Cdc2; therefore keeping it in an inactive state so that cells cannot enter mitosis. Chk1 also upregulates Mik1, which is thought to have a minor role in maintainance of DNA damage checkpoint. Cds1, a homolog of mammalian Chk2, is phosphorylated on Threonine-11 (Thr-11) by Rad3, which leads to its activation (Tanaka, Boddy et al. 2001). Cds1 binds and phosphorylates Wee1 (Rhind and Russell 2001). Cds1 also upregulates Mik1 to a greater extent than Chk1 (Rhind and Russell 2001). Similarly to Chk1, Cds1 inhibits dephosphorylation of Cdc2 by phosphorylating Cdc25 and rendering it inactive.

Figure 3-2

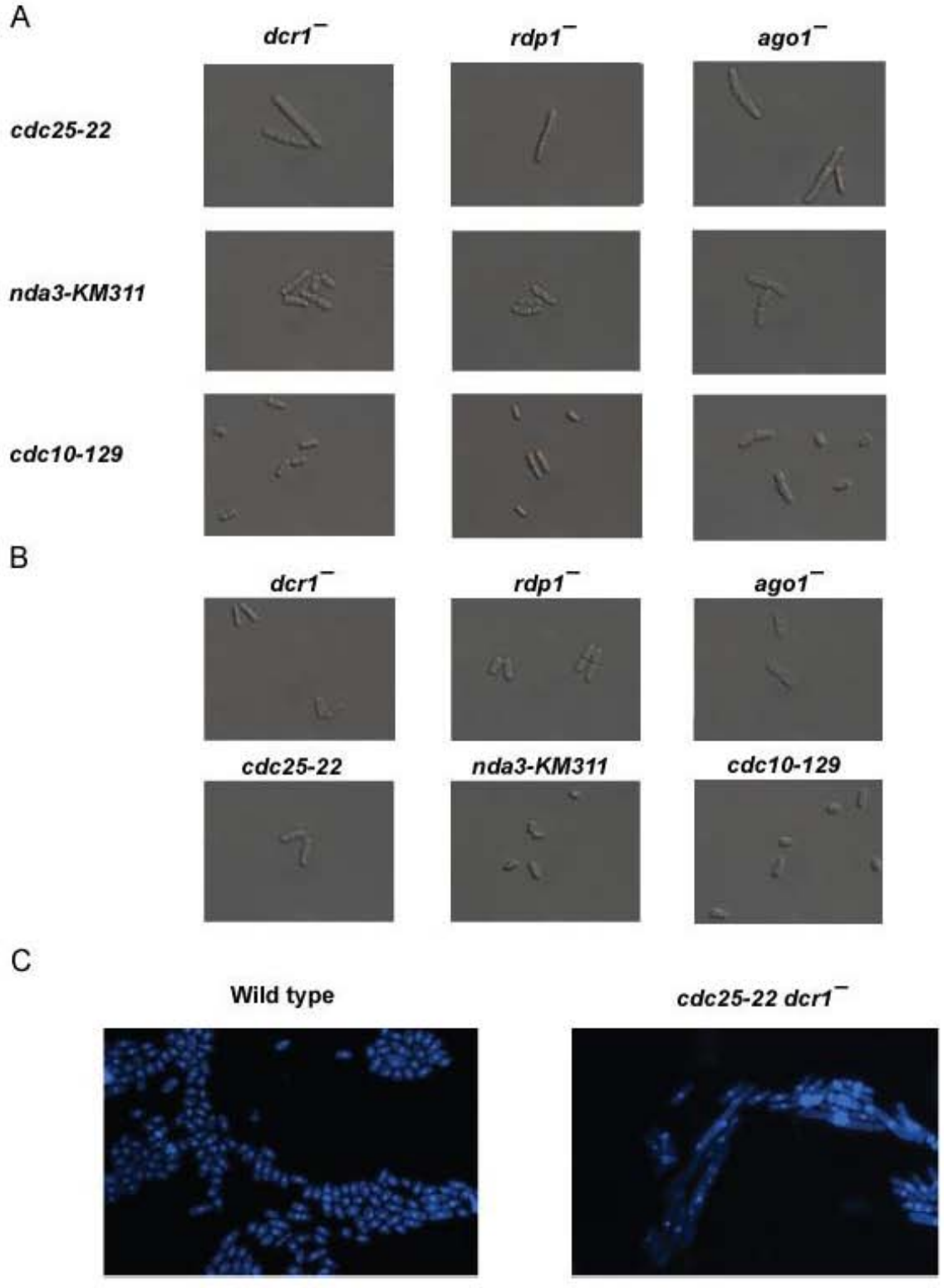

A
dcr1⁻
rdp1⁻
ago1⁻
cdc25-22
nda3-KM311
cdc10-129
B
dcr1⁻
rdp1⁻
ago1⁻
cdc25-22
nda3-KM311
cdc10-129
C
Wild type
cdc25-22 dcr1⁻

Fig. 3-2 Analysis of temperature sensitive *cdc25-22*, *nda3-KM311* and *cdc10-129* in RNAi background

A. Comparison of *cdc25-22*, *nda3-KM311* and *cdc10-129* mutants in either *dcr1*$^{-}$, *ago1*$^{-}$ or *rdp1*$^{-}$ background. *Cdc25-22 RNAi* double mutants have the most severe phenotype.

B. Images of single mutants show no abnormal phenotype.

C. DAPI staining of wild type cells and *cdc25-22 dcr1*$^{-}$ double mutants grown at permissive temperature. *Cdc25-22 dcr1*$^{-}$ exhibit an abnormally elongated phenotype and chromosome segregation defects.

Figure 3-3

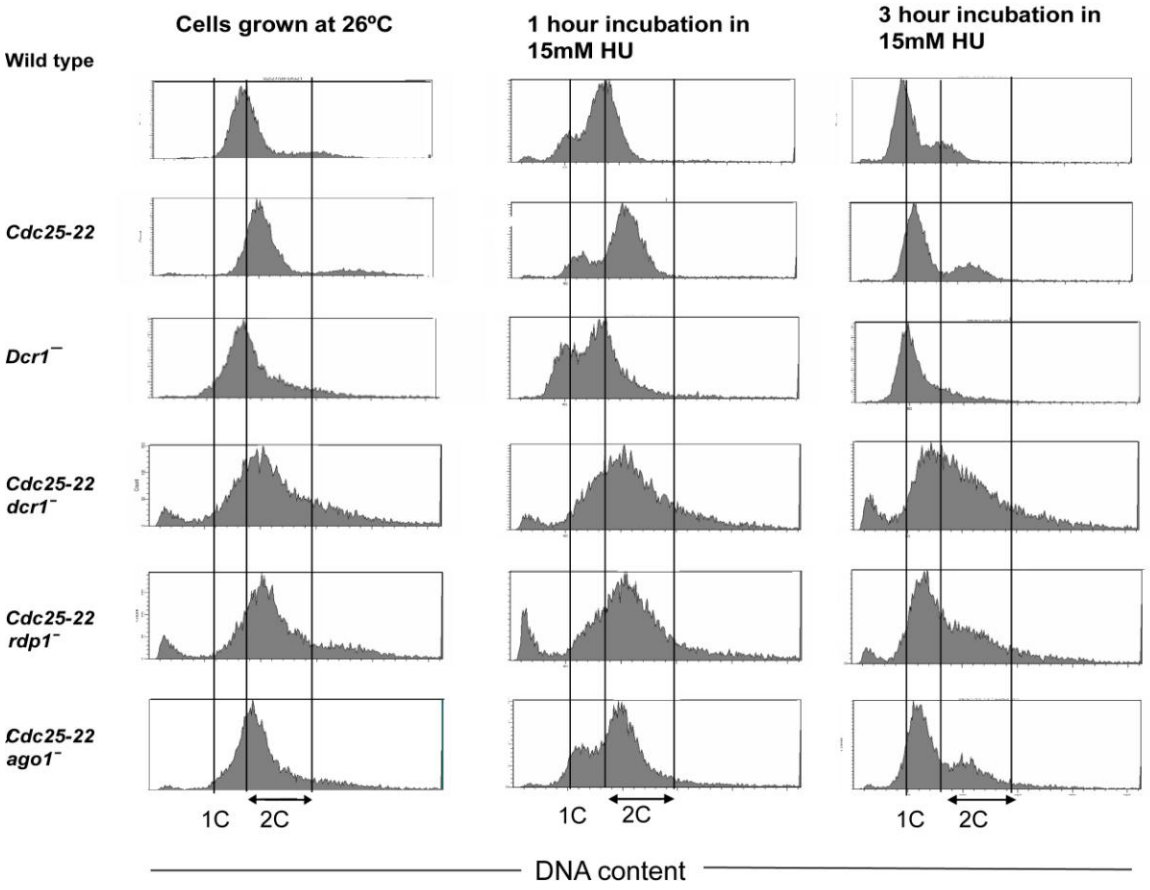


Fig. 3-3 Hydroxyurea effect on cell cycle progression
Exponentially growing samples were treated with 15 mM HU for 1 and 3 hours to induce arrest in S phase at 26˚C. All samples were analyzed by FACS. *Cdc25-22 dcr1*$^{-}$ mutant does not fully synchronize in S phase. *Cdc25-22 rdp1*$^{-}$ mutant has an intermediate phenotype with most cells arresting in S phase, which can be seen by shifting of the 2C peak. *Cdc25-22 ago1*$^{-}$ has a very minor defect and arrests in S phase.

Figure 3-4

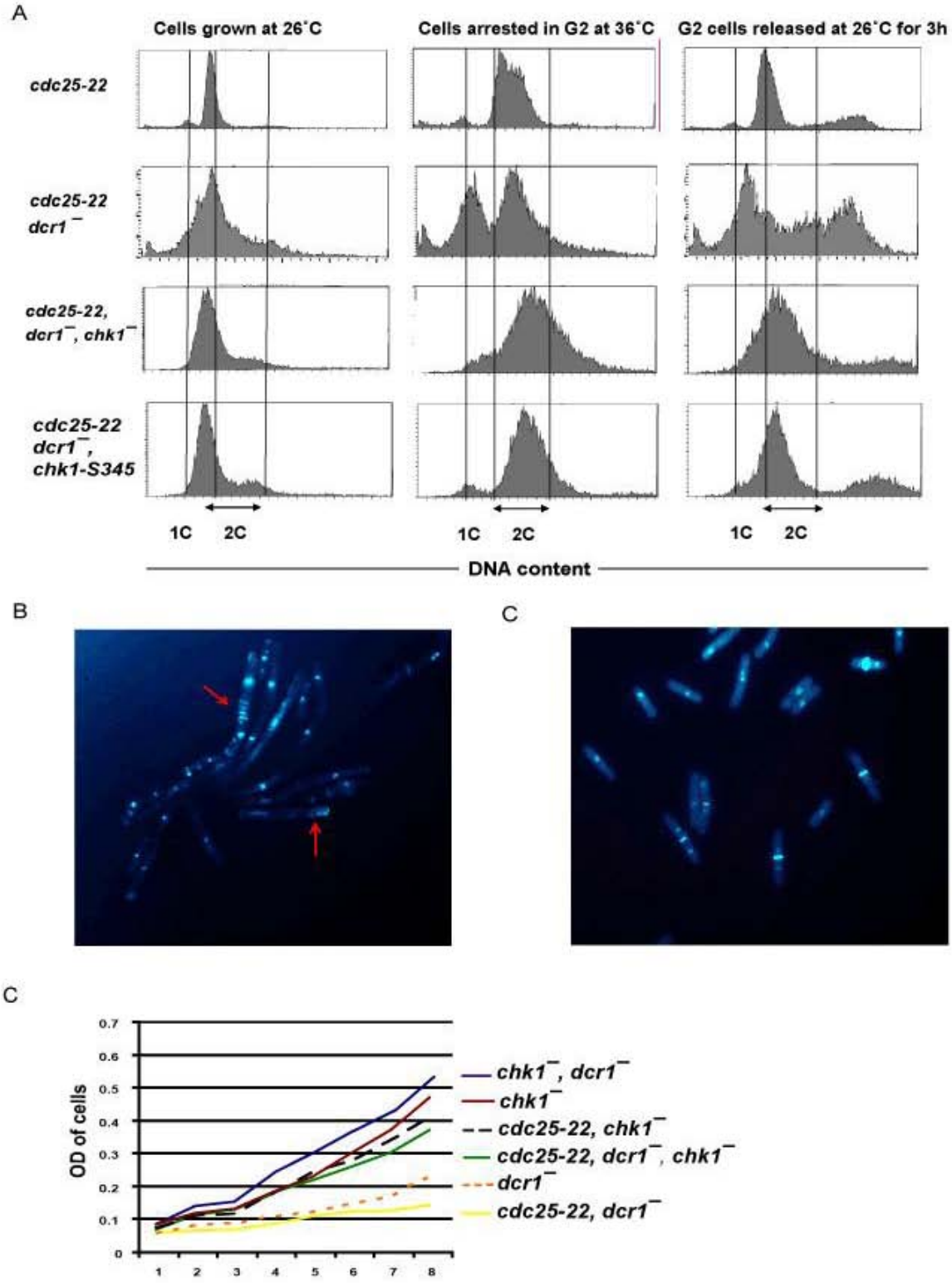

A
Cells grown at 26˚C
Cells arrested in G2 at 36˚C
G2 cells released at 26˚C for 3h
cdc25-22
cdc25-22 dcr1⁻
cdc25-22, dcr1⁻, chk1⁻
cdc25-22 dcr1⁻, chk1-S345
1C
2C
DNA content
B
C
C
OD of cells
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
chk1⁻, dcr1⁻
chk1⁻
cdc25-22, chk1⁻
cdc25-22, dcr1⁻, chk1⁻
dcr1⁻
cdc25-22, dcr1⁻

Fig. 3-4 Comparative analysis of *cdc25-22 dcr1⁻* and *cdc25-22 dcr1⁻ chk1⁻* mutants
A. FACS analysis of *cdc25-22 dcr1⁻, cdc25-22 dcr1⁻ chk1⁻, cdc25-22 dcr1⁻ chk1-S345* and *cdc25-22* mutants. The mutants were grown at 26˚C, arrested at 36˚C and released back to permissive temperature to induce cycling. The samples were taken at 26˚C, after 4 hours at 36˚C and 3 hours after the release to 26˚C.
B. Image of *cdc25-22 dcr1⁻* mutant released from the arrest. The two arrows show multiple septa and fractionated DNA.
C. Image of *cdc25-22 dcr1⁻ chk1⁻* mutants grown at permissive temperature. The triple mutant does not have a severe growth defect that can be seen in *cdc25-22 dcr1⁻*.
D. Growth curve analysis. The strains were grown at 26˚C for 8 hours. OD was measured every 2 hours. *Cdc25-22 dcr1⁻* has the most severe growth defect, which is rescued in *cdc25-22 dcr1⁻ chk1⁻* mutant.

Figure 3-5

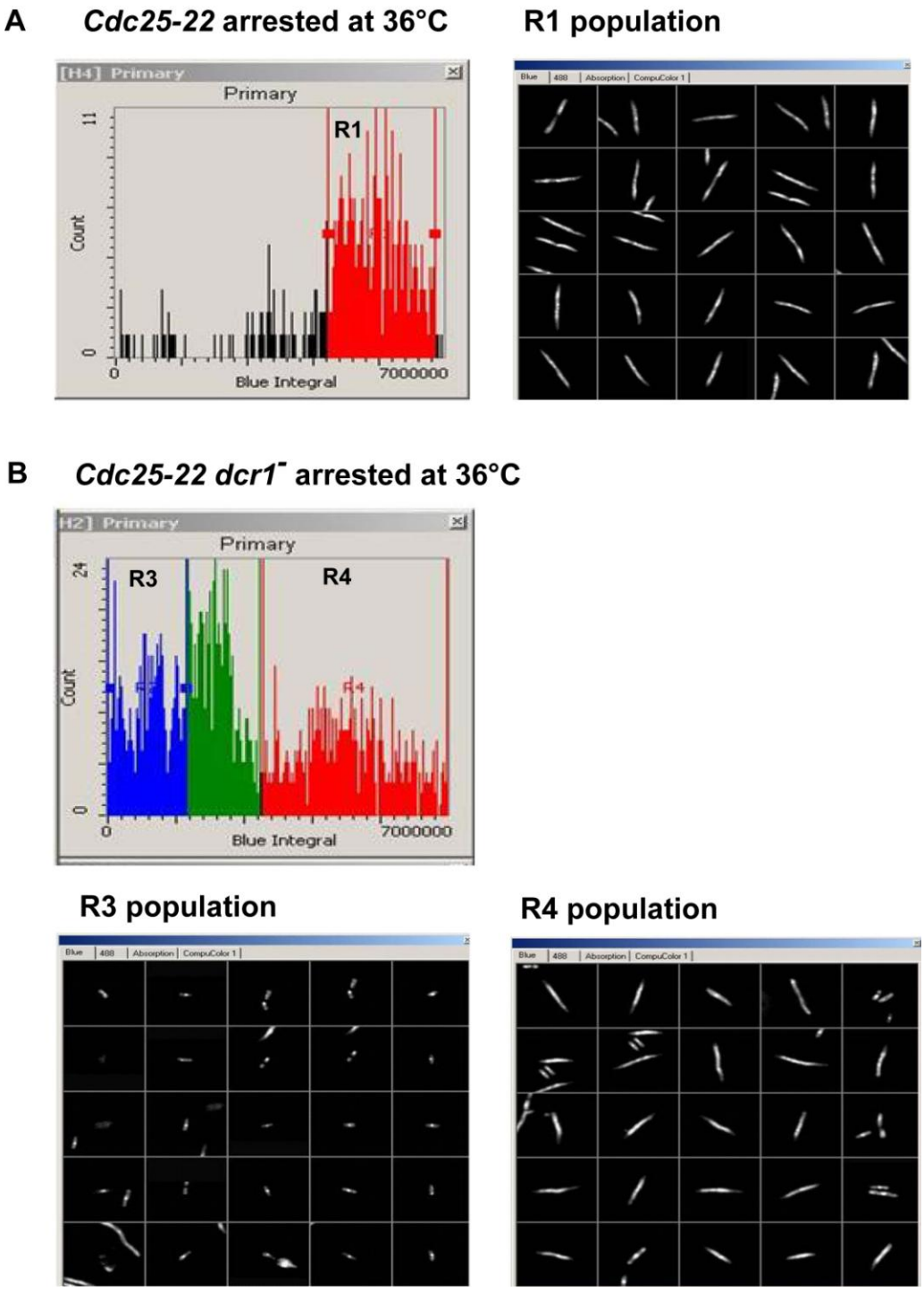

A
Cdc25-22 arrested at 36°C
R1 population
[H4] Primary
Primary
R1
Count
11
0
0
7000000
Blue Integral
B
Cdc25-22 dcr1- arrested at 36°C
[H2] Primary
Primary
R3
R4
Count
24
0
0
7000000
Blue Integral
R3 population
R4 population

Fig. 3-5 iCys Research Imaging Cytometer analysis of *cdc25-22* and *cdc25-22 dcr1*$^{-}$ mutants

A. Temperature induced arrest of *cdc25-22* results in uniform population of elongated G2 cells. The cells were stained with DAPI and analyzed by iCys. The "Blue Integral" (X axis) is a measure of total fluorescence, whereas the Y axis represents cell count. R1 population stained in red represents G2 cells.

B. Arrest of *cdc25-22 dcr1*$^{-}$ gives rise to two populations of cells shown as R3 (blue) and R4 (red) diagrams. The populations of G2 and G1 cells were first established using G2 arrested cells control (A) and G1 population control (data not shown).

Figure 3-6

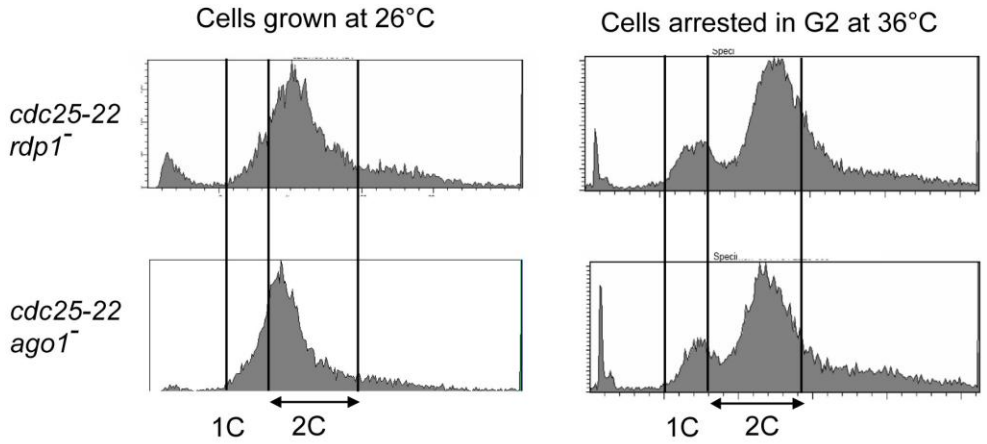


Fig. 3-6 Analysis of *cdc25-22 ago1*$^{-}$ and *cdc25-22 rdp1*$^{-}$ mutants at permissive and restrictive temperatures
Both mutant were grown at 26°C and shifted to 36°C to induce arrest in G2. Unlike *cdc25-22 dcr1*$^{-}$, both *cdc25-22 rdp1*$^{-}$ *cdc25-22 ago1*$^{-}$ arrest in G2, with a minor population of lagging cells in 1C-2C.

Figure 3-7

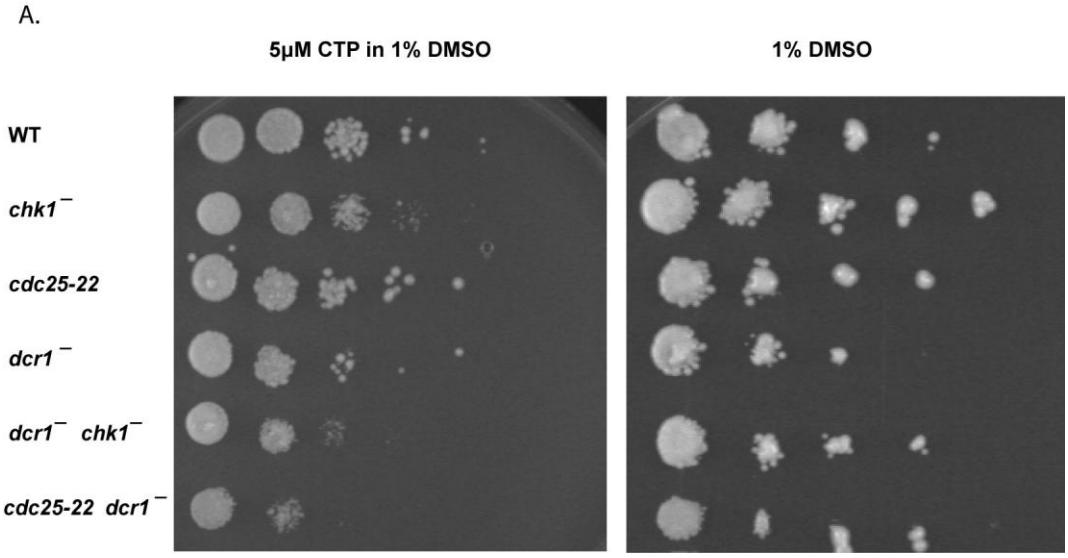


Fig. 3-7 Camptothecin mediated damage assay
10^5 cells were diluted 1:10, plated on DMSO and 5μM camptothecin (CPT) plates and analyzed for growth defects. CPT causes Chk1 phosphorylation, growth defects and slows down progression of S phase. *Cdc25-22 dcr1*$^-$ grow very slow on 5μM camptothecin, indicating damage. *Dcr1*$^-$ *chk1*$^-$ mutant cells also grow slower compared to *chk1*$^-$, which suggests that damage is amplified in the absence of Dcr1 and it occurs even in *cdc25*$^+$ background.

Figure 3-8

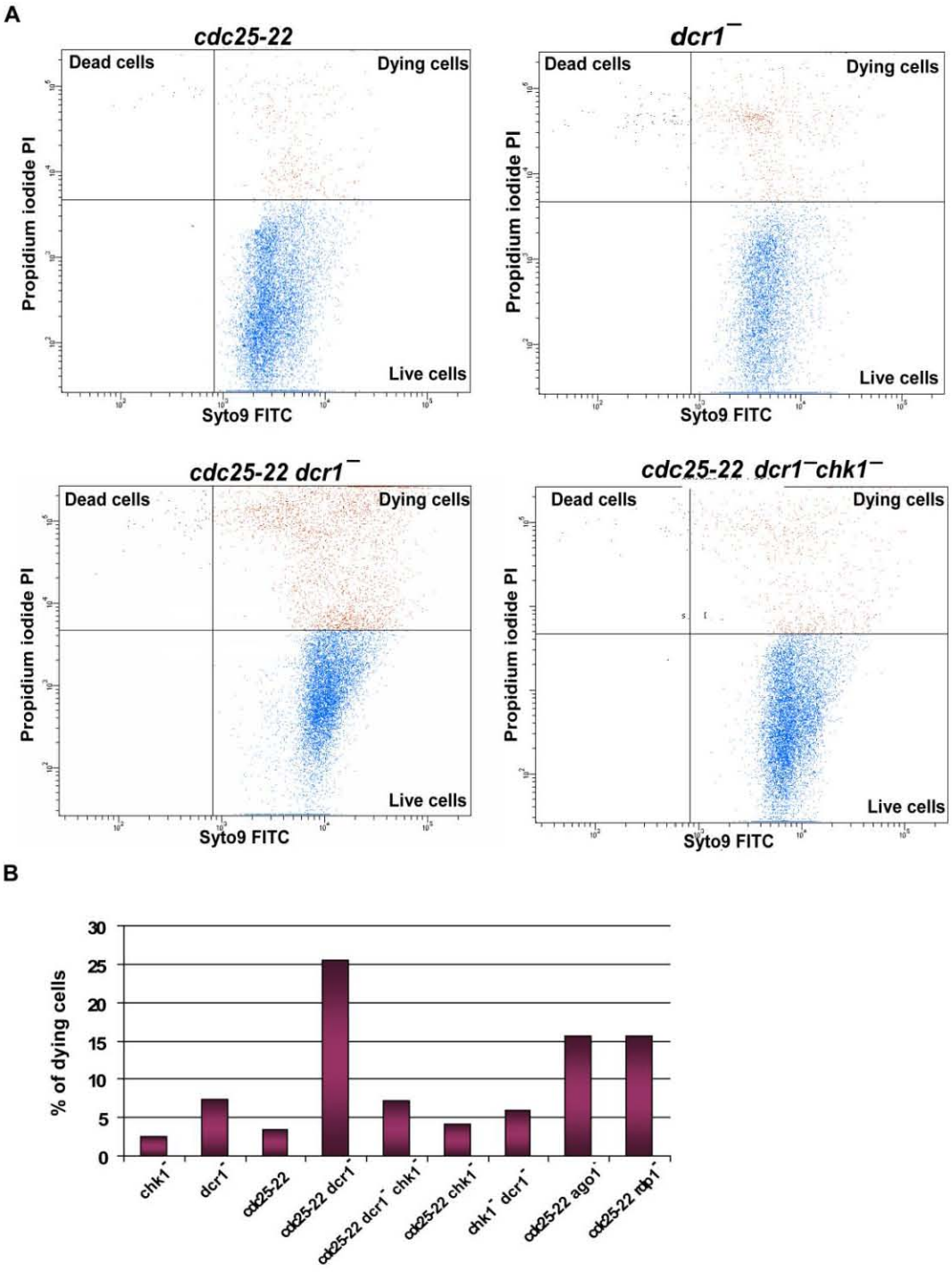

A
cdc25-22
dcr1⁻
cdc25-22 dcr1⁻
cdc25-22 dcr1⁻chk1⁻
Dead cells
Dying cells
Live cells
Propidium iodide PI
Syto9 FITC
B
% of dying cells
30
25
20
15
10
5
0
chk1⁻
dcr1⁻
cdc25-22
cdc25-22 dcr1⁻
cdc25-22 dcr1⁻ chk1⁻
cdc25-22 chk1⁻
chk1⁻ dcr1⁻
cdc25-22 ago1⁻
cdc25-22 rdp1⁻

Fig. 3-8 Analysis of cell viability by dual Syto9 and Propidium Iodine staining
A. All mutants were grown at 26° C to exponential stage, washed and stained with Syto9 and Propidium Iodine (PI) to assess dead and alive cells. The staining was visualized using FACS. Syto9 stains DNA of both live and dead cells, whereas PI stain can only penetrate dead cells. Severe damage in the *cdc25-22 dcr1*$^{-}$ mutant causes cell death. 25% of *cdc25-22 dcr1*$^{-}$ mutant die whereas only 6.5% *cdc25-22 chk1*$^{-}$*dcr1*$^{-}$ die.
B. Quantitative representation of dead (PI stained), dying (PI and Syto9 stained) and live (Syto9 stained) cells.

Figure 3-9

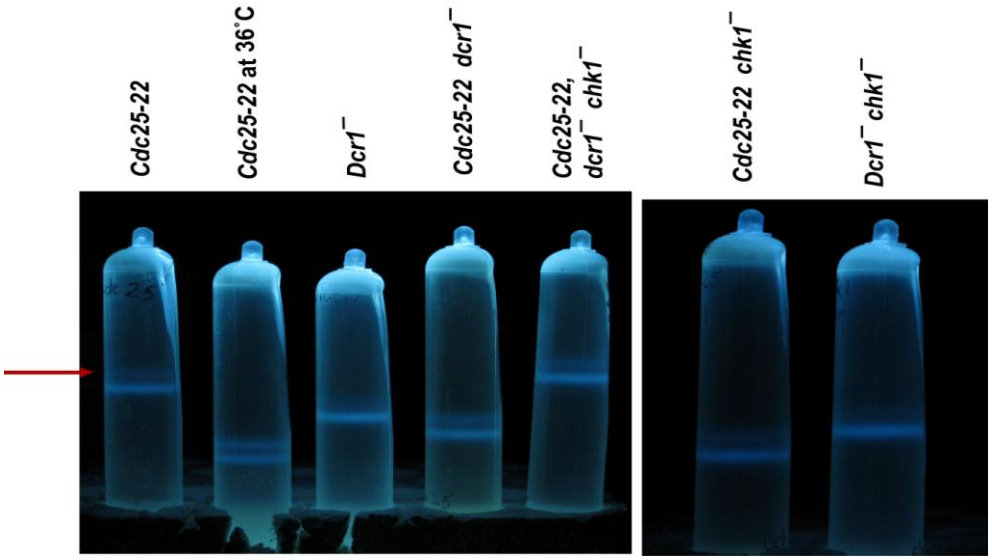


Fig. 3-9 Cesium chloride DNA preparation analysis
Two distinct band can be visualized in DNA preparations in all *cdc25-22* mutants: chromosomal DNA band (lower) and mitochondrial DNA band (upper, shown by red arrow). *Cdc25-22* grown at 36°C and *cdc25-22 dcr1*$^{-}$ grown at 26°C accumulate most mitochondrial DNA.

Figure 3-10

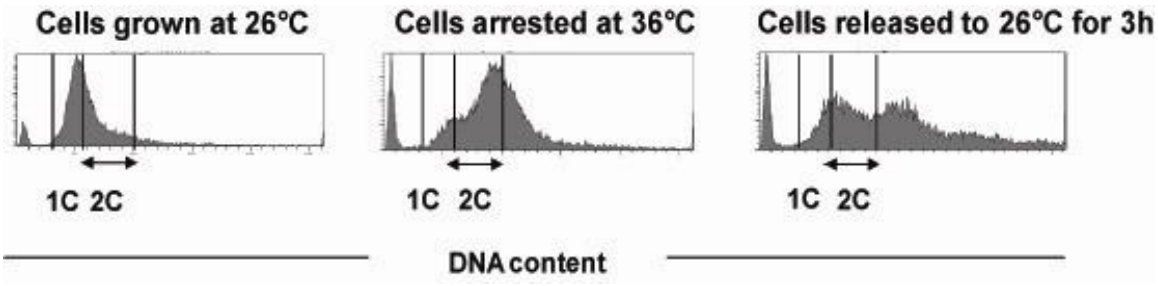


Fig. 3-10 Arrest and release of *cdc25-22 dcr1⁻ rad3⁻* mutant
The mutants were grown at 26˚C, arrested at 36˚C and released back to permissive temperature to induce cycling. Both arrest and release enhances a number of dead cells, as seen in a peak moved to the left.

Figure 11

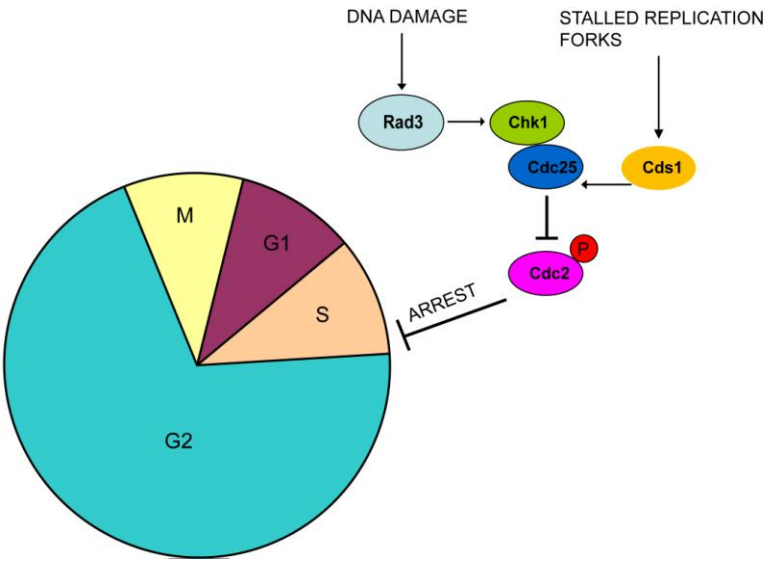


Fig. 3-11 A potential model for Dicer1 involvement in Cdc25 regulation of cell cycle control

DNA damage signal in S phase activates a Cds1 dependent checkpoint response and slows down the cell cycle. The damage is sensed by Chk1, which causes a Chk1-dependent DNA damage signaling that results in arrest in G2. Lack of Dcr1 elicits a Chk1 dependent DNA damage signal when Cdc25 is temporarily inactive, causing permanent damage and cell death. In the absence of Cds1 the cells bypass the S phase. Therefore, *cdc25-22 dcr1*$^{-}$ *cds1*$^{-}$ mutants die due to accumulation of severe damage without repairing it.

3.4 Materials and Methods

3.4.1 FACS analysis of *cdc25,* RNAi double mutants

The mutants were grown at 26˚C (permissive temperature) and shifted to 36˚C (restrictive temperature) for 4 hours. Samples were collected at both 26˚C and 36˚C, spun down, washed with distilled water and resuspended in 70% ethanol. The fixed samples were stored at 4˚C or immediately processed and prepared for analysis. Before FACS analysis, the cells were rehydrated by resuspending in 50uM sodium citrate and treated with 0.1mM RNAseA for 2 hours. Propidium Iodide (PI) was added to the final concentration of 2µM. Samples were processed using LSRII.

3.4.2 iCys Research Imaging Cytometry analysis

Logarithmically grown cells were fixed in 70% ethanol and stored at 4˚C. The cells were rehydrated by washing with water twice and resuspended in 12.5% glycerol solution containing 10µM DAPI. iCys scans were done at 60X magnification and compared to G1 and G2 controls.

3.4.3 Cesium DNA preparation

All preparations were done as described in Cotterill, S "Eukaryotic DNA Replication: a practical approach", Oxford University Press, 1999.

3.4.4 Cell viability assay

A growing population of cells was stained according to instructions of LIVE/DEAD Funga Light Yeast Viability Kit from Invitrogen. Briefly, a live population of exponentially growing *S.pombe* cells was spun down, washed twice with water, resuspended in 1ml of water containing 1µl of Syto9 fluorescence stain to label all DNA within cells and 1 µl of PI stain to label dead cells. The samples were then analyzed by FACS. The number of dead and live cells was determined using a dot plot of forward scatter vs side scatter.

3.4.5 Hydroxyurea induced S phase arrest

Log phase wild type *S.pombe* cells were treated with 15mM hydroxyurea (Sigma) for 3 hours to induce synchronization in S phase. Samples were collected at 1h and 3h and analyzed by FACS.

Chapter IV: Loss of silencing and its potential dependence on inheritance of DNA template

4.1 Introduction

In 1994 Robin Allshire presented an interesting, yet puzzling, result. A *ura4* reporter gene was inserted at centromere I (*cen1*) of *S. pombe* giving rise to variegated expression (Allshire, Javerzat et al. 1994). In addition, an *ade6+* gene placed in *cen1* or *cen3* resulted in variegated expression, seen as white colonies with red sectors. Since the cells of a colony were genetically identical, it was obvious that the variegated pattern of expression was due to an epigenetic mechanism. Epigenetic variegation is a widely recognized phenomenon that has been observed in a large number of species. Today we know that position effect variegation (PEV) is a result of unstable expression or aberrant silencing. Heterochromatin is a central player in PEV (Chapter I 1.9, Fig. 1-1) and RNAi has been proven crucial for heterochromatin assembly. Nonetheless, the variegated phenotype observed upon insertion of the *ade6+* reporter gene at the centromere indicated that RNAi induced silencing is unstable.

The instability of RNAi induced heterochromatin formation may depend on lineage. An *S.pombe* mother cell divides around 20 times before it dies. Lineage dictated developmental fate is familiar in *S.pombe* thanks to Amar Klar and colleagues who showed that switching at the mating type locus depends on inheritance of an imprinted DNA strand (Klar 1990). Furthermore, the DNA replication machinery is involved in both imprinting and initiation of switching (Dalgaard and Klar 1999). The model

presented by Klar and colleagues involves a strand specific nick that is formed by the lagging-strand replication complex (Dalgaard and Klar 1999) and leads to the formation of a double stranded DNA break on one sister chromatid. Homologous recombination leads to a repair of the break by transferring one of the inactive mating type cassettes (either *mat2P* or *mat3M*) to the active *mat1* locus (Klar, Bonaduce et al. 1991; Thon and Klar 1993). This process results in switched mating type. Because of the strand specificity, only one of the two daughter cells has a potential to switch and one out of four granddaughter cells switches its mating type, which is commonly referred to as "one in four rule". This pattern of switching, giving rise to only one switched cell, resembles stem cell lineages in higher eukaryotes.

Provided there is a non-random distribution of DNA templates at cell division, could RNAi be involved in "marking" one sister chromatid, so that it would be distinguished when a cell divides? If this was indeed the case, the "mother" cell that carries the original, "immortal" DNA template would never lose silencing, and therefore an *S.pombe* lineage it gives rise to would never become variegated. The silencing could on the other hand be lost in a daughter cell.

4.1.1 The Immortal Strand Hypothesis

The immortal strand hypothesis, originally presented by John Cairns in 1975 (Cairns 1975), is based on experiments which showed that cultured mouse embryonic cells labeled with tritriated thymidine (Lark, Consigli et al. 1966) were able to retain the label without diluting it. Accordingly, the hypothesis states that during division, one

DNA template gets selectively retained in a single stem cell population, therefore avoiding DNA replication errors. The hypothesis offers a tempting explanation for how an organism could prevent propagation of mutations, especially in cancer cells, since it would always retain an original, intact copy of the DNA template in a population of stem cells.

One notion that supports the non-random distribution of DNA templates suggests processing of DNA strands as a mechanism that may underlie preferential segregation. The two DNA strands are processed differently due to directionality of the replication fork machinery (Lansdorp 2007). The forward, or the leading strand, is replicated continuously, whereas the reverse, or lagging, strand is replicated in short intervals. Once an RNA primer is deposited on the leading strand, replication follows smoothly. On the contrary, lagging strand processing requires formation of Okazaki fragments, recruitment of multiple RNA primases and ligases. Therefore, it would not be surprising if differently processed strands were also differently segregated. Another property of stem cells that distinguishes them from other cells is their reduced capacity to repair DNA. Paradoxically, this property may actually prevent accumulation of mutations that could result in cancer (Cairns 2002). An aberrant stem cell will undergo apoptosis or senescence, reducing the probability of transmitting the damage to daughter cells (Rando 2007).

There is growing evidence for the immortal strand hypothesis in certain cells and tissues. Studies of mouse neuronal stem cells (NSC) at a single cell level revealed that a population of NSC labeled with BrdU was able to retain the signal over multiple divisions (Karpowicz, Morshead et al. 2005). On the other hand, no retention was

observed in mouse fibroblasts (Karpowicz, Morshead et al. 2005). Synchronizing the cell cycle with nocodazole arrest allowed visualization of binucleated NSC with only one BrdU labeled nucleus (Karpowicz, Morshead et al. 2005), a phenomenon not observed in the fibroblasts. Satellite cells, which have stem cell like properties and are important for growth of skeletal muscle and repair, also retain of BrdU label in a "mother, or stem cell" (Shinin, Gayraud-Morel et al. 2006; Conboy, Karasov et al. 2007). However, Numb, a factor important for binary cell fate determination (Uemura, Shepherd et al. 1989; Petersen, Zou et al. 2002) was shown to co-segregate with the mother centrosome in satellite cells, suggesting that multiple factors may contribute to asymmetric segregation (Shinin, Gayraud-Morel et al. 2006). Recently, a study in *S.cerevisiae* proposed a that a kinetochore specific mechanism is involved in asymmetric segregation of DNA templates (Thorpe, Bruno et al. 2008).

On the other hand, there is substantial evidence to disprove the immortal strand hypothesis. For instance, the fact that DNA is constantly subjected to challenge (such as UV, mutagens) increases the chance of affecting the original template (Lansdorp 2007). Unless the probability of stem cells to acquire genetic mutations would be somehow lower compared to other cells, the aberrant stem cells would result in a very high number of mutations. Analysis of individual haematopoietic stem cells (HSCs) stained with BrdU, ClDU and IdU showed no preferential DNA template segregation (Kiel, He et al. 2007). Very few stem cells retained the labeling, which was not consistent with the predicted amounts. Therefore, it was suggested that immortal strand retention is not a general property of stem cells.

Currently, the mechanism responsible for asymmetric strand segregation remains unknown. If there is a non-random segregation of DNA templates, the cell would need a means of differentiating an original strand in order to retain it. It has been postulated that an epigenetic mechanism may facilitate the strand recognition and asymmetric segregation. This epigenetic mark could be deposited on a DNA strand shortly after DNA replication and retained until mitosis. Alternatively, a transcript tethered to one chromosome after replication could temporarily distinguish two DNA strands, impacting variegation.

In the following experiment I explored the idea that RNAi could potentially be involved in distinguishing DNA templates. The presence of single stranded transcripts tethered to a centromere by PolII and the RNAi complex RITS (Martienssen, Zaratiegui et al. 2005; Buhler, Verdel et al. 2006) may impact the establishment of variegation, if the transcripts remain tethered to a single strand following chromosomal replication. This could provide a transient distinction between the two sister chromatids, when tethering, and silencing, is lost from one chromatid. Once the silencing is lost it cannot be regained. Meanwhile, the replication of the other strand would be completed and the strand would be once again able to be transcribed. This strand would remain silenced.

4.2 Results

4.2.1 Measuring mother cell silencing loss

To test if silencing is dependent on lineage I performed an analysis of the strains AP259 and AP263, which were transformed with a construct that contained a *cen2* homology region inserted in a forward and reverse orientation at the *ura4* locus, respectively (Fig. 4-2). I analyzed multiple generations of daughter cells (DA) derived from individual mother cells. Each mother cell was randomly picked from a growing population of either AP259 or AP263. Using a tetrad dissector, I placed the mother cell in position one, and as it divided, I transferred the newly formed daughter cell to a position adjacent to mother cell (Fig. 4-1). I was able to successfully separate around 10-15 daughter cells per one mother cell. It was imperative to look at many generations of daughters derived from a single mother to properly determine the epigenetic state of the mother cell.

The AP259 and AP263 strains, kindly provided by Amikam Cohen (Hebrew University of Jerusalem), were analyzed for lineage specific loss of silencing. Both of these constructs have a 3.6 kB part of the K region fused with *ade6+* reporter gene and inserted at the *ura4* locus on chromosome III (Ayoub, Goldshmidt et al. 2000) (Fig. 4-2). This part of the K region contains the heterochromatic *dg* and *dh* repeats (*cen2* homology), which allowed me to assess their influence on the expression of *ade6+*. In the AP259 construct the *cen2* homology is inserted in the forward orientation, whereas in AP263, *cen2* region is in the reverse orientation with respect to the *ura4* gene and the nearest ARS. Both AP259 and AP263 have a mutation in the endogenous *ade6*, which leads to accumulation of red pigment on low adenine medium due to aberrant adenine biosynthesis. Therefore, if *ade6+* reporter gene linked to *dg* and *dh* repeats is silenced the

colonies are red. White color of colonies indicates expression of *ade6+* and hence loss of silencing.

If the loss of silencing is dependent on single strand tethering of a *dg* or *dh* transcript I would see a difference between the two constructs that would be dependent on their orientation with respect to the direction of the replication fork. The rate of reestablishment of silencing once it is lost is less than 1%, so if a mother cell loses silencing the silenced state would be transmitted to daughter cells. In this case, red or variegated colonies followed by a row of white colonies would be observed (Fig. 4-3, lines 16 and 18). If the mother cell does not lose silencing all colonies derived from it would be red or variegated (Fig. 4-3, lines 19 and 29). If a daughter cell loses silencing, I would see a completely white individual colony, followed by all red/variegated colonies. If the loss of silencing is not dependent on the tethering of the transcript to the chromosome, or if tethering is not strand specific, I will observe a random distribution of the loss of silenced state between mothers and daughters.

Several assumptions were made during the course of the analysis. Ayoub *et al* have shown that loss of silencing occurred at the rate of 30%. Only 1% of cells that lost silencing could revert back to the silenced state. Therefore, I assumed that all white colonies lost silencing during cell division, whereas red colonies never lost silencing. I also assumed that every colony that appeared completely white was derived from a single cell that lost silencing. A white colony could be theoretically derived from a silenced, or red cell that lost silencing after few divisions; however, it is unlikely that all of the daughter cells that came from a silenced mother cell would lose silencing at the same

time. Finally, I assumed that the rate of the silencing loss in an overall population of cells was the same for both AP259 and AP263 constructs.

I have analyzed 38 lineages of AP263 and 29 lineages of AP259 (Appendix 1, Appendix 2). Thirteen AP259 and twenty one AP263 lineages had both red and white colonies, which allowed me to assess the rate of loss of silencing in mother cells. Statistical analysis was performed to determine the rate of loss of silencing per cell division (Appendix 3). There was a significant difference in the loss of silencing in daughter cells between the two strains. 15 daughter cells coming from AP263 lineages lost silencing compared to 6 daughter cells of AP259. On the other hand, the loss of silencing in a mother cell was not significant. 9 out of 14 AP259 lineages showed loss of silencing in a mother cell, compared to only 6 out of 19 AP263 lineages, but when the number of divisions was taken into account (Appendix 3) no statistical difference was detected.

4.3 Discussion

Based on the lineage analysis of AP259 and AP263, loss of silencing in daughter cells is dependent at least in part on orientation of the construct and the *ade6+* reporter gene with respect to the ARS. Therefore the rate of the loss of silencing could be dependent perhaps on inheritance of DNA templates. No statistically significant difference was observed in mother cells. Nonetheless, further lineage analysis could provide insight into the mechanism of strand specific loss of silencing. Differential

timing of expression of RNAi regulated centromeric transcripts during S phase (Chapter II) is also an attractive model of how variegation may arise.

The major limitation of the lineage experiment is proper differentiation of mother and daughter cells. Even though a mother cell's diameter becomes considerably larger after a few divisions (Barker and Walmsley 1999) distinguishing the two newly divided cells is not always trivial during the first 2 or 3 divisions. Since silencing is lost at about 30% rate, a lot of lineages had only white colonies, making those lineages non-informative for the overall experiment. It would be necessary to analyze more lines for each particular construct to be able to draw accurate conclusions. The preliminary observations suggest that the loss of silencing is not all-or-non. Even though the daughters of AP263 lineages lose silencing more frequently, silencing is also lost in the AP259 lines. Nonetheless, the loss of silencing appears to be influenced by the orientation of *cen2* homology with respect to the ARS. In addition, the different timing of the single stranded centromeric transcripts during early and mid S phase may result in transient tethering, recognition of a particular strand and preferential segregation. These hypotheses need to be explored in more detail.

Strand specific tethering of transcripts to the centromere may impact our perception of RNAi induced variegation. In a larger study, we could also investigate age-dependent silencing loss. McMurray *et al* showed that loss of heterozygosity in *S.cerevisiae* increases with the age of mother cells (McMurray and Gottschling 2003). Any potential correlation with age could impact our understanding of the aging process.

Figure 4-1

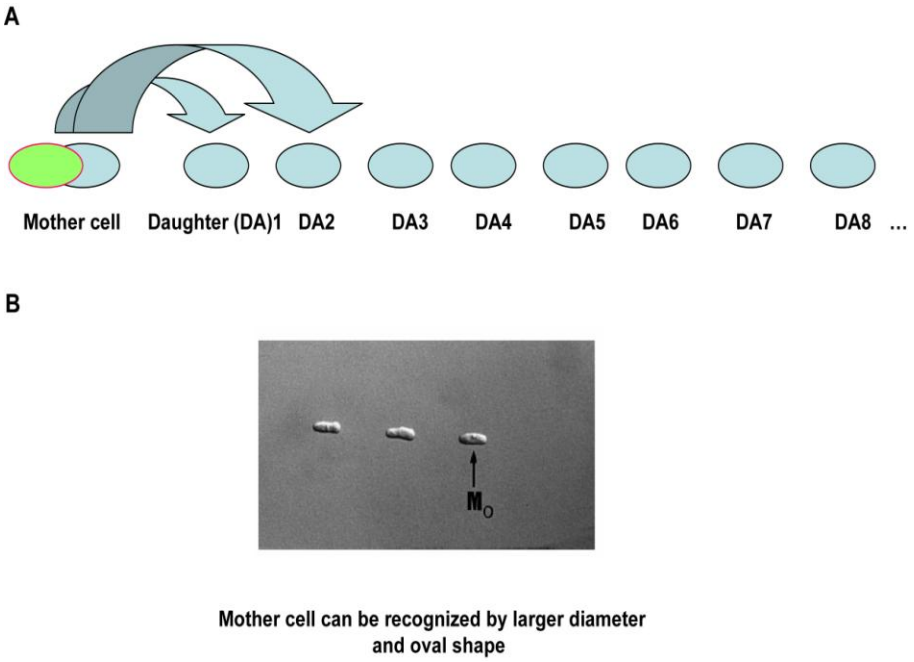


Fig. 4-1 Experimental analysis of loss of silencing
A. Experimental approach to mother-daughter isolation after division. As a mother cell divides, the single newly formed daughter cells are placed to the right, in position 1, 2, 3….
B. Graphic representation of differences between mother (right) and daughter (left and middle) cells [reproduced with permission from (Barker and Walmsley 1999)]. As a mother cell (Mo in the picture) divides, its diameter increases and the cell shape becomes somewhat oval and smaller than newly formed daughter cell, as can be seen in the image. An old mother cell, which has divided around 15-20 times becomes almost completely round.

Figure 4-2

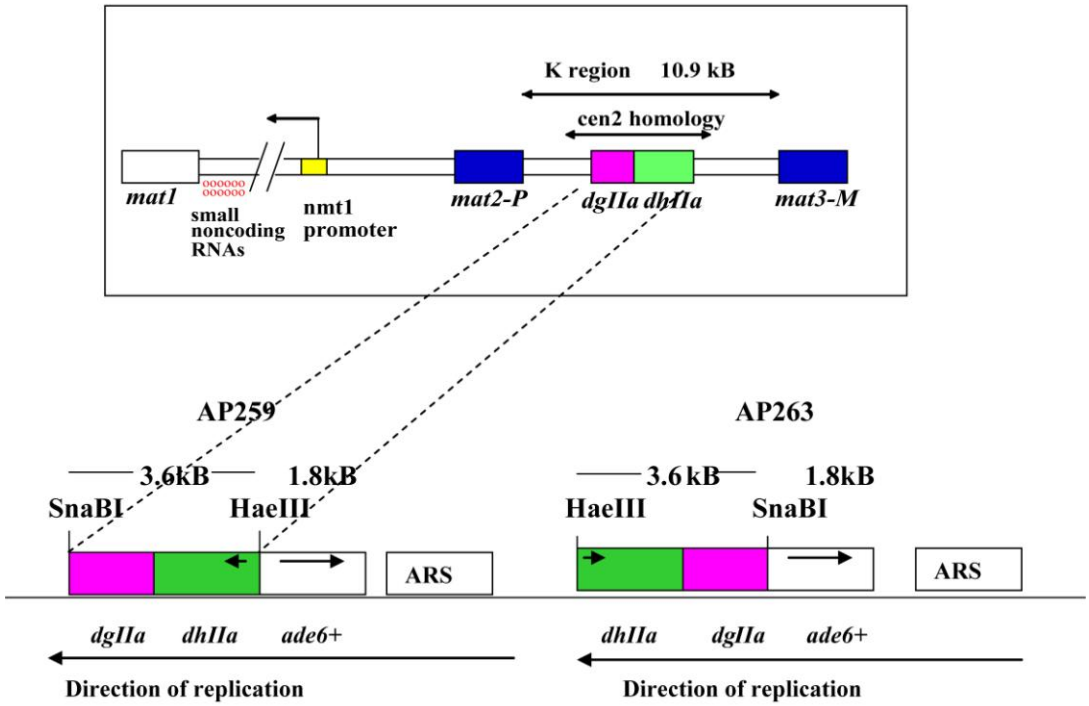


Fig. 4-2 Design of AP259 and AP263 constructs
Cen2 homology, derived from the silent mating type locus (above panel) was fused to the *ade6+* reporter gene and inserted in both forward (AP259) and reverse (AP263) orientation with respect to the ARS at *ura4* on chromosome III. Arrows indicate promoters and their directions.

Figure 4-3

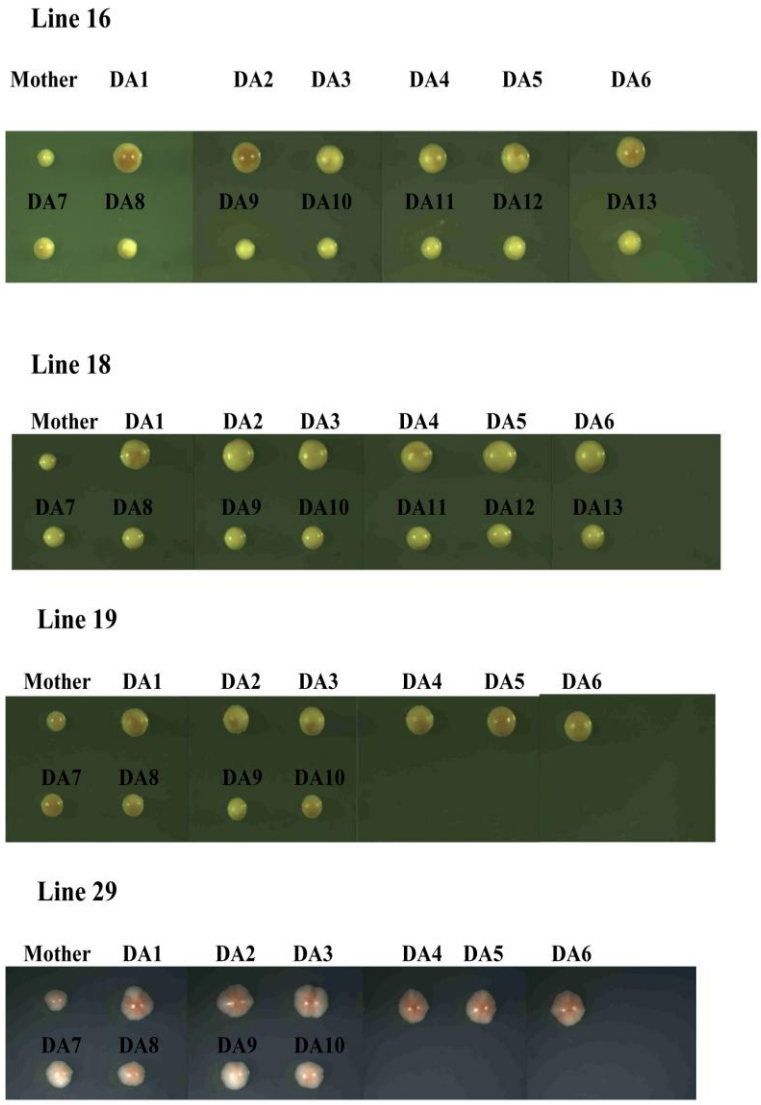


Figure 4-3 Analysis of loss of silencing in AP259
The first colony of each line derives from the mother cell after the last daughter selection. Each successive daughter cell (DA) was separated from the mother cell upon division and placed to its right. Lines 16 and 18 show loss of silencing in the mother cell, which is indicated by a row of white daughter cells. Because all previous daughters were derived from a red or variegated mother cell, the mother cell must have lost silencing. In lines 19 and 29 the mother cell has not lost silencing, as it still has some red pigmentation.

Figure 4-4

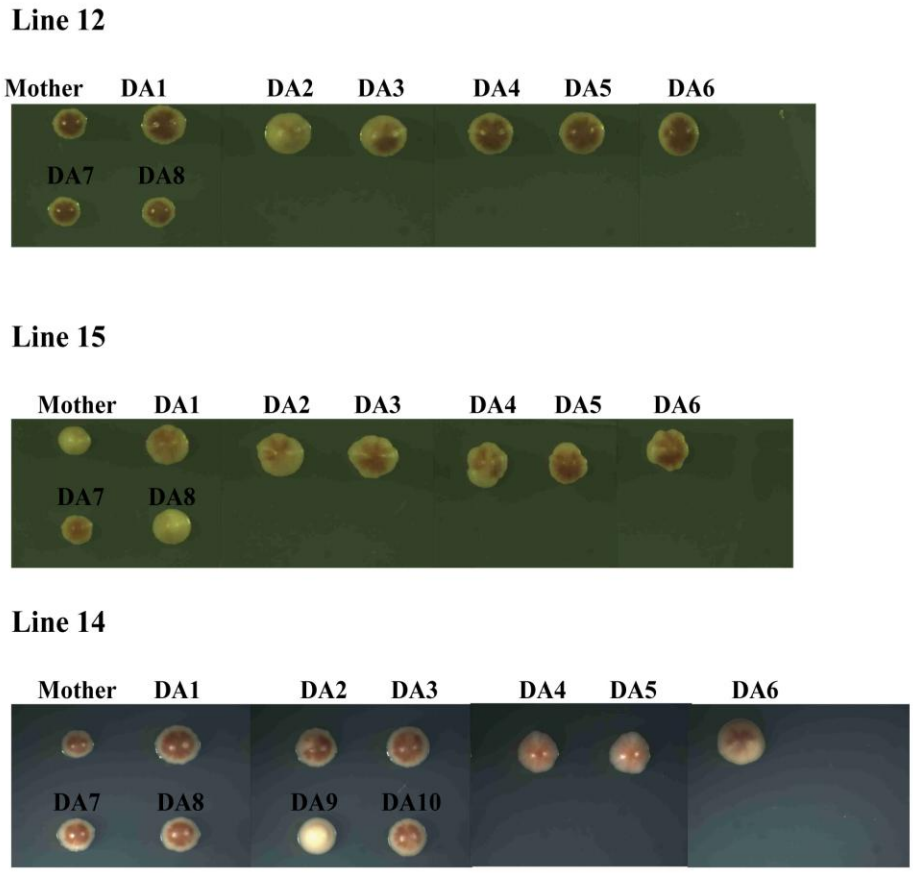


Fig. 4-4 Analysis of loss of silencing in AP263
The first colony of each line derives from the original mother cell after the last daughter selection. Each successive daughter cell (DA) was separated from the mother cell upon division and placed to its right. In Line 15, the mother cell has lost silencing. In Lines 12 and 14, the mother cell retains pigmentation, and so has not lost silencing. However, silencing is lost in DA9 (line 14).

4.4 Materials and Methods

4.4.1 Fission yeast strains

AP259 and AP263 strains were a kind gift from Amikam Cohen. The strains were grown in YE medium with low adenine concentrations.

4.4.2 Experimental approach

A lineage analysis was performed using a single “mother” cell picked randomly from a growing population of either AP259 or AP263 strain. The mother cell was separated using a tetrad dissector and allowed to divide on YE medium. A new daughter cell was separated from the original mother cell upon division and placed in an appropriate position on plate. A lineage derived from one mother cell was then incubated at 30°C for 4 days. The colonies were scored for white, red or variegated color and photographed.

Chapter V: Future directions and conclusions

5.1 Summary

During my studies I have pursued a variety of projects related to epigenetic inheritance, cell cycle control and variegation. I explored functions of RNAi that are different from its well established role in silencing, and I showed that RNAi mediated silencing is cell cycle dependent. The analysis of RNAi regulated centromeric transcripts, siRNA and histone modifications provided evidence for a mechanism of inheritance of epigenetic marks and uncovered a role of the phospho-methyl switch in maintaining them. I also showed that RNAi ensures cell cycle integrity. Analysis of cell cycle checkpoints revealed that Dcr1 is likely involved in replication fork stability in S phase.

5.1.1 Epigenetic inheritance: what is next?

Inheritance of epigenetic marks has been an intriguing question of molecular biology. The S phase of the cell cycle must be properly controlled to ensure the transmission of a faithfully replicated genome to both daughter cells. Therefore, it is not surprising that the inheritance of epigenetic states would occur at this time. Various studies have shown that heterochromatin regulation is a dynamic process that participates in cell cycle regulation, transposon control and epigenetic inheritance. The role of RNAi in facilitating epigenetic inheritance in the S phase of the cell cycle might be helpful in

elucidating the role of RNAi in XCI or imprinting, which also rely on heterochromatic modifications and on replication.

The dependence of RNAi on the S phase of the cell cycle, as manifested by S phase specific double stranded RNA transcription and siRNA generation, raises a question of potential reliance of epigenetic inheritance on S phase. It is well established that histones can be deposited in a manner that is both dependent and independent of DNA replication. Does it mean that the epigenetic modifications associated with such histones have to wait for the next S phase to get reestablished, or is there a mechanism that allows more timely incorporation of the epigenetic marks? Recent studies in *Xenopus* have shown that histone variant 3.3 (H3.3), which is deposited outside of S phase is involved in epigenetic memory (Ng and Gurdon 2008), which implies importance of other, yet to be discovered, pathways in maintaining "epigenetic identity". This observation is further supported by the fact that quiescent cells also have an epigenetic memory. Therefore, even though we can view the S phase of the cell cycle as a "window of opportunity" for not only DNA replication, but also for the transmission of epigenetic states to subsequent generations, other stages of cell cycle are likely to play a role in inheritance of epigenetic marks. These fundamental questions of the epigenetic field await further clarification.

5.1.2 RNAi and epigenetic inheritance: what else could be involved?

When it comes to replication, pericentric regions in *S.pombe* are unusual. Contrary to most heterochromatin regions, they replicate early during S phase, before

euchromatic regions. Recent analysis suggests that heterochromatic proteins could actively regulate DNA replication initiation. Interaction between Swi6 and DDK (Dbf4-dependent Cdc7 kinase) has been shown to recruit DDK to pericentric heterochromatin regions, as well as *mat1* locus to ensure its replication (Hayashi, Takahashi et al. 2009). Swi6 may also interact with the replication protein Cdc18 (S. Forsburg, personal communication). I detect transient Swi6 increase at the G1/S boundary, which supports this idea.

5.2 RNAi and cell cycle

Cell cycle analysis of RNAi induced silencing revealed a strong correlation between these two processes. It is therefore not entirely surprising to observe that RNAi components participate in cell cycle control. Specifically, the S phase of the cell cycle appears to be regulated by Dcr1. My current data suggests that this regulation is crucial for genome stability.

5.2.1 Genome stability and cell cycle: why Dcr1….

Genetic analysis of *cdc25-22 dcr1*$^{-}$ analysis by FACS, camptothecin treatment and growth curves indicate that lack of Dcr1 triggers the Chk1 dependent DNA damage response. Further, Dcr1 and Cds1 are required for maintenance of the S phase and proper response to damage.

These observations are supported by the analysis of *Drosophila* rasiRNA (Chapter III, 3.3.2). It is suggested that rasiRNA pathway suppresses DNA damage signaling, as

the phenotypes associated with mutations in *armi* and *aub* genes are suppressed by mutants in the ATR/Chk2 pathway (Klattenhoff, Bratu et al. 2007). The phenotype of the *cdc25-22 dcr1*$^{-}$ mutant also implies that Dcr1 is involved in suppressing DNA damage. In addition, the triple *cdc25-22 dcr1*$^{-}$*chk1*$^{-}$ mutant suppresses the *cdc25-22 dcr1*$^{-}$ phenotype. However, *cdc25-22 dcr1*$^{-}$ mutants are slightly enhanced by Chk2 homolog Cds1, rather than suppressed, perhaps reflecting species specificity in checkpoint control.

In order to elucidate the role of Dcr1 in DNA damage response and S phase integrity future work should focus on whether the phenotype observed by both FACS and growth analysis depends on Dicer's catalytic RNase III domain, which is specifically involved in processing of the double stranded RNA transcripts. If the catalytic mutant rescued the phenotype of *cdc25-22 dcr1*$^{-}$ cells, it would suggest a role of Dcr1 that is independent of its function in siRNA biogenesis. No rescue would indicate that the catalytic domain of Dcr1 is indeed responsible for the DNA damage response and slow growth. This result would imply a role of siRNA in DNA replication and S phase integrity. This hypothesis is further supported the fact that *cdc25-22 rdp1*$^{-}$ mutant are also significantly elongated, and Rdp1 amplifies dsRNA and siRNA. The *cdc25-22 ago1*$^{-}$ mutant has the least defects and it grows fastest compared to other double mutants. Therefore, these results imply an additional function for siRNA, which is independent of Ago1.

5.2.2 Involvement of Dcr1 in Cds1 dependent replication checkpoint

The phenotypes of *cdc25-22 dcr1*$^{-}$ and *cdc25-22 dcr1*$^{-}$*cds1*$^{-}$ mutants imply that Dcr1 could be involved in S phase regulation. Cds1 is a mediator of DNA replication

checkpoint during S phase, and it also plays a role in stabilizing stalled replication forks (Chapter III, 3.1.4). The triple mutant does not slow down S phase because it lacks Cds1. Therefore it does not have an opportunity to recover from S phase specific damage, which leads to cell death. *Dcr1*$^{-}$ could trigger a Cds1 response. To test this, I would perform a Cds1 kinase activity assay using an antibody against phosphorylated Cds1. I would also test *rdp1*$^{-}$, *ago1*$^{-}$, *cdc25-22 dcr1*$^{-}$, *cdc25-22 rdp1*$^{-}$ and *cdc25-22 ago1*$^{-}$ to check for Cds1 kinase activity. Because *cdc25-22 rdp1*$^{-}$ and *cdc25-22 ago1*$^{-}$ mutants have a mild phenotype compared to *cdc25-22 dcr1*$^{-}$, I would expect to see none or very little phosphorylation of Cds1 in these mutants. Increased Cds1 kinase activity in *dcr1*$^{-}$ would further imply that Dcr1 plays a role in S phase by stabilizing replication forks. It would also confirm that Dcr1 is functionally different from other RNAi mutants.

5.2.3 RNAi and cell cycle: could it be DNA repair?

DNA repair is fundamental to maintaining genome integrity of an organism. Two types of repair pathways- homologous recombination (HR) and Non-Homologous End Joining (NHEJ) specifically repair double stranded breaks. HR occurs in S and G2 phases of the cell cycle and repairs DSBs by copying genetic information from the sister chromatid, whereas NHEJ acts in G1 and ligates breaks together, resulting in loss of DNA sequence (Dinant, Houtsmuller et al. 2008). *S.pombe* can distinguish different types of DNA damage (Rhind and Russell 1998) and invoke an appropriate response that is specific to a cell cycle stage (Rhind and Russell 1998). The double arrest phenotype could be of significance in understanding the role of RNAi in DNA repair. A potential

role of Dcr1 in repair of naturally occurring DSBs could result in inability to complete the S phase in *cdc25-22 dcr1*$^{-}$ mutants. Failure to repair DSBs could result in a DNA damage response observed in the double mutant. Recent studies have shown that DNA damage checkpoints are involved in repair of heterochromatin. Heterochromatin repair proceeds much slower than euchromatin repair due to presence of heterochromatic factors, such as KAP-1 or HP1 (Goodarzi, Noon et al. 2008). Repair of heterochromatic DSBs involves the Rad3 homolog ATM (Goodarzi, Noon et al. 2008). Phosphorylation of Kap-1 by ATM is thought to diminish its localization with heterochromatin and make it more permissible for repair. The repetitive nature of many heterochromatic regions could indicate that heterochromatic DNA may employ different regulatory mechanism during repair.

S.pombe contains Rik1, a DDB1 (DNA damage binding protein) ortholog associates with Cul4, and is thought to be important for gene stability and RNAi (Fig. 1-2) (Jia, Kobayashi et al. 2005). In humans, DDB1-Cul4 complex has been implicated in nucleotide excision repair (NER) (Holmberg, Fleck et al. 2005). Even though Ago1 is required for Rik1 spreading, it does not seem to have a role in DNA damage according to our analysis (Chapter III). This could further support a hypothesis that the role of Dcr1 in DNA damage is different from other RNAi proteins.

Posttranslational modifications of histones also contribute to DNA repair. Histone 4 acetylation has been shown to be involved in NHEJ and DNA replication coupled repair (Bird, Yu et al. 2002). Both types of repair are mediated by ESA1 histone acetyl transferase. Recruitment of repair proteins to the site of damage is a key aspect of repair (Bradbury and Jackson 2003; Wurtele and Verreault 2006). Arp4, a histone tail binding

protein, which is a part of ESA1 complex is recruited directly to DSB sites (Bird, Yu et al. 2002). HMGM1, a nucleosome binding protein, affects the response to irradiation induced HDSB by facilitating ATM interaction with chromatin (Kim, Gerlitz et al. 2009). Recent studies have shown that histone modifications are crucial for reestablishment of chromatin after DNA damage. Acetylation of histone 3 lysine 56, mediated by histone chaperone Asf1 and histone acetyl transferase Rtt109, repairs DSBs and promotes chromatin assembly (Chen, Carson et al. 2008; Li, Zhou et al. 2008). Further studies will help elucidate any role of RNAi in maintaining genome integrity via histone modifications.

5.3 Reconciling Immortal Strand Hypothesis: what have we learned from the analysis of AP259 and AP263 constructs?

The lineage analysis of loss of silencing in strains with different orientations of the *cen* repeat relative the a reporter gene (AP259 and AP263) reveals that there is a weak correlation between the rate of loss of silencing and "strandedness" in daughter cells, as daughters of AP263 strain lost silencing more frequently than the daughters of AP259 strain. The lineage analysis of both strains showed no statistically significant loss of silencing in mother cells. However, there are few experiments, which could be informative in reconciling the role of "strandedness" in loss of silencing, and perhaps provide evidence for loss of silencing in mother cells.

The current experimental observations of loss of silencing are based on the analysis of mother cells randomly picked from a growing cell culture. We cannot exclude

a possibility that this randomly picked mother cell is a daughter cell that lost silencing in a course of a few divisions. Instead, the lineage analysis could be performed on *S.pombe* spores, which might fix the lineage following the "erasure" at meiosis.

Another experiment that could be useful in assessing non-random segregation involves BrdU labeling analysis. A fission yeast FY2316 strain was engineered to take up BrdU from the growth medium (Hodson, Bailis et al. 2003). Preferential segregation could be visualized if an exponentially growing population of cells is incubated with BrdU for one cell cycle, washed and be prepared for immunohistochemistry using anti-BrdU antibody. Asymmetric labeling of one nucleus and not the other in recently divided cells would indicate preferential "immortal" strand segregation, as has been demonstrated in mammalian cells (Tannenbaum, Sherley et al. 2005; Armakolas and Klar 2006).

In order to investigate the strandedness of the tethered transcripts, strand specific qRT-PCR could be performed in synchronized cells. It is well established that RITS tethers transcripts to the template (Buhler, Verdel et al. 2006). An analysis of single stranded tethered transcripts, combined with its expression during S phase could provide insight into the rate of loss of silencing observed during lineage experiments and whether RNAi may be involved.

5.4 RNAi- final thoughts

During the last decade, RNAi has emerged as a conserved mechanism that controls gene silencing. Its effects have been widely studied in organisms ranging from *S.pombe* to mammals, as its implications for understanding silencing and hence diseases

are enormous. This work and the work of others have shown other significant functions of this conserved mechanisms that stretch beyond its role in silencing. Further work on RNAi in various organisms will certainly uncover other components of RNAi pathways, clarify the involvement of RNAi's in regulation of cell cycle checkpoints and perhaps provide evidence for a direct role in DNA replication and epigenetic inheritance.

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Appendix 1 Analysis of AP 263 lineages

	Mother	Daughter 1	Daughter 2	Daughter 3	Daughter 4	Daughter 5	Daughter 6	Daughter 7	Daughter 8	Daughter 9	Daughter 10	Daughter 11	Daughter 12	Daughter 13	Daughter 14
Line 1	V	V	V	V		V	V	V	R	V	V	R	W	W	
Line 2	W	W	W	W	W	W	W	W	W	W	W	W			
Line 3	W	W	W	W	W	W	W	W	W	W	W				
Line 4	W	W	W	W	W	W	W	W	W						
Line 5	V	V	V	V	V	V	V	V	V	W	V	V	V		
Line 6	W		R	V	V	R	V	V	V	V					
Line 7	R	R	R	R	R	R	R								
Line 8	R	R	R	R	R	R	R	R	R	R	R	R	R		
Line 9	W	W	W	W	W	W	W	W							
Line 10	V	V	V	V	V	V	V		V	V					
Line 11	V	V	V	V	V	V	V	V	V	V					
Line 12	R/V	V	V	V	V	V	V	R	V						
Line 13	W	W	W	W	W	W	W	W							
Line 14	R	V	V	V	V	V	V	R	V	W	V	V			
Line 15	W	V	V	V	V	V	V	R/V	W						
Line 16	V	W	W	V	V	V	W	V	V	V	V	V			
Line 17	W	W	V	W	W	W	W	W	W	W	W				
Line 18	W	V	V	W	W	W	W	W	W						
Line 19	R	V	V	R	R	R	V	V	R	V	R	R	R		
Line 20	V		R	V	V	R	V	V	V	V					
Line 21	R	V	V	R	V	V	V	V	V	V	R	V			
Line 22	W	W	W	W	W	W	W	W	W	W	W	W	W		
Line 23	W	W	W	W	W	W	W	W	W	W	W	W	W		
Line 24	W	W	W	W	W	W	W	W	W	W	W	W	W		
Line 25	V	V	V	V	V	W	W	W	W	W	W	W	W	W	
Line 26	W	V	V	V	V	V	V	V	V	W					
Line 27	W	W	W	W	W	W	W	W	W	W	W	W	W	W	
Line 28	W	W	W	W	W	W	W	W	W	W	W	W			
Line 29	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
Line 30	W	V	V	V	V	V	W	W	W	W	W	W	W	W	
Line 31	W	W	W	W	W	W	W	W	W	W	W	W	W		
Line 32	W	W	W	W	W	W	W	W	W	W	W	W	W		
Line 33	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
Line 34	W	W	W	W	W	W	W	W	W	W	W				
Line 35	W	W	W	W	W	W	W	W	W	W	W	W	W		

Line 36	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**		
Line 37	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**		
Line 38	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**	**W**		

Appendix 1 and 2 Lineage analysis of AP259 and AP263 constructs

A red colony of either AP259 or AP263 strain was isolated on YE plate. It was imperative to start with a colony that was silenced to assess if silencing can occur in a lineage derived from a single mother cell. The red colony was spread on YE medium and a single, random cell was picked up using a tetrad dissector. The first cell of each lineage was a mother cell. As the mother cell divided, a newly formed daughter cell was placed to its right (daughter 1, daughter 2, daughter 3, etc.). After the analysis, the lineages were grown on YE plates at 30˚C. Overall, I analyzed 29 lineages of AP259 construct and 38 lineages of AP263 construct.

The silencing state is expressed as color coded W (black color), V (pink color) and R (red color) letter. These letters correspond to the silencing state of a particular colony. “W” indicates white color of a colony and hence loss of silencing. “V”, or pink, indicates variegated state, hence the colonies had a mixture of red and white. “R” corresponds to completely red colony, which was silenced.

Because the rate of loss of silencing is over 30%, some original mother cells were silenced to begin with. The lineages that arose from that mother cell were not informative for the overall experiment, as the loss of silencing cannot be observed in a lineage that already lost silencing. These lineages have only white colonies (for instance lineages 31-38 of AP263 construct). Loss of silencing in a mother cell can be seen as a red or pink colonies followed by only white colonies (line 30 of AP 263 lineage). Loss of silencing in daughter cell can be seen as a single white colony in a lineage that is red (silenced) or pink (variegated) (daughter 9 line 5 of AP263 lineage).

Appendix 2 Analysis of AP259 lineages

	Mother	Daughter 1	Daughter 2	Daughter 3	Daughter 4	Daughter 5	Daughter 6	Daughter 7	Daughter 8	Daughter 9	Daughter 10	Daughter 11	Daughter 12	Daughter 13	Daughter 14
Line 1	W	W	W	W	W	W	W	W							
Line 2	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
Line 3	W	W	W	W	W	W	W	W	W	W	W	W	W	W	
Line 4	W	W	W	W	W	W	W	W	W	W	W	W	W		
Line 5	W	W	W	W	W	W	W	W	W	W	W				
Line 6	R	R	R	R	R	R	R	R	V	V	V	V			
Line 7	W	W	W	W	W	W	W	W	W	W	W	W	W		
Line 8	W	W	W	W	W	W	W	W	W	W	W	W			
Line 9	W	W	W	W	W	W	W	W	W	W	W				
Line 10	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
Line 11	W	W	W	W	W	W	W	W	W	W	W	W	W	W	
Line 12	W	V	V	W	W	W	W	W	V	W	W	W	W	W	W
Line 13	W	V	W	W	V	V	V	V	V	W	W	W	W	W	W
Line 14	V	V	V	R	V	V	V	V	V	V	V	V	V		
Line 15	W	W	W	W	W	W	W	W	W	W	W	W			
Line 16	W	V	V	W	V	V	V	V	W	W	W	W	W	W	
Line 17	W	W	V	W	V	W	W	V	V	W	W	W	W	W	
Line 18	W	V	W	V	V	W	W	W	W	W	W	W	W	W	W
Line 19	V	V	V	V	V	V	V	V	V	V					
Line 20	W	W	W	W	W	W	W	W	W	W	W				
Line 21	W	W	W	W	V	W									
Line 22	W	W	W	W	W	W	W	W	W	W	W	W	W	W	
Line 23	W	V	V	V	V	W	V	V	W	W	W	W	W	W	
Line 24	W	W	W	W	W	W	W	W	W	W					
Line 25	W	W		W	W	W	W	W	W	W	W	W	W	W	
Line 26	W	V	W	W	W	W	W	W	W	W	W	w			
Line 27	W	V	W	V	V	W	W	W	W	W	W	W			
Line 28	V	V	V	V	V	V	V	V	V	V	V				
Line 29	V	V	V	V	V	V	V	V	V	V	V				

Appendix 3 Statistical analysis of loss of silencing in mothers and daughters

A

Statistical analysis of loss of silencing in mothers and daughter cells

	259	263	Raw total
Loss of silencing in a mother cell	9	6	15
No loss of silencing	102	157	259
Total	111	163	274

Loss of silencing in a daughter cell	15	6	21
No loss of silencing	96	157	253
Total	111	163	274

1) Expected frequencies for 259: 6.72

Expected frequencies for 263: 8.27

2) X^2= ((O - E)2/E)

3) Degree of freedom (df) : 1

4) Yates correction: x^2 if df =1

5) X^2= ((IO – EI-0.5)2/E)

6) X^2= 2.37

For X^2 of p=0.05 with a df of 1, the table value is 3.84

2.37<3.84, therefore the difference between the silencing of a mother cell is not statistically significant.

Loss of silencing in a daughter cell:

X^2= 8.76

8.76>3.84, therefore the difference in the loss of silencing observed in a daughter cell is statistically significant.

B

Calculation details- loss of silencing in a mother cell

- Loss of silencing in a mother cell: AP259 and AP263
- 15x111/274=6.1 -expected frequency for loss of silencing in AP259
- 15x163/274=8.9 -expected frequency for loss of silencing in AP263
- 259x111/274=104.9 -expected frequency for no loss of silencing in AP259
- 259x163/274=154.1 –expected frequency for no loss of silencing in AP263

- X^2= ((IO – EI-0.5)2/E) YATES CORRECTION
- AP259 loss of silencing x^2= (9 -6.1 -0.5)^2/6.1=0.94
- AP259 no loss of silencing x^2=(102 - 104.9-0.5)^2/104.9=0.11
- AP263 loss of silencing x^2= (6 -8.9 -0.5)^2/8.9=1.29
- AP263 no loss of silencing x^2=(157 -154.1-0.5)^/154.1=0.03

- Total sum = 0.94+0.11+1.29+0.03=2.37
- 2.37<3.84, therefore loss of silencing in a mother cell is not statistically significant

C

Calculation details- loss of silencing in a daughter cell

- Loss of silencing in a daughter cell: AP259 and AP263
- 21x111/274=8.5 -expected frequency for loss of silencing in AP259
- 21x163/274=12.4 -expected frequency for loss of silencing in AP263
- 253x111/274=102.5 -expected frequency for no loss of silencing in AP259
- 253x163/274=150.5 –expected frequency for no loss of silencing in AP263

- X^2= ((O – E-0.5)2/E) YATES CORRECTION
- AP259 loss of silencing x^2= (15 - 8.5 -0.5)^2/8.5=4.23
- AP259 no loss of silencing x^2=(96-102.5-0.5)^2/102.5=0.47
- AP263 loss of silencing x^2= (6 -12.4 -0.5)^2/12.4 =3.83
- AP263 no loss of silencing x^2=(157 -150.5-0.5)^2/150.5=0.23

- Total sum = 4.23+0.47+3.83+0.23=8.76
- 8.76>3.84, therefore loss of silencing in a daughter cell is statistically significant

Appendix 3 Statistical analysis of loss of silencing

In order to estimate the loss of silencing in mother cells a total amount of divisions was taken into consideration (B). Only lineages that had red or variegated colonies were counted, as white lineages were not informative for loss of silencing. For instance, line 5 of AP263 divided five times before losing silencing (Mother-Daughter 5). After the fifth division, the silencing was lost; therefore subsequent divisions were not taken into consideration.

Expected frequency was calculated based on the number of mothers that lost silencing. The total number of divisions between two constructs was taken into consideration. The frequencies of loss of silencing and no loss of silencing were calculated. For example, in order to calculate the expected frequency of loss of silencing in a mother cell of AP259 lineages, I multiplied the observed value corresponding to mother cells that lost silencing by the number of divisions of AP259 lineage and then divided the number by the total number of divisions of both AP259 and AP263 lineages.

The same formula was used to calculate the frequency of loss of silencing of AP263, as well as the frequency of no loss of silencing in AP259 and AP263.

Then, using the Chi Square formula, I calculated the observed frequency of loss of silencing based on total counts for both construct. Yates correction was used to correct for the Chi Square, because our frequency was below 1 (there are only two lines analyzed). The following formula was used:

$\chi 2$ Yates = Σ = [(Observed-Expected)-0.5]2/Expected

Finally, the Chi Square numbers corresponding to AP259 loss of silencing, AP259 no loss of silencing, AP263 loss of silencing and AP263 no loss of silencing were added. Because the known value corresponding of Chi Square for a degree freedom of 1 is 3.84 and my expected frequency value is 2.37, the loss of silencing observed in mother cells is not statistically significant.

The observed and expected frequencies of loss of silencing in daughter cells were calculated as described above. Because the observed frequency number: 8.76, is greater than the expected frequency number: 3.84, the loss of silencing in a daughter cells between the two constructs is statistically significant (C).

Appendix 4 Strain list

Strain name	Genotype	Source
DG21	*h-, otr1R (Sph1)::ura4 ura4-DS/E ade6-216 his7-366 leu1-21*	(Li, Goto et al. 2003)
DG124	*h- Δrdp1::kanMX6 otr1R(Sph1)::ura4 leu1-32 ade6-216 his7-366 uraDS/E*	(Li, Goto et al. 2003)
DG445	*h+ Δrdp1::kanMX6 otr1R(Sph1)::ura4 leu1-32 ade6-216 his+ ura4-DS/E*	this study
DG478	*h+ Δago1::kanMX6 otr1R(Sph1)::ura4 ade6-216 leu1-32 his-*	this study
ZB20	*h- Δago1::kanMX6 otr1R(Sph1)::ura4 ura4-DS-E ade6-216 leu1-32 his-*	(Li, Goto et al. 2003)
DG690	*h- Δdcr1::kanMX6 otr1R(Sph1)::ura4 ade6-210 leu1-32 his7-366*	(Li, Goto et al. 2003)
DG691	*h+ Δdcr1::kanMX6 otr1R(Sph1)::ura4 ade6-210 leu1-32*	this study
DG704	*h+ cdc25-22 leu1*	(Hiraoka, Toda et al. 1984)
	h+ nda3-KM311 leu	(Hiraoka, Toda et al. 1984)
DG706	*h- cdc10-129 leu1*	(Hiraoka, Toda et al. 1984)
FY6071	*h?, h3.2-S10A Δh3.1/h4.1::his3+ Δh3.3/h4.3::arg3+ leu1-32 ura4D18 his3D1 arg3D3 ade6-210 otr1R(SphI):ade6+*	(Mellone, Ball et al. 2003)
IH2452	*h+ ark1. Δ 1::LEU2 ura4D18, ade6-+pRep82Ark1.PkC*	(Petersen and Hagan, 2003)
AK01	*cdc25-22 Δago1::kanMX6*	this study
AK02	*cdc25-22 Δdcr1::kanMX6*	this study
AK03	*cdc25-22 Δrdp1::kanMX6*	this study
AK04	*nda3-KM311 Δago1::kanMX6*	this study
AK05	*nda3-KM311 Δdcr1::kanMX6*	this study
AK06	*nda3-KM311 Δrdp1::kanMX6*	this study
AK07	*cdc10-129 Δago1::kanMX6*	this study
AK08	*cdc10-129 Δrdp1::kanMX6*	this study
AK09	*cdc10-129 Δdcr1::kanMX6*	this study
AK29	*h- leu1 ura4 Dcds1::ura4*	FY11110 from Yeast Genetic Resource Center, Japan
AK30	*h- leu1 ura4 Dcds1 Dchk1::ura4*	FY11040 from Yeast Genetic Resource Center, Japan
AK31	*h- leu1 ura4 Dchk1::ura4*	From Yeast Genetic Resource Center, Japan
AK32	*Δdcr1::kanMX6, Δchk1*	this study
AK33	*Δdcr1::kanMX6 Δcds1*	this study

AK34	*cdc25-22 Δchk1*	this study
AK35	*cdc25-22 Δdcr1::kanMX6 Δchk1*	this study
AK38	*cdc25-22 Δrdp1::kanMX6 Δcds1*	this study
AK39	*cdc25-22 Δago1::kanMX6 Δcds1*	this study
AK 63	*cdc25-22 Δdcr1::kanMX6 chk1-S345*	this study

Appendix 5 List of Abbreviations

Ade6+- adenine 6 reporter gene

BrdU- 5-bromo-2-deoxyuridine, a synthetic nucleoside

cenII- S.pombe centromere II

ChiP- Chromatin Immunoprecipitation

CPT- Camptothecin

DAPI- 4',6-diamidino-2-phenylindole

DNA- deoxyribonucleic acid

DSB repair- DNA double-stranded break repair

dsRNA- double stranded RNA

FACS- Fluorescence Activated Cell Sorting

HU- hydroxyurea

HP1- heterochromatin protein 1

HR- homologous recombination

H3K9me- Histone 3 Lysine 9 methylation

H3K10ph- Histone 3 Lysine 10 phosphorylation

imr- innermost repeats of *S.pombe* centormere

K-lysine, an aminoacid

mat- mating type locus of *S. pombe*

mESC- mouse embryonic stem cells

MEFs- Mouse Embryonic Fibroblasts

miRNAs- micro RNAs

NHEJ- Non-Homologous End Joining

otr- outermost repeats of *S.pombe* centromere

PCR- Polymerase Chain Reaction

PEV- Position Effect Variegation

PI- Propidium Iodine

Pol II- polymerase II

Phospho-methyl switch- Phosphorylation-methylation swich

rasiRNA- repeat associated small interfering RNA

rDNA- ribosomal DNA

RNA- ribonucleic acid

RNAi- RNA interference

S-serine, an aminoacid

siRNAs- small interfering RNAs

ts- temperature sensitive

WCE- Whole Cell Extract

XCI- X chromosome inactivation