Mitotic Regulators and Their Effects on Drosophila Chromosome Structure during Development

by

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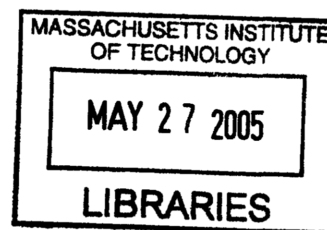

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ABSTRACT

Variants of the canonical cell cycle are frequently used in nature to accomplish specific developmental goals. In one such variant, the endocycle, synthesis phase alternates with a gap phase without an intervening mitosis, producing cells that have multiple copies of the genome. These cells show diversity in their chromosome structure; at one extreme, the sister chromatids are separate (polyploid) and at the other extreme, the sisters are held together (polytene). The endocycle itself can be modified and these variations are speculated to correlate with the observed differences in chromosome structure. In this thesis, we have analyzed the contribution of mitotic regulators to the endocycle and polytene chromosome structure in Drosophila. We show that *morula*, a gene required for the transition from polytene to polyploid chromosome structure in Drosophila nurse cells, is a subunit of the anaphase-promoting complex/cyclosome. Increasing levels of *cyclin B*, a known mitotic target of the APC/C, does not alter the timing of the transition, indicating that CYCLIN B is not the only APC/C target at the polyteny-polyploidy transition. In mitosis, activity of APC/C and POLO lead to the loss of sister-chromatid cohesion and we find that mutants in *polo* are unable to progress through the polyteny-polyploidy transition. Finally, we find that the cohesin complex, a complex required for the physical attachment of sister chromatids in mitosis, is required for proper polytene chromosome structure in the salivary gland. These results describe a requirement for the cohesin complex in a variant of the cell cycle lacking mitosis and indicate that sister-chromatid cohesion differentiates polytene and polyploid chromosome structures.

Thesis Supervisor: Terry L. Orr-Weaver
Title: Professor of Biology

Dedicated to Mom, Dad and Matt
(and Manny Ortiz)

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Finally, last August Jerry Remy asked the readers of his website, remyreport.com, what they would do or give up to help the Red Sox win the World Series. While many of the nation described cleaning toilets in Yankee Stadium or eating broken glass, I wrote that I would dedicate my PhD thesis to Manny Ortiz, John Kerry's favorite Red Sox and the most elusive member of the 2004 Red Sox. The rest, as they put it, is history and, foolishly, I am sticking to my word. This thesis is dedicated to Manny Ortiz, my beloved Cubbies and my cherished Red Sox for teaching me that no matter how bad it gets, there is always next year. It is nice to win though.

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Chapter One

Introduction

The mitotic cell cycle is driven by oscillations of mitotic regulators

The ability to duplicate a cell's genome and equally segregate the genetic material is essential for the production of genetically identical sister cells. These events must proceed in a specific order because segregation of the DNA cannot occur prior to replication of the genome. To ensure the proper sequence of events, cells utilize a cycle that consists of four distinct stages. The mitotic cell cycle consists of a synthesis phase (S) during which the DNA is replicated and a mitosis phase (M) during which the DNA is segregated. These phases are separated by two gap phases; the first gap phase (G1) is a period of growth and preparation for DNA replication. During the second gap phase (G2), which follows S phase, the cell's organelles replicate and the cell prepares for mitosis. Entry into and exit from each stage is precisely regulated by enzymatic reactions, as are the physical events of each stage. Multiple regulators ensure that these events occur in a specific temporal pattern.

The mitotic cell cycle is characterized by the activity of cyclin-dependent kinases (CDKs), one type of cell cycle regulator that ensures events occur in the right order. DNA replication and segregation occur in periods of high CDK activity, while exit from mitosis and G1 require low levels of CDK activity. The activity of a particular CDK kinase is controlled in several ways. First, CDKs are activated by their association with specific cyclins. In *Saccharomyces cerevisiae*, a single CDK, Cdc28, is bound by different cyclins throughout the cell cycle. The association with different cyclins controls substrate specificity of Cdc28 during specific stages of the cell cycle. In S phase, Cdc28 associates with Clb5 and Clb6 to phosphorylate substrates involved in DNA replication. During mitosis, Clb1 and Clb2 associate with Cdc28, directing the kinase towards mitotic substrates. In higher eukaryotes, cyclins associate with multiple CDKs, adding another layer of complexity and regulation. The S phase

kinase CDK2 associates with CYCLIN E and, in mammalian cells it also associates with CYCLIN A. The mitotic kinase CDK1 can be bound by different mitotic cyclins, including CYCLIN A and CYCLIN B. Second, in addition to CDK association with cyclin, CDK kinase activity is also controlled by posttranslational modifications. During G2, mitotic CDK activity is inhibited by phosphorylation of a tyrosine residue by the kinase WEE1. By the G2/M transition, this inhibitory phosphate must be removed by CDC25 phosphatase and, in many organisms, an activating phosphate at a nearby threonine residue must be added by Cyclin-Activating Kinase (CAK).

CDK activity is also controlled by cyclin-dependent kinase inhibitors (CKIs) that bind CDK complexes and inhibit their activity (for review see Sherr and Roberts 1999). CKIs belong to two classes; CIP/KIP family members, such as p21, p27 and p57 in mammals, bind to and inhibit all CDK1, CDK2, CDK4 and CDK6 complexes, while the INK4 family, including p16 in mammals, specifically bind and inhibit CDK4/6-CYCLIN D complex (Sherr and Roberts 1999). Many CKIs act primarily in G1; INK4 proteins inhibit transcription of G1-S genes by restricting the activity of CDK4/6 and CIP/KIP proteins generally inhibit CDK activity that promotes entry into S phase. In *S. cerevisiae*, the CKI SIC1 promotes G1 by downregulating mitotic CDK activity at the M-G1 transition and by inhibiting S phase CDK activity (Donovan *et al.* 1994, Nugroho and Mendenhall 1994, Schwob *et al.* 1994).

In *Drosophila melanogaster*, two CKIs have been characterized and shown to inhibit CDK activity. *roughex* (*rux*) is required for the G1 phase in the developing eye; mutants in *rux* accumulate high levels of CYCLIN A in early G1 and enter S phase prematurely (Thomas *et al.* 1994, Thomas *et al.* 1997). *rux* was determined to be a bona fide CKI by demonstration that it interacted *in vitro* and *in vivo* with CYCLIN A, that overexpression of *rux*, reduced CDK1

activity and that, *in vitro*, RUX can directly inhibit CYCLIN A/CDK1 activity (Foley *et al.* 1999). RUX has also been shown to inhibit CYCLIN A/CDK1 activity in mitosis and thus has been speculated to assist in exit from mitosis in Drosophila embryos (Foley and Sprenger 2001). A second Drosophila CKI, *dacapo* (*dap*), encodes a CIP/KIP family member that inhibits CYCLIN E/CDK2 activity (de Nooij *et al.* 1996, Lane *et al.* 1996). *dap* mutants do not exit from the cell cycle normally in embryogenesis and thus proceed through an additional cell cycle (de Nooij *et al.* 1996, Lane *et al.* 1996). Ectopic expression of *dap* leads to a G1 arrest in embryogenesis and eye development, suggesting that *dap* regulates G1-S progression (de Nooij *et al.* 1996, Lane *et al.* 1996). Multiple mechanisms of regulation highlight the importance of controlling CDK activity and suggest a model where oscillations in CDK activity drive progression of the cell cycle.

Destruction of mitotic regulators is controlled by the APC/C

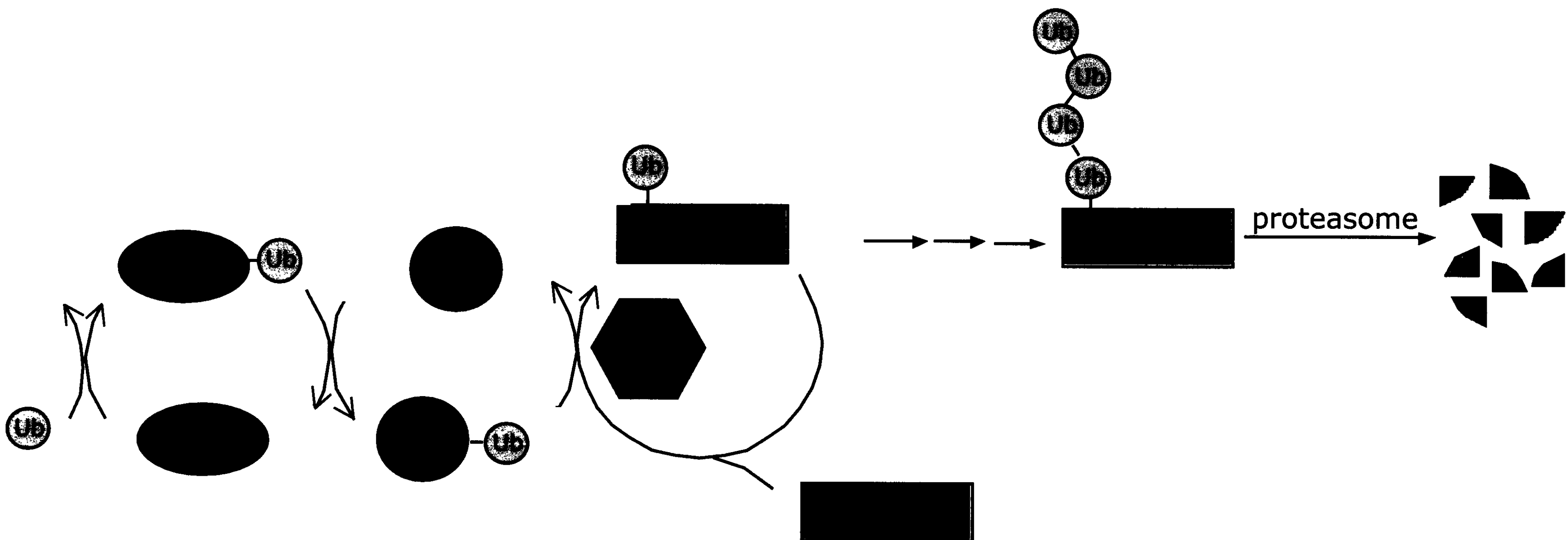
In addition to regulation by CKIs, the activity window of a particular cyclin/CDK is also temporally controlled by the presence of the cyclin. Cyclins are transcribed at certain stages during the cell cycle; *cyclin E* is transcribed at the G1/S transition, while *cyclin A* and *B* are transcribed prior to mitosis. Temporal regulation of cyclin transcription ensures that CDK activity is turned on at a specific time and the subsequent cellular activities occur quickly. CDK activity must also be turned off with the same precision and speed. Cyclin/CDK activity is inactivated at a specific time by a rapid decrease in protein level of the cyclin. Early observations of sea urchin eggs demonstrated that, upon fertilization, protein levels of cyclins accumulated prior to mitosis and then suddenly declined (Evans *et al.* 1983). The discovery of cyclin-ubiquitin conjugates in mitotic extracts combined with the observation that cyclin degradation is

sensitive to inhibitors of the ubiquitin degradation system implicated degradation as the mechanism for the decline (Glotzer *et al.* 1991, Hershko *et al.* 1991). These studies and others have led to the following model: cyclins are tagged with an ubiquitin chain, a proteinacous signal that is specifically recognized by the cytosolic 26S proteasome. The proteasome then degrades these marked cyclins, resulting in the rapid decrease in cyclin protein levels seen in mitosis.

Since these early observations, more details about the ubiquitin degradation system and the role of this system in the cell cycle have been elucidated. Ubiquitin is a small protein that is covalently conjugated through its carboxyl terminus directly to a protein substrate or to an ubiquitin chain on a protein substrate. Three major enzymes are required to transfer an activated ubiquitin to its target: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase that confers substrate specificity (E3) (Figure 1 and for review, see Hershko and Ciechanover 1998). First, ubiquitin is activated by a high-energy thioester bond between a glycine residue in its carboxyl terminus and an active site cysteine residue in the ubiquitin-activating enzyme. Second, the activated ubiquitin is transferred to an active site cysteine residue in the ubiquitin-conjugating enzyme (E2) and forms a second thioester bond. Finally, the activated ubiquitin is transferred to the substrate either through the E2 directly or in combination with a third enzyme, the ubiquitin ligase (E3). An amide isopeptide bond is formed between the carboxyl terminus of ubiquitin and a lysine residue in the substrate. Multiple rounds of ubiquitination lead to the formation of a polyubiquitin chain, which is recognized by the 26S proteasome. This mechanism of proteolysis is used throughout eukaryotic biology to achieve specific protein degradation of a diversity of substrates and multiple E3 ubiquitin ligases have been identified and characterized (for review see Pickart and Eddins 2004).

Figure 1: The ubiquitin-mediated proteolytic pathway involves three enzymes and marks substrates for degradation.

A single ubiquitin protein is activated at its carboxyl-terminus by a high-energy thioester bond with a cysteine residue of the ubiquitin-activating enzyme (E1). This activated ubiquitin is then passed on to a cysteine residue on the ubiquitin-conjugating enzyme (E2). The E2, in combination with the ubiquitin-ligating enzyme (E3), transfers the ubiquitin to a lysine side chain on the substrate, like CYCLIN B in the cell cycle. The E3 ubiquitin-ligating enzyme responsible for degradation of mitotic cyclins and other cell cycle substrates is named the anaphase-promoting complex/cyclosome (APC/C). Iterations of this pathway result in a substrate that is tagged with a chain of ubiquitins, a signal that is recognized by the 26S proteasome. The proteasome then degrades the substrate into small peptides and intact ubiquitin proteins.


Ub
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Ub
proteasome
1. activation
2. conjugation
to substrate
3. ligation to substrate

A combination of biochemical and genetic studies identified the E3 required during mitosis to degrade the mitotic cyclins and its regulators. A 20S multisubunit complex named the anaphase-promoting complex/cyclosome (APC/C) was purified from clam and *Xenopus laevis* egg extracts and demonstrated to support ubiquitination and degradation of CYCLIN B (King *et al.* 1995, Sudakin *et al.* 1995). Additionally, genetic experiments in *S. cerevisiae* identified several mutants that arrested with high levels of Clb2 mitotic cyclin in G1 and many of the proteins encoded by these genes were found in a complex (Irniger *et al.* 1995, Zachariae and Nasmyth 1996). Homologs of these subunits have since been identified in a number of organisms and the APC/C appears to be a highly conserved mechanism for cell cycle control in eukaryotes (Tugendreich *et al.* 1995, Yamashita *et al.* 1996, Golden *et al.* 2000, Bentley *et al.* 2002). We now know that the APC/C is highly regulated and has a number of substrates in the cell cycle. The APC/C is controlled by conserved activating factors; in the mitotic cell cycle these regulators are FIZZY/CDC20 and FIZZY-RELATED/CDH1 (see below). In addition to the mitotic cyclins, another major substrate of the APC/C is SECURIN, a regulator of sister-chromatid cohesion (see below). The APC/C has also been implicated in the degradation of several other substrates in the cell cycle including spindle proteins, mitotic protein kinases, and regulators of DNA replication.

Subunit composition of the APC/C and functions

Biochemical purification has permitted the identification of a number of APC/C subunits. Vertebrate and yeast APC/Cs consist of at least 11 core subunits that are highly conserved and are stably associated throughout the cell cycle (for reviews see Zachariae and Nasmyth 1999, Peters 2002, Harper *et al.* 2002 and Castro *et al.* 2005). Genetic screens and homology searches

have begun to identify APC/C subunits in *Arabidopsis thaliana*, *Caenorhabditis elegans* and Drosophila, although the complete composition of these APC/Cs remains undetermined. Although many of the details of APC/C regulation have been discovered, less is known about the functions of the individual APC/C subunits. A number of structural motifs found in APC/C subunits are also found in other E3 ubiquitin ligases, providing information as to how these subunits function in the complex (see Table 1 and text below). The list of APC/C subunits, however, may not yet be complete, further complicating our understanding of APC/C function. Additionally, the identification of organism specific subunits, such as APC7 found only in vertebrates or Apc9, Apc13/Swm1 and Apc15/Mnd2 found only in *S. cerevisiae*, suggests that the core functions and subunits of the APC/C may be modified for different goals (Yu *et al.* 1998, Zachariae *et al.* 1998, Yoon *et al.* 2002).

The discovery of an APC/C subunit with a cullin domain, APC2, and an APC/C subunit with a RING finger, APC11, revealed similarities between the APC/C and other E3 ubiquitin ligases (Zachariae *et al.* 1998, Yu *et al.* 1998, Ohta *et al.* 1999, Gmachl *et al.* 2000). Cullins are a protein family that includes Cdc53, a subunit of the Skp1-cullin-F box (SCF) protein complex. The SCF is an E3 ubiquitin ligase that targets CKIs and G1 cyclins for degradation in the cell cycle (for review see Deshaies 1999, Vodermaier 2004). In the SCF, the carboxyl terminus of Cdc53, which contains the cullin domain, recruits the Rbx1/Roc1/Hrt1 (a RING-H2 finger domain protein) to SCF, stimulating the binding of the E2 ubiquitin-conjugating enzyme Cdc34 (Patton *et al.* 1998, Kamura *et al.* 1999, Seol *et al.* 1999, Skowyra *et al.* 1999). RING-H2 fingers coordinate two zinc ions and are speculated to mediate protein-protein interactions (Borden and Freemont 1996). The amino terminus of Cdc53 binds Skp1, a protein that binds the substrate associated F box proteins (Bai *et al.* 1996, Patton *et al.* 1998). Therefore, the cullin-containing

Table 1: Mitotic subunits of the APC/C

H. Sapiens	*S. cerevisiae*	Drosophila[a]	Structural Motifs	Proposed Function in APC/C
APC1	Apc1	SHATTERED	Rpn1/2	structural,
APC2	Apc2	MORULA	cullin	catalytic
APC3	Cdc27	MÁKOS	TPR	activator binding, structural
APC4	Apc4	CG32707	-	structural
APC5	Apc5	IDA	-	structural
APC6	Cdc16	CDC16	TPR	structural
APC7	-	CG14444	TPR	activator binding, structural
APC8	Cdc23	CG2508	TPR	structural
-	Apc9	-	-	structural
APC10	Doc1	CG11419	DOC	substrate binding
APC11	Apc11	LEMMING	RING-H2	catalytic
CDC26	Cdc26	-	-	structural

a: Predicted Drosophila genes were identified as APC/C subunits by sequence homology in Harper *et al.* 2002 and were confirmed for this study.

protein is proposed to bring the substrate, RING-H2 protein and E2 ligase into close proximity (Zheng *et al.* 2002). By analogy, APC2 and APC11 have been proposed to interact in a similar manner and perform a similar function.

Direct studies of APC11 have demonstrated its requirement for APC/C function and its interaction with the carboxyl terminus of APC2 (Zachariae *et al.* 1998, Ohta *et al.* 1999). *In vitro* biochemical experiments suggest that APC11 may be the crucial catalytic subunit for the ubiquitination activity of the APC/C. Both human and *S. cerevisiae* APC11 have been identified as the minimal requirement for ubiquitination of APC/C substrates in the presence of the E2 ubiquitin-conjugating enzyme UBC4 (Gmachl *et al.* 2000, Leverson *et al.* 2000). These two studies demonstrate a requirement for the RING-H2 domain for ubiquitination of two APC/C substrates in mitosis, SECURIN (see below) and CYCLIN B, and for viability in *S. cerevisiae.* In a similar study, however, APC2 was identified as a requirement for ubiquitination activity of APC substrates *in vitro* (Tang *et al.* 2001). In this study, UBCH10 was added as the E2 ubiquitin-conjugating ligase as opposed to UBC4. Tang *et al.* showed that UBCH10 binds directly to the cullin domain of APC2, while UBC4 binds directly to APC11, explaining the differences in these studies (Tang *et al.* 2001). The APC/C can use two classes of the E2 ubiquitin conjugating enzymes *in vitro*, members of the Ubc4 family or the UBCx/UbcH10/E2-C family (King *et al.* 1995, Aristarkhov *et al.* 1996, Yu *et al.* 1996, Osaka *et al.* 1997, Townsley *et al.* 1997). Recent *in vivo* studies in *Schizosaccharomyces pombe* and Drosophila suggest that these enzymes are not redundant and that different E2 ligases add to the regulation of APC/C activity (Seino *et al.* 2003, Mathe *et al.* 2004). APC2 and APC11, therefore, perform the catalytic function within the APC/C, similar to their family members in other E3 ubiquitin ligases.

The DOC domain found in APC10/DOC1 is another conserved domain that is found in other ubiquitin ligases (Grossberger *et al.* 1999, Kominami *et al.* 1998). *doc1* was genetically identified in *S. cerevisiae* in a screen for mutants that prevent degradation of mitotic cyclins and has been demonstrated to exist in the APC/C complex (Hwang and Murray 1997, Zachariae *et al.* 1998). APC10/DOC1 has subsequently been identified as an APC/C subunit in other organisms as well (Grossberger *et al.* 1999, Kominami *et al.* 1998). APC10/DOC1 is essential for viability in *S. pombe,* and *S. cerevisiae* mutants show a severe growth delay, suggesting a requirement for APC10/DOC1 in APC/C function (Hwang and Murray 1997, Kominami *et al.* 1998). Interestingly, a mutation in APC10/DOC1 that disrupts APC/C degradation of mitotic cyclins and the E3 ligase activity of APC/C does not destabilize the complex, indicating that APC10/DOC1 plays a key role in APC/C function but not in complex formation or stability (Kominami *et al.* 1998, Grossberger *et al.* 1999, Carroll and Morgan 2002, Passmore *et al.* 2003). APC10/DOC1's essential role in APC/C function is being elucidated; APC10/DOC1 has recently been demonstrated to be required for APC/C's interactions with substrates, but not with APC/C activators (Passmore *et al.* 2003, Carroll *et al.* 2005). Thus far, APC10/DOC1 is the only core APC/C subunit implicated in substrate binding.

APC3/CDC27, APC6/CDC16 and APC8/CDC23 were the first APC/C subunits identified and, with APC7, contain ten repeated copies of a degenerate 34 amino acid motif known as the tetratricopeptide (TPR) motif (Lamb *et al.* 1994, King *et al.* 1995, Irniger *et al.* 1995). The distribution of the repeats is conserved among these homologs; nine TPR repeats are found in the carboxyl terminus of these subunits and are thought to mediate protein-protein interactions (Lamb *et al.* 1995, reviewed in Blatch and Lassle 1999). True to the proposed function of their main structural motif, these core subunits have been shown to interact with

several other APC/C subunits. Human APC3/CDC27 and APC7 interact with the carboxyl terminus of APC10/DOC1, suggesting that these subunits could connect various APC/C subdomains (Wendt *et al.* 2001, Vodermaier and Peters 2004). The TPR repeats of APC3/CDC27 and APC7 have been demonstrated to bind the carboxyl terminus of an APC/C activator, FZR/CDH1, implicating these domains in regulating the substrate specificity of APC/C (Vodermaier *et al.* 2003). Additionally, APC3/CDC27, APC6/CDC16, APC7 and APC8/CDC23 are all phosphorylated in mitosis, further suggesting that they may play an important role in regulation of the APC/C (Peters *et al.* 1996, Kraft *et al.* 2003).

Less is known about other APC/C subunits and their contributions to APC/C function and regulation. APC1 is the largest subunit of the APC/C identified and it is phosphorylated in mitosis (Peters *et al.* 1996, Yamashita *et al.* 1996, Zachariae *et al.* 1996). APC1 contains an Rpn1/2 motif that is found in subunits of the 19S cap complex of the 26S proteasome (Lupas *et al.* 1997). This domain has been speculated to serve as a scaffold for complex assembly, although the function of this domain in APC1 has not been discovered (Lupas *et al.* 1997). APC4, 5, 9 and CDC26 have no known protein motifs and are currently speculated to act in stabilizing the complex. APC4 and APC5 have been isolated as part of an APC/C subcomplex with APC1, 2 and 11 in mammalian cells suggesting that they may play a structural role bringing APC/C subdomains in contact (Vodermaier *et al.* 2003). Both Apc9 and CDC26 have been implicated in stabilizing interactions between APC/C subunits. Apc9 is a nonessential, yeast specific subunit, but APC/C immunoprecipitated from an *apc9* deletion stain has reduced activity and lower levels of APC/C-associated Cdc27 (Zachariae *et al.* 1998, Passmore *et al.* 2003). CDC26, on the other hand, has been identified in both yeast and vertebrates (Yamada *et al.* 1997, Zachariae *et al.* 1998, Gmachl *et al.* 2000. In a temperature-sensitive *cdc26* deletion strain, levels of Cdc16, Cdc27 and

Apc9 are reduced in immunoprecipitated APC/C, suggesting that Cdc26 is necessary to recruit or stabilize these subunits specifically at high temperatures (Zachariae *et al.* 1998, Passmore *et al.* 2003).

In Drosophila, ten APC/C subunits have been identified by sequence homology, although mutants in only a limited number of these have been characterized (Harper *et al.* 2002). Genetic studies of APC/C subunit mutants have contributed to our understanding of subunit functions and suggested that the APC/C may have varying compositions for different functions. *shattered* (*shtd*) encodes APC1 and strong mutants in *shattered* die during larval stages and do not develop imaginal discs (Tanaka-Matakatsu 2003, reviewed in Lee and Orr-Weaver 2003). Additionally, weaker alleles of *shtd* are viable and display small rough eyes. Further analysis of eye discs reveals that *shtd* mutants display defects in maintaining a G1 arrest in eye discs, accumulating high levels of mitotic cyclins and prematurely entering S phase. These studies reveal a requirement for APC/C in maintaining a developmental arrest in differentiated tissues.

The *morula* (*mr*) locus encodes the APC2 homolog and has been demonstrated to contain a cullin domain like previously identified APC2 subunits (Kashevsky *et al.* 2002). Strong mutants in *mr* die at the larval-pupal boundary and show a metaphase arrest with highly condensed chromosomes in the mitotically dividing larval neuroblasts (Reed and Orr-Weaver 1997). Anaphase figures were never observed in *mr* mutant neuroblasts, and it was not determined whether the sister chromatids had separated in the metaphase arrest (Reed and Orr-Weaver 1997). Hypomorphic alleles in *mr* are female sterile, and the mutant females exhibit arrest in oogenesis. Escapers, in which the defect in oogenesis has been suppressed, show a metaphase arrest in the rapid S-M cycles of embryogenesis (Reed and Orr-Weaver 1997). Additionally, mutants in *mr* show defects in the endocycle, a cell cycle variant consisting of synthesis and gap

phases (see below), suggesting a previously unidentified role for the APC/C in modified cell cycles in development (Reed and Orr-Weaver 1997, Kashevsky *et al.* 2002). APC2's proposed catalytic partner, APC11, is encoded by *lemming* (*lmg*), and mutations in *lmg* have been reported to lead to abnormal mitoses and apoptosis in imaginal discs (Taylor 2001). Overexpression of *lmg* leads to defects in axon guidance and synaptogenesis in larvae, suggesting that APC/C has a role in neuronal development in Drosophila (Kraut *et al.* 2001). A role for the APC/C in Drosophila synaptic growth has since been described, and the APC/C has also been demonstrated to regulate postmitotic neuronal growth and functions in *C. elegans* and mammals (van Roessel *et al.* 2004, Konishi *et al.* 2004, Juo and Kaplan 2004).

Two TPR containing APC/C subunits have been identified in Drosophila; *mákos* (*mks*) encodes APC3/Cdc27 and *cdc16* encodes APC6/Cdc16 (Deak *et al.* 2003, Huang and Raff 2002). Localization studies and RNAi experiments of Drosophila APC6/CDC16 and APC3/CDC27 revealed differences between these two core APC/C subunits and suggest that, in Drosophila, the APC/C may have a varying composition for different functions or different locations (Huang and Raff 2002). Transgenic flies expressing CDC16- and CDC27-GFP fusion proteins were generated, demonstrated to be incorporated into the APC/C and observed live in both early syncytial and later cellularized embryos. In both cases, CDC16- and CDC27-GFP were excluded from the nucleus during interphase and entered the nucleus upon entry into mitosis. Interestingly, a fraction of CDC27-GFP accumulated on mitotic chromosomes and remained there until exit from mitosis. CDC16-GFP, however, appeared to be dramatically excluded from the chromosomes. This difference in subunit localization during mitosis may reflect the presence of multiple APC/C with varying subunit compositions. It is also possible though that these two localizations reflect differences in CDC16 and CDC27 localization when not incorporated into

APC/C. The fact that the majority of CDC16- and CDC27-GFP appears to be incorporated into a larger complex argues against this, although it is formally a possibility.

Differences in the roles of CDC16 and CDC27 were further suggested by phenotypes resulting from RNAi knockdown of these subunits in Drosophila cell culture (Huang and Raff 2002). Protein levels of CDC16 and CDC27 were reduced by 90% in both *cdc16* and *cdc27* RNAi experiments. Depletion of CDC16 and CDC27 each resulted in an increase in the mitotic index, although the cells were not arrested in mitosis. Chromosomes in *cdc16* RNAi expressing cells displayed a tight chromosome alignment in metaphase while the chromosomes appeared more disorganized in *cdc27* RNAi expressing cells. Immunofluorescence experiments with a centromere protein revealed that sister chromatids were rarely separated in *cdc16* RNAi expressing cells, but were frequently separated in *cdc27* RNAi expressing cells. Additionally, the chromosome-associated fraction of CYCLIN A was efficiently degraded in *cdc16* RNAi expressing cells, but remained at high levels in *cdc27* RNAi expressing cells. Although there are considerable caveats with using fusion proteins and tissue culture, the striking differences in *cdc16* and *cdc27* depleted cells suggest that these two subunits have different roles in APC/C function and may exist in different APC/C isoforms.

The identification of mutations in Drosophila *cdc27* has allowed *in vivo* studies of this subunit and confirmed some of the observations from the RNAi experiments. A strong hypomorphic mutation in *mákos*, mks^1, is pharate adult lethal, which is defined by pupae that die with fully developed imaginal discs (Deak *et al.* 2003). Mitotically cycling larval neuroblasts display a high mitotic index with overcondensed chromosomes in a metaphase arrest (Deak *et al.* 2003). Unlike *cdc27* depleted tissue culture cells, mks^1 larval neuroblasts arrest in mitosis with high levels of both CYCLIN A and CYCLIN B, suggesting that CDC27 is required for the

degradation of both of these mitotic cyclins *in vivo*. Like the cells depleted for *cdc27* by RNAi however, sister chromatid arms and centromeres appear to be separated in these arrested neuroblasts, as determined by multiple techniques (Deak *et al.* 2003). It remains possible that some residual MÁKOS function is able to separate the sister chromatids and that APC/C function for cyclin degradation and for sister separation have different thresholds. Thus, CDC27 clearly contributes to APC/C function in mitosis in Drosophila, though it remains to be conclusively demonstrated whether CDC27 is necessary for only cyclin degradation or for sister-chromatid separation as well.

Finally, *ida* encodes the Drosophila homolog of APC5, a subunit without a known motif to indicate its function (Bentley *et al.* 2002). *ida* mutants are prepupal-lethal and show proliferation defects in mitotic larval tissues such as the imaginal discs and optic lobes. These mutants behave as genetic nulls and the *ida* transcript is not detected in extracts from most of the homozygous larvae, suggesting that these mutations disrupt expression of *ida* and that little, if any, IDA protein exists in these larvae. The generation of *ida* germline clones revealed a requirement for IDA in oogenesis, as very few eggs were produced in *ida* mutant germlines. Like the mks^1 and strong *mr* mutants, in *ida* mutant larval neuroblasts the mitotic index is increased and the chromosomes appear highly condensed, suggesting that IDA is required for proper progression through mitosis. Chromosomes in *ida* mutants are never observed to fully align on the metaphase plate and sisters are often separated. Additionally, *ida* mutants appear to enter anaphase with frequent lagging chromosomes and high levels of CYCLIN B. These results suggest that IDA may only be required for some APC/C functions in mitosis, as CYCLIN B degradation is blocked, but sister chromatid separation is not. The authors, therefore, suggest that IDA/APC5 may not participate in all APC/C activities and may not act as a core subunit.

These results are interesting in light of the discovery in mammalian cells that APC5 interacts with the essential catalytic subunits APC2/APC11 in a stable subcomplex with APC1 and APC4, suggesting a core role for APC5 (Vodermaier *et al.* 2003). Although it is possible that these contradictions in interpretation may be due to nuances of genetic and biochemical studies, it is also likely that the APC/C is not exactly the same in every organism. As we learn more about the APC/C, these differences will be revealed and provide a greater understanding of how this E3 ubiquitin ligase can be modulated. These *in vivo* studies in Drosophila are essential to our understanding of the APC/C as they reveal differences in subunit functions, APC/C composition, and the role of APC/C in developmental contexts that are not obvious *in vitro*.

Regulation of APC/C activity and substrate specificity

The identification of the mitotic E3 ubiquitin ligase as APC/C began to reveal how the activities of mitosis were controlled. The APC/C, however, could not be active throughout the cell cycle, otherwise CDKs would never reach a threshold of activity. It seemed likely, therefore, that activity the APC/C would be tightly regulated. Genetic and biochemical experiments have since identified two key APC/C regulators in the mitotic cell cycle: CDC20/FIZZY and CDH1/FIZZY-RELATED. Although many of the mechanistic details have been observed *in vitro*, the first identification of APC/C regulators came from mutant analysis in Drosophila and *S. cerevisiae*. *fizzy* (*fzy*) was identified in Drosophila and *fzy* mutants display a metaphase arrest in late embryonic cycles (Dawson *et al.* 1995). This metaphase arrest was later correlated to high levels of the mitotic CYCLINS A, B and B3 (Sigrist *et al.* 1995). Additionally, *fizzy-related* (*fzr*) mutants progress through an extra mitotic cycle in late embryogenesis and display high levels of mitotic cyclins as well (Sigrist and Lehner 1997). These phenotypes suggest that *fzy* and *fzr* are

required for proteolysis of mitotic cyclins. Both *fzy* and *fzr* have seven tandem WD40 repeats at their carboxyl-termini, a motif that is proposed to generate a protein-protein interaction face (Lambright *et al.* 1996, Dawson *et al.* 1993, Sigrist and Lehner 1997). Homologs of FZY and FZR have been identified in many eukaryotes and the presence of the WD40 repeats is conserved (Sethi *et al.* 1991, Weinstein *et al.* 1994, Visintin *et al.* 1997, Schwab *et al.* 1997, Lorca *et al.* 1998, Kallio *et al.* 1998, Fang *et al.* 1998).

Although both *fzy* and *fzr* mutants displayed high levels of mitotic cyclins, differences in their phenotypes suggested that *fzy* and *fzr* had non-overlapping functions in Drosophila development. Though *fzy* mutants arrest in metaphase, displaying a requirement in mitosis, *fzr* appears to be required when cells exit the mitotic cell cycle and stop proliferating (Dawson *et al.* 1993, Dawson *et al.* 1995, Sigrist *et al.* 1995, Sigrist and Lehner 1997). In Drosophila, therefore, both FZY and FZR contribute to the degradation of mitotic cyclins, but only FZY seems to be required during mitosis. This appears to be similar in *S. cerevisiae*; in G1, *cdc20/fzy* is required to degrade a target regulating sister-chromatid separation (Pds1), but not the mitotic cyclin Clb2 (Visintin *et al.* 1997). *cdh1/fzr* shows the opposite specificity; in G1, it is required for Clb2 degradation, but not Pds1 (Visintin *et al.* 1997). These results were both dependent upon the APC/C, suggesting that CDC20/FZY and CDH1/FZR may confer substrate specificity to the APC/C (Visintin *et al.* 1997). Substrate specificity is also conferred by the timing of CDC20/FZY and CDH1/FZR activity; CDC20/FZY activates the APC/C at the onset of anaphase and CDH1/FZR becomes active at the mitosis-G1 transition (reviewed in Castro *et al.* 2005). CDH1/FZR is phosphorylated in S, G2, and M and this modification blocks CDH1/FZR binding to the APC/C outside of G1 (Zachariae *et al.* 1998, Jaspersen *et al.* 1999, Lukas *et al.* 1999, Kramer *et al.* 2000). Additionally, CDC20/FZY is degraded by APC/C-CDH1/FZR,

which ensures that APC/C-CDC20/FZY activity does not persist and allows the substrates of APC/C-CDC20/FZY to accumulate for another round of mitosis (Prinz *et al.* 1998, Shirayama *et al.* 1998, Pfleger and Kirschner 2000). Several mechanisms, therefore, contribute to the specificity and timing of substrate degradation by each APC/C complex.

CDC20/FZY and CDH1/FZR are speculated to generate substrate specificity for the APC/C by binding and recruiting substrates. This model combines several pieces of converging data. Firstly, CDC20/FZY and CDH1/FZR appear to stimulate Xenopus and human APC/C activity *in vitro*, proposing a role for these regulators in APC/C activation (Fang *et al.* 1998, Lorca *et al.* 1998). Secondly, the APC/C and human homologs of these regulators have been demonstrated to interact *in vitro* and *in vivo*, suggesting that CDC20/FZY and CDH1/FZR act directly on the APC/C (Fang *et al.* 1998, Kallio *et al.* 1998, Kramer *et al.* 1998). Finally, CDC20/FZY and CDH1/FZR bind specific APC/C substrates in the absence of the APC/C both in *vitro* and *in vivo* (Ohtoshi *et al.* 2000, Burton and Solomon 2001, Pfleger *et al.* 2001, Schwab *et al.* 2001, Sorensen *et al.* 2001, Hilioti *et al.* 2001). This interaction is speculated to be mediated through the WD40 interaction face, as this motif has been identified in a subset of SCF subunits that mediate substrate binding (Patton *et al.* 1998).

Additionally, phosphorylation likely plays a role in regulating both the CDC20/FZY and CDH1/FZR activators and the core APC/C itself. Phosphorylated CDC20/FZY appears to have increased affinity for the APC/C *in vitro*, although this does not seem to be required for APC/C activation (Kramer *et al.* 2000). In contrast, phosphorylated CDH1/FZR is blocked from interacting with the APC/C (Zachariae *et al.* 1998, Jaspersen *et al.* 1999). Phosphorylation of APC/C subunits remains more controversial; in some studies phosphorylation of the APC/C appears to be required for APC/C activity whereas in other cases it does not appear to be

required (Lahav-Baratz *et al.* 1995, Yamada *et al.* 1997, Patra and Dunphy 1998, Fang *et al.* 1998, Kotani *et al.* 1999, Shteinberg *et al.* 1999, Rudner and Murray 2000, Kraft *et al.* 2003). Although our knowledge of APC/C composition, regulation, and its substrate specificity has greatly increased in the last few years, clearly many details remain to be elucidated.

Sister-chromatid cohesion assists in proper chromosome segregation

Proper segregation of sister chromatids in mitosis requires that the sisters remain attached following replication in S phase and align at metaphase with each sister kinetochore attached to microtubules emanating from a different pole. This bipolar attachment assures that once cohesion of the sister chromatids is lost, the sisters will segregate to opposite poles. It is crucial therefore, that the sister chromatids remain connected until proper bipolar attachment has been made for each chromatid pair. In classical cytology experiments, the association of sister chromatids was observed and analysis by FISH in *S. cerevisiae* suggested that sister chromatids remain associated along their lengths from the time of DNA replication until anaphase when the sisters were observed to dramatically separate (Guacci *et al.* 1994). Again, a combination of genetic and biochemical studies have revealed a number of proteins essential for sister-chromatid cohesion in mitosis (Table 2). Four of these factors form an evolutionarily conserved complex that localizes to sister chromatids; this complex has been named cohesin for its essential role in sister-chromatid cohesion (Guacci *et al.* 1997, Michaelis *et al.* 1997, Losada *et al.* 1998, Toth *et al.* 1999, Losada *et al.* 2000, Sumara *et al.* 2000, Tomonaga *et al.* 2000, Vass *et al.* 2003). Additionally, several factors have been implicated in the loading of this complex onto chromosomes, and in the establishment and maintenance of cohesion until anaphase.

Table 2: Factors Implicated in Sister-Chromatid Cohesion

S. cerevisiae	*S. pombe*	Vertebrates	*D. melanogaster*
Cohesin Subunits			
SCC1/MCD1	RAD21	RAD21	RAD21
SCC3	PSC3, REC11[a]	SA1, SA2, SA3[a]	SA
SMC1	PSM1	SMC1	SMC1
SMC3	PSM3	SMC3	CAP
REC8[a]	REC8[a]	REC8[a]	-
Cohesin Loading Proteins			
SCC2	MIS4	SCC2A, SCC2B	NIPPED-B
SCC4	-	-	-
Cohesion Establishment Proteins			
ECO1/CTF7	ESO1	ECO1, ECO2	DECO
-	-	-	SAN
Other Proteins Involved in Cohesion			
PDS5	PDS5	PDS5	CG17509
SGO1	SGO1, SGO2	SGO1	MEI-S332[a]
-	-	-	ORD

a: These factors are required specifically in meiosis (for review see Marston *et al.* 2004).

The cohesin complex consists of four subunits: two SMC family members, SMC1 and SMC3, and two non-SMC subunits, SCC1/MCD1/RAD21 and SCC3 (Table 2). *scc1/mcd1* was first identified in *S. cerevisiae* in genetic screens for mutants that were defective in sister-chromatid cohesion, resulting in premature sister-chromatid separation (PSCS), and it was shown to be essential for proper chromosome segregation (Guacci *et al.* 1997, Michaelis *et al.* 1997). SCC1/MCD1 was observed to bind chromosomes during S phase and dissociate at the metaphase-anaphase transition with the loss of sister-chromatid cohesion, implicating SCC1/MCD1 in physically holding the chromatids together (Michaelis *et al.* 1997). *smc1* and *smc3* were also identified by this screen and SCC1/MCD1 and SMC1 were shown to physically interact by co-immunoprecipitations, suggesting that these factors may form a complex (Michaelis *et al.* 1997, Guacci *et al.* 1997). Another *S. cerevisiae* cohesin gene, *scc3*, was also identified by its mutant phenotype of PSCS (Toth *et al.* 1999). These four proteins were demonstrated to physically interact, to co-localize onto chromosomes and to be interdependent for localization, implying that they acted together in a complex (Toth *et al.* 1999).

The Xenopus SMC1 and SMC3 homologs were identified by their sequence similarity to *S. cerevisiae* SMC1 and SMC3 and were shown to exist in two distinct cohesin complexes with SCC1/MCD1/RAD21 (Losada *et al.* 1998). Immunodepletion of cohesin subunits, particularly SMC1 and SMC3, in interphase resulted in unattached chromatids in mitosis, indicating that the cohesin complex acted similarly in vertebrates (Losada *et al.* 1998). Genetic deletion of SCC1 in chicken cells also led to PSCS and chromosome segregation defects (Sonoda *et al.* 2001). The presence of two cohesin complexes seems to be a feature of vertebrates, as two complexes were purified from human cells as well (Sumara *et al.* 2000). These complexes were shown to differ by the SCC3 homolog incorporated; complexes consist of either stromalin 1 (SA1) or stromalin 2

(SA2) (Losada *et al.* 2000, Sumara *et al.* 2000). The predominant cohesin complex in Xenopus contains SA1, while in humans the predominant complex contains SA2 (Losada *et al.* 2000). Another key difference between yeast and metazoan cohesin came from cytological studies of the cohesin complex on chromosomes. While in *S. cerevisiae*, cohesin appears to remain on chromosome arms and centromeres until anaphase, in Xenopus, Drosophila and human cells, the bulk of cohesin dissociates from chromatin early in mitosis, specifically in late prophase (Losada *et al.* 1998, Losada *et al.* 2000, Sumara *et al.* 2000, Warren *et al.* 2000b). Importantly, a small amount of SCC1, presumably as part of the cohesin complex, was noted to remain associated with the centromere until anaphase, explaining how sister chromatids remained attached in the absence of cohesin along the arms (Waizenegger *et al.* 2000, Warren *et al.* 2000b).

The subunits of the cohesin complex and its behavior have been characterized in other organisms as well. In Drosophila, homologs of these subunits have been identified by genomic approaches and by biochemical purification of the cohesin complex (Valdeolmillos *et al.* 1998, Warren *et al.* 2000a, Cobbe and Heck 2000, Vass *et al.* 2003, Valdeolmillos *et al.* 2004). DSMC1 and DSMC3/CAP can be immunopurified in a 1:1 heterodimer from embryo extracts using antibodies to DRAD21 (Vass *et al.* 2003). DSCC3/SA1 is also found in this complex and associates more closely with DRAD21 than the SMC subunits, demonstrating that the composition of Drosophila cohesin is similar to cohesin in *S. cerevisiae* and vertebrates (Vass *et al.* 2003). RNAi studies in S2 cells and embryos also suggest that the Drosophila cohesin complex acts in a manner similar to that of the other characterized cohesin complexes. S2 cells depleted of DRAD21 show several mitotic defects including abnormal chromosome alignment at metaphase, abnormal spindle morphology and PSCS (Vass *et al.* 2003). In embryos treated with dsRNA to *rad21*, a range of mitotic abnormalities result including delays in condensation and

congression in prophase and aberrant chromosome segregation, suggesting an *in vivo* role for RAD21 and the cohesin complex (Vass *et al.* 2003). Cytological studies with antibodies to DRAD21 and DSCC3/SA1 demonstrate that the two proteins have a localization pattern similar to that of vertebrate cohesin. Both DRAD21 and DSCC3/SA1 colocalize along condensing chromosomes in prophase, specifically at centromeres in metaphase and are lost from centromeres in anaphase (Warren *et al.* 2000b, Valdeolmillos *et al.* 2004). Studies in *S. pombe* have revealed that the composition of the cohesin complex is conserved in this organism as well, although the majority of cohesin appears to bind chromatids throughout the cell cycle (Tomonaga *et al.* 2000). Again, a small fraction is removed at the metaphase-anaphase transition and this removal is essential for progression in anaphase (Tomonaga *et al.* 2000). Finally, genetic studies of cohesin homologs in *C. elegans* have revealed these genes are essential for proper chromosome segregation and are required for embryonic viability (Mito *et al.* 2003). The cohesin complex, therefore, is a key regulator of sister-chromatid cohesion and is essential for proper sister-chromatid segregation in many organisms.

In addition to the cohesin complex, proteins have been identified that are required for the establishment and maintenance of cohesion (Table 2). Two factors, SCC2 and SCC4, have been proposed to assist in loading the cohesin complex onto chromatids. SCC2 is evolutionarily conserved and homologs required for cohesion have been identified in *S. pombe*, *S. cerevisiae*, Xenopus, humans and Drosophila, while SCC4 has only been identified in *S. cerevisiae* thus far (Michaelis *et al.* 1997, Furuya *et al.* 1998, Rollins *et al.* 1999, Ciosk *et al.* 2000, Gillespie and Hirano 2004, Tonkin *et al.* 2004, Krantz *et al.* 2004). SCC2 associates with chromosomes during DNA replication and loss of *scc2* leads to defects in sister-chromatid cohesion in mitosis and loss of viability (Furuya *et al.* 1998, Ciosk *et al.* 2000, Gillespie and Hirano 2004, Rollins *et al.* 2004).

Mutants in the Drosophila SCC2 homolog, Nipped-B, show PSCS in mitotically dividing larval neuroblasts and die as late 2nd instars (Rollins *et al.* 2004). Intriguingly, *Nipped-B* has also been implicated in other chromatin activities; NIPPED-B facilitates transcriptional activation of the *cut* and *Ubx* genes by remote enhancers, suggesting that NIPPED-B may also participate in organizing functional chromatin domains (Rollins *et al.* 1999, Rollins *et al.* 2004).

Factors involved in the establishment and maintenance of sister-chromatid cohesion

In *S. cerevisiae* SCC2 has been shown to form a complex with SCC4 that is required during DNA replication (Ciosk *et al.* 2000). Importantly, in mutants of these factors, cohesin complexes form properly, but do not bind chromatin (Ciosk *et al.* 2000). Additionally, in Xenopus extracts, SCC2 is not required for maintenance of sister-chromatid cohesion once it has been established. These data suggest that SCC2 (and SCC4 in *S. cerevisiae*) facilitate cohesin's association with chromatids. Further details of this requirement have been described in Xenopus egg extracts. Gillespie and Hirano observed that "replication licensing" was required for SCC2's association with chromatin, but that initiation of DNA replication was not (Gillespie and Hirano 2004). These observations were furthered by the demonstration that binding of SCC2 to chromatin is dependent upon MCM2-7, the putative replication helicase. Additionally, the recruitment of cohesins to chromatids requires the origin recognition complex (ORC), and other replication initiation factors such as CDC6, CDT1 and MCM2-7 (Takahashi *et al.* 2004). The function of this requirement and whether or not it is an evolutionarily conserved mechanism remains to be determined, but previous observations in *S. cerevisiae* have demonstrated that cohesion must be established during DNA replication and the authors suggested that this might occur following passage of the replication fork (Uhlmann and Nasmyth 1998). It seems likely,

therefore, that there are links between the DNA replication machinery, the loading of the cohesin complex and the establishment of sister-chromatid cohesion.

The acetyltransferase ECO1/CTF7 is also required for the establishment of sister-chromatid cohesion and homologs have been identified in a number of organisms (Toth *et al.* 1999, Skibbens *et al.* 1999, Tanaka *et al.* 2000, Ivanov *et al.* 2002, Bellows *et al.* 2003, Williams *et al.* 2003, Vega *et al.* 2005). Like *scc2* mutants, disruption of *eco1* results in PSCS and loss of cell viability (Skibbens *et al.* 1999, Toth *et al.* 1999, Tanaka *et al.* 2000, Williams *et al.* 2003, Vega *et al.* 2005). In *eco1* mutants in *S. cerevisiae*, SCC1 and SCC3 were shown to associate with chromosomes with the proper timing but to separate their centromeres prematurely (Skibbens *et al.* 1999, Toth *et al.* 1999). Additionally, *eco1* was shown to be required exclusively in S phase in both *S. cerevisiae* and *S. pombe* and, therefore, in the establishment but not maintenance of sister-chromatid cohesion (Toth *et al.* 1999, Skibbens *et al.* 1999, Tanaka *et al.* 2000). In Drosophila, two acetyltransferases, *san* and Drosophila *eco1* (*deco*), have been identified and are required for proper sister-chromatid cohesion (Williams *et al.* 2003). Mutants in these genes are lethal at the larval/pupal boundary and show PSCS in squashes of mitotically dividing neuroblasts (Williams *et al.* 2003). Unlike cohesin in the *S. cerevisiae* mutants, RAD21/SCC1 was not observed on the centromeres of separated chromatids in *san* and *deco* mutant prometaphases (Williams *et al.* 2003). The authors note that localization of RAD21/SCC1 to the interphase nucleus is not altered in *san* and *deco* mutants and they conclude, therefore, that cohesin is loaded properly in these mutants, but cannot be maintained (Williams *et al.* 2003). The role for this acetyltransferase in cohesion in any organism is still unclear; *S. cerevisiae* and human ECO1 have been demonstrated to have acetyltransferase activity although the *in vivo* substrates of this enzyme have not been identified (Ivanov *et al.* 2002,

Bellows *et al.* 2003). It remains, therefore, to be shown how these acetyltransferases contribute to establishing sister-chromatid cohesion and whether the mechanism is evolutionarily conserved.

Finally, three additional proteins have been identified and characterized as having a role in maintaining sister-chromatid cohesion. PDS5 is an essential and conserved factor that interacts with cohesin complexes in *S. cerevisiae* and Xenopus, but is not a core component of the cohesin complex (Hartman *et al.* 2000, Panizza *et al.* 2000, Sumara *et al.* 2000). In *S. cerevisiae*, the *pds5* mutant phenotype demonstrates a requirement for PDS5 in sister-chromatid cohesion and proper chromosome segregation (Hartman *et al.* 2000, Panizza *et al.* 2000). Temporal studies revealed that the function of PDS5 is required from S phase until mitosis and that PDS5 co-localizes with the cohesin complex on chromatids (Hartman *et al.* 2000, Panizza *et al.* 2000). As PDS5 localization is dependent on SCC1/MCD1, but not vice versa, it has been suggested that PDS5 acts to stably maintain cohesin until its dissociation from chromosomes (Hartman *et al.* 2000, Panizza *et al.* 2000, Stead *et al.* 2003). In *S. pombe*, mutants in *pds5* also show PSCS and interact with the cohesin complex (Tanaka *et al.* 2000, Wang *et al.* 2002). Interestingly, the PDS5 homolog also physically interacts with ESO1, the ECO1/CTF7 homolog, suggesting a role for *S. pombe* PDS5 with ESO1 in establishment of cohesion (Tanaka *et al.* 2001). The requirement for *eso1* in cohesion establishment is relieved when *pds5* is mutated, suggesting that, in *S. pombe*, PDS5 may act to block the establishment of cohesion until counteracted by ESO1 (Tanaka *et al.* 2001). It seems, therefore, that PDS5 may perform slightly different functions in *S. cerevisiae* and *S. pombe*, a notion that is supported by the observation that PDS5 is essential for viability in *S. cerevisiae*, but not in *S. pombe* (Hartman *et al.* 2000, Tanaka *et al.* 2001, Wang *et al.* 2002).

Currently, PDS5 homologs have been identified in Xenopus, human and *C. elegans*, Sordaria and Apergillius; in vertebrates, PDS5 associates with the cohesin complex and,

importantly, has been demonstrated to dissociate from chromosomes with the bulk of cohesin in late prophase (Sumara *et al.* 2000). Mutants in the *C. elegans pds5* homolog, *evl-14/pds-5*, demonstrate a requirement for *pds5* in viability, displaying defects in sister-chromatid cohesion in both mitosis and meiosis (Wang *et al.* 2003). *pds5* has not been described in Drosophila, although a sequence homolog, encoded by *CG17509*, does exist by BLAST searches (J.A. Wallace and T.L. Orr-Weaver, unpublished observations). The characterization of Sordaria PDS5, SPO76, has provided crucial insight into the function of this protein. Mutants in *spo76* show defects in both mitotic and meiotic chromosome morphology, cohesion, DNA repair and recombination (Moreau 1985, Huynh *et al.* 1986, van Heemst *et al.* 1999). In mitotic prometaphase, both chromosome cohesion and condensation are coordinately affected in *spo76* mutants, although the chromosomes look wild-type at metaphase/anaphase and segregation is normal (van Heemst *et al.* 1999). In meiosis, *spo76* mutants show aberrant, diffuse chromosome morphology at prophase and PSCS at metaphase I, indicating that *spo76* is required for maintenance of sister-chromatid cohesion in meiosis (Moreau 1985, van Heemst *et al.* 1999). SPO76 was also shown to associate with both meiotic and mitotic chromosomes and is lost from chromosomes by metaphase (van Heemst *et al.* 1999). Based on the localization and mutant analysis, it has been proposed that SPO76 coordinates sister-chromatid cohesion and chromosome condensation at distinct stages of chromosome morphogenesis in meiosis and mitosis (van Heemst *et al.* 1999).

A second factor involved in the maintenance of sister-chromatid cohesion was originally identified by its essential role in meiosis in Drosophila (Davis 1971, Goldstein 1980). Mutants in *mei-S332* show a high frequency of nondisjunction in meiosis II, and *mei-S332* was shown to be required for the persistence of centromeric cohesion in meiosis I (Kerrebrock *et al.* 1992). The

observation that MEI-S332 localizes to both meiotic and mitotic centromeres until their separation suggests that this factor likely maintains centromeric cohesion in the presence of factors promoting the dissolution of arm cohesion in either meiosis I or prophase in mitosis (Kerrebrock *et al.* 1995, Moore *et al.* 1998, Tang *et al.* 1998, LeBlanc *et al.* 1999). Importantly, although *mei-S332* is essential for meiosis, it is not necessary for mitosis in Drosophila, but does contribute to mitotic cohesion (Kerrebrock *et al.* 1992, Kerrebrock *et al.* 1995, LeBlanc *et al.* 1999).

Recently, homologs of MEI-S332 have been characterized in *S. cerevisiae*, *S. pombe*, Xenopus and humans and have been named members of the SHUGOSHIN family (Kitajima *et al.* 2004, Marston *et al.* 2004, Rabitsch *et al.* 2004, Salic *et al.* 2004, Indjeian *et al.* 2005, McGuinness *et al.* 2005). In *S. pombe* there are two SHUGOSHIN family members, Sgo1 and Sgo2, while in *S. cerevisiae* there appears to only be one SHUGOSHIN, Sgo1 (Kitajima *et al.* 2004, Marston *et al.* 2004, Rabitsch *et al.* 2004, Indjeian *et al.* 2005). Consistent with the role of MEI-S332 in Drosophila, Sgo1 is required for maintaining centromeric cohesin in meiosis I in *S. cerevisiae* and in *S. pombe* (Kitajima *et al.* 2004, Marston *et al.* 2004, Rabitsch *et al.* 2004). Sgo2 in *S. pombe* has been reported to have a role in mitosis, as *sgo2* mutants are viable but demonstrate missegregation of chromosomes at mitosis (Kitajima *et al.* 2004). However, in another study, *sgo2* mutants did not show such defects, so the details of *S. pombe* Sgo2 in mitosis remain controversial (Rabitsch *et al.* 2004). In *S. cerevisiae*, *sgo1* mutants are viable, but show defects in mitotic progression and chromosome segregation (Kitajima *et al.* 2004, Marston *et al.* 2004). Additionally, *sgo1* mutants are sensitive to disruption of microtubules, suggesting that Sgo1 might be involved in kinetochore function (Kitajima *et al.* 2004, Indjeian *et al.* 2005). Vertebrate Sgo is required in mitosis to prevent PSCS and, intriguingly, may regulate kinetochore

microtubule stability *in vivo* (Salic *et al.* 2004, McGuinness *et al.* 2005). A recent study noted that depletion of Sgo1 in human cell culture lead to a reduction of SCC1 association with centromeres in prophase, demonstrating that Sgo1 is essential to maintain centromeric cohesin when the bulk of the cohesin complex is removed from cohesin arms in vertebrates (McGuinness *et al.* 2005). The SHUGOSHIN family, therefore, plays a role in maintaining cohesion and may regulate other processes in mitosis as well.

A third factor, ORD, is required to maintain meiotic cohesion and proper meiotic chromosome segregation in both males and females in Drosophila (Mason 1976, Miyazaki and Orr-Weaver 1992, Bickel *et al.* 1997). Null alleles in *ord* display random chromosome segregation in both meiosis I and II and PSCS has been observed cytologically in *ord* oocytes and spermatocyctes (Goldstein 1980, Lin and Church 1982, Miyazaki and Orr-Weaver 1992, Bickel *et al.* 1997, Bickel *et al.* 2002). By analysis with FISH in *ord* mutant spermatoctyes, Balicky *et al.* observed that meiotic cohesion defects become evident in late G2, when centromeric cohesion is lost prematurely (Balicky *et al.* 2002). Localization studies of a GFP-ORD fusion protein demonstrated that ORD becomes associated with chromosome arms and centromeres during G2 in spermatocytes and remains only at centromeres from prophase until anaphase II (Balicky *et al.* 2002). Intriguingly, *ord* mutants show defects in chromosome condensation and ORD associates with meiotic chromosomes before the initiation of condensation (Goldstein 1980, Miyazaki and Orr-Weaver 1992, Bickel *et al.* 1997, Balicky *et al.* 2002). It has been suggested; therefore, that ORD may maintain centromeric cohesion during chromosome morphogenesis and compaction in prophase in spermatocytes (Balicky *et al.* 2002). In oocytes, ORD localizes to both chromosome arms and centromeres and promotes proper meiotic homolog recombination in Drosophila females (Webber *et al.* 2004).

Structure of the cohesin complex and mechanism of sister-chromatid cohesion

The identification of the cohesin complex and it subunits has allowed examination of the mechanism of cohesion, particularly how this complex confers cohesion to two sister chromatids. Initial structural information about the cohesin complex came from studies of the SMC family, members of which are involved in cohesion, condensation, DNA repair and recombination, and dosage compensation (for review see Hirano 2002). SMC proteins contain globular domains at both their amino- and carboxyl-terminus, which are separated by a long coiled-coil region (Melby *et al.* 1998). The coiled-coil region is broken in the middle by a non-coiled coil domain, referred to as the hinge domain (Melby *et al.* 1998). Two bacterial SMC proteins were purified and examined by electron microscopy to determine the conformation of these proteins (Melby *et al.* 1998). These observations revealed that these SMC proteins were able to homodimerize and form rod-shaped molecules with the globular domains at one end and the hinge domain at the other, bringing the two antiparallel coiled-coil regions into close proximity (Melby *et al.* 1998, see SMC1 and SMC3 in Figure 2A). Visualization of human cohesin complexes by electron microscopy demonstrated that this conformation was not unique to bacterial SMC proteins and that the non-SMC subunits associated with the globular ends of the SMC dimer (Anderson *et al.* 2002). Additionally, the SMC dimers formed a "V-shape" and, in the presence of non-SMC subunits, the cohesin complex took on the appearance of a ring (Melby *et al.* 1998, Anderson *et al.* 2002).

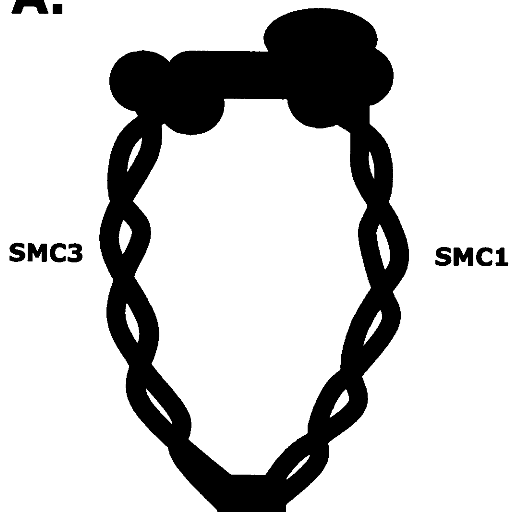
Several biochemical experiments of *S. cerevisiae* cohesin confirmed these observations and provided further details of the structure of the cohesin complex. First, it was determined that the observed SMC coiled-coil regions form an intramolecular coil-coil and that SMC1 and SMC3 proteins are not intertwined by this region (Haering *et al.* 2002). Second, Haering *et al.*

Figure 2: Structure of the cohesin complex and possible models for sister-chromatid cohesion via the cohesin complex.

A. The cohesin complex consists of two SMC proteins, SMC1 (green) and SMC3 (blue), and two non-SMC proteins, SCC1/MCD1/RAD21 (purple) and SCC3 (pink). SMC1 and SMC3 form a heterodimer attached by their hinge domains at one end and attached by their association with SCC1 at the other end. The SMC proteins each form an intramolecular coiled-coil, bringing their globular amino- and carboxyl-termini together to form an active ATPase. Experiments suggest that SCC1 binds the globular ends of SMC1 and SMC3 and SCC3 forming a ring-like structure.

B. Many models for how the ring structure of cohesin promotes cohesion of two sister-chromatids have been suggested. In the simplest model, the ring encloses the two sister-chromatids at several points along their length, holding them together until the release of cohesion (ring model). In a second model, the ring binds and bridges the two sister chromatids, bringing them together (direct binding model). Finally, it has been suggested that each ring may contact a single sister chromatid and that the rings may then be interlinked or covalently attached to provide cohesion between the sisters (double ring model). It is important to note that each of these models has considerable concerns; it has not been demonstrated that two chromatids can fit within a single cohesin ring nor has it been shown that any of the cohesin subunits can directly contact DNA. Finally, multimers of the cohesin complex have not been isolated. These models, therefore, should be considered suggestions and will be refined, as more details are understood.

A.



B.

Ring Model

Direct Binding Model

OR

Double Ring Model

determined that SMC1 and SMC3 interacted through their hinge domains. Third, it was shown that SCC1/MCD1 binds to the globular domains of SMC1 and SMC3 and only to this domain, linking the SMC proteins by their globular domains as well (Haering *et al.* 2002). Fourth, SCC3 was demonstrated to bind SCC1, bringing SCC3 to the SMC heterodimer (Haering *et al.* 2002). These observations led to a structural model for the cohesin complex as a ring formed by the SMC heterodimer and closed by the binding of SCC1/MCD1 to the globular domains of the heterodimer (see Figure 2A). Additional evidence for the role of the cohesin ring came from *in vivo* studies of modified cohesin subunits (Gruber *et al.* 2003). Gruber *et al.* created a modified *S. cerevisiae* SMC3 protein with a TEV protease cleavage site in the coiled-coil region and showed that this altered SMC3 protein complemented deletion mutants of *smc3* (Gruber *et al.* 2003). Inducing cleavage of SMC3 led to the release of cohesin from chromatin in metaphase, as evidenced by absence of SCC1 staining on chromosome spreads, and loss of sister-chromatid cohesion *in vivo* (Gruber *et al.* 2003). As cleavage of SMC3 was sufficient to destroy cohesion, the authors conclude that the ring structure must be essential for cohesin function. Finally, two studies in *S. cerevisiae* demonstrate that ATP is required for cohesin function (Arumugam *et al.* 2003, Weitzer *et al.* 2003). By bringing together the amino- and carboxyl- globular domains in the SMC proteins, a functional ATPase of the ABC family is generated (Hopfner *et al.* 2000, Lowe *et al.* 2001, reviewed in Hirano 2002). Mutations that abolish ATP binding or ATP hydrolysis are lethal, suggesting a requirement for these activities in SMC functions (Arumugam *et al.* 2003). Specifically, the binding of ATP to SMC1 was shown to be required for SCC1/MCD1's association with the SMC1/SMC3 heterodimer (Arumugam *et al.* 2003, Weitzer *et al.* 2003). If ATP hydrolysis was prevented, this abolished cohesin's association with chromatin but did not disrupt SCC1/MCD1's interaction with the SMC1/SMC3 heterodimer (Arumugam *et al.* 2003,

Weitzer *et al.* 2003). These studies have provided significant insight into the structure and mechanism of the cohesin complex and have prompted many models that wait testing.

As the cohesin complex subunits are evolutionarily conserved, it seems likely that the cohesin complex forms a ring structure in many organisms. How, then, does the cohesin complex confer cohesion between sisters? Many models for sister-chromatid cohesion via the cohesin ring have been presented (Figure 2B, for reviews see Campbell and Cohen-Fix 2002, Haering and Nasmyth 2003). As disruption of the ring structure leads to loss of cohesin from chromatids, the simplest explanation is that the ring encloses the sister chromatids (ring model in Figure 2B). It is not clear, however, that two chromatids could physically fit inside the ring and, given that cohesin loads onto chromatids in G1, if the replication fork and machinery would be able to progress through the closed ring. A second model, the direct binding model, therefore, suggests that the two sister chromatids are not held inside the ring, but that the ring binds both chromatids, thus providing cohesion (direct binding model in Figure 2B). This model, however, is contradicted by the fact that direct binding of DNA by cohesin has only been observed *in vitro*, in extracts from vertebrate cells, and is not consistent with the current mechanism for cohesin loss at the metaphase-anaphase transition (see below, Losada and Hirano 2001). Another proposed model is that of the double ring (double ring model in Figure 2B). This model incorporates the ring structure of the cohesin complex and the current mechanism for cohesin loss (see below). This model suggests that two rings could intertwine, with each containing a sister chromatid, or the two rings could be covalently associated, given the symmetry of the SMC1/SMC3 heterodimer (Campbell and Cohen-Fix 2002, Haering and Nasmyth 2003). Multimers of the cohesin complex, however, have not been isolated or detected in biochemical experiments in *S. cerevisiae* (Haering *et al.* 2002). Finally, it remains to be determined how the

cohesin ring associates with each chromatid, whether the chromatid is simply enclosed by the ring or whether the chromatin is wrapped around the cohesin ring in a more complex structure.

Loss of sister-chromatid cohesion is triggered by the APC/C and POLO

The field has made significant advances not only in the identification of cohesin factors and the establishment of cohesion, but also in understanding the dissociation of cohesin from chromatids. Early studies of ubiquitin-mediated proteolysis and the APC/C in mitosis revealed the requirement for degradation of a non-cyclin substrate to separate the sister chromatids at the metaphase-anaphase transition (Holloway *et al.* 1993, Surana *et al.* 1993). This factor was first identified in genetic screens as *pds1* in *S. cerevisiae*, *cut2* in *S. pombe* and *pimples* in Drosophila and by functional homology as PTTG in vertebrates (Yamamoto *et al.* 1996b, Yamamoto *et al.* 1996a, Funabiki *et al.* 1996a, Stratmann and Lehner 1996, Zou *et al.* 1999). These proteins are known as SECURINS and have little sequence homology amongst the family members. In *S. cerevisiae*, *pds1* mutants were identified by their PSCS phenotype and inviability after treatment with a microtubule drug (Yamamoto *et al.* 1996b, Yamamoto *et al.* 1996a). *pds1* was shown to be required for proper chromosome segregation and growth, but only at high temperatures (Yamamoto *et al.* 1996b). Interestingly though, the stability of PDS1 protein was observed to change during the cell cycle and it was noted that PDS1 possesses a recognition sequence for the APC/C. Additionally, *pds1* mutants genetically interact with mutants in APC/C subunits, suggesting that PDS1 is a substrate of the APC/C (Yamamoto *et al.* 1996b, Yamamoto *et al.* 1996a). Demonstration of this hypothesis in *S. cerevisiae* came from observations that PDS1 is ubiquitinated by the Xenopus APC/C *in vitro* (Cohen-Fix *et al.* 1996). The importance of this degradation was illustrated by introduction of nondegradable forms of PDS1 *in vivo*; without the

ability to degrade PDS1, cells fail to initiate anaphase and do not separate their sister chromatids, although the APC/C is still able to degrade other substrates (Cohen-Fix *et al.* 1996). As one of the key events at the metaphase-anaphase transition is the separation of sister chromatids, it was hypothesized that PDS1 may regulate sister-chromatid cohesion. By comparing the kinetics of sister-chromatid separation in mutants of *pds1* and *cdc26*, an APC/C subunit, it was determined that destruction of PDS1 was the sole role for APC/C in triggering the dissociation of SCC1 from sister chromatids, a key component of sister-chromatid cohesion (Ciosk *et al.* 1998).

Studies of SECURINS in *S. pombe*, Drosophila and vertebrates demonstrated functional similarities among these homologs. CUT2, PIMPLES and PTTG levels were observed to decrease at anaphase in an APC/C-dependent manner, and the introduction of nondegradable SECURIN or excess wild-type SECURIN blocks sister-chromatid separation (Funabiki *et al.* 1996a, Funabiki *et al.* 1996b, Stratmann and Lehner 1996, Zou *et al.* 1999, Leismann *et al.* 2000). These studies also revealed that SECURIN in *S. pombe* and Drosophila differs from *S.cerevisiae*, because PSCS is not observed in *S. pombe* and Drosophila *securin* mutants. Furthermore, *securin* is essential for viability in these organisms (Funabiki *et al.* 1996a, Stratmann and Lehner 1996). Additionally, in the absence of *securin*, these mutants continue to progress through the cell cycle, demonstrating that loss of *securin* does not inhibit the cell cycle in these systems, but does block anaphase (Funabiki *et al.* 1996a, Stratmann and Lehner 1996, Zou *et al.* 1999, Leismann *et al.* 2000). The mechanism for SECURIN's role in regulating sister-chromatid cohesion was discovered via its physical association with a member of the SEPARASE family, ESP1 in *S. cerevisiae* and vertebrates, CUT2 in *S. pombe* and THREE ROWS and SEPARASE in Drosophila (Funabiki *et al.* 1996a, Ciosk *et al.* 1998, Zou *et al.* 1999, Leismann *et al.* 2000, Jager *et al.* 2001). *separase* is required for separation of sister chromatids as well (Funabiki *et al.*

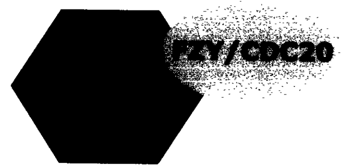
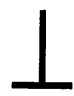
1996a, Ciosk *et al.* 1998, Jager *et al.* 2001). As overexpression of ESP1 in *S. cerevisiae* leads to sister-chromatid separation in the presence of wild-type PDS1, it was hypothesized that SEPARASE promotes sister-chromatid separation, but is held inactive by SECURIN until the metaphase-anaphase transition (Figure 3, Ciosk *et al.* 1998). At the metaphase-anaphase transition, and in the presence of excess SEPARASE, SEPARASE is active and promotes separation of the sister chromatids.

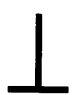
The mechanism for dissociation of cohesin complexes from chromatids by SEPARASE was elucidated by the discovery that SCC1 protein is cleaved *in vivo* by SEPARASE and that this event is necessary and sufficient to trigger sister-chromatid separation (Figure 3, Uhlmann *et al.* 1999, Uhlmann *et al.* 2000). This mechanism has been described in other systems as well and is likely to be evolutionarily conserved (Waizenegger *et al.* 2000, Hauf *et al.* 2001, Siomos *et al.* 2001). SCC1 is cleaved at the initiation of anaphase and cleavage-resistant SCC1 does not dissociate from chromosomes at the onset of anaphase (Uhlmann *et al.* 1999, Hauf *et al.* 2001). In *S. cerevisiae*, premature cohesin cleavage by an inducible protease triggers dissociation of SCC1 from chromatids and progression into anaphase (Uhlmann *et al.* 2000). *In vitro*, purified SEPARASE effectively cleaves SCC1, and SEPARASE was subsequently identified as a cysteine protease, containing two conserved residues that are "hallmarks" of cysteine proteases (Uhlmann *et al.* 1999, Uhlmann *et al.* 2000). Mutation of these residues leads to stable SCC1, and this mutant cannot rescue other *esp1* mutants, indicating that the function of these residues is critical *in vivo* (Uhlmann *et al.* 2000).

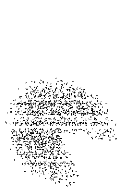
In Drosophila, PIMPLES and SEPARASE are associated with a third protein, THREE ROWS. *three rows* was initially isolated by its embryonic cuticle phenotype and is required for sister-chromatid separation in mitotic cycles in the embryos (Nusslein-Volhard 1984,

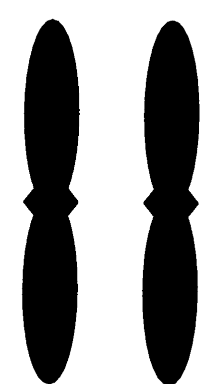
Figure 3: Model for the dissociation of the cohesin complex from sister chromatids in vertebrates and Drosophila.

There are two events during which the cohesin complex is dissociated from chromatids in vertebrates and Drosophila. Unlike in *S. cerevisiae*, the bulk of the cohesin complex is removed from chromatid arms in prophase and it is currently thought that this event is mediated by phosphorylation of the SCC3 homolog by the mitotic kinase, POLO. Cohesin at the centromere is protected from this dissociation and persists until the metaphase-anaphase transition. At the onset of anaphase, the APC/C becomes active by association with FZY/CDC20 and targets SECURIN for degradation by the 26S proteasome. Until this transition, SECURIN binds the protease enzyme SEPARASE holding it inactive. Proteolysis of SECURIN releases active SEPARASE, which cleaves the SCC1/MCD1/RAD21 subunit of the cohesin complex, an event facilitated by phosphorylation of SCC1/MCD1/RAD21 by POLO. This cleavage leads to removal of cohesin from the centromere in vertebrates and Drosophila and from the entire length of the chromatids in *S. cerevisiae*. The removal of the cohesin complex from sister chromatids releases cohesion, allowing the sisters to be segregated to opposite poles.






FZY/CDC20
P
cohesin phosphorylated, dissociates from arms
separase cleaves SCC1 at centromeres
sister-chromatid cohesion lost

D'Andrea *et al.* 1993, Philp *et al.* 1993). PIMPLES, THREE ROWS, and SEPARASE physically interact *in vivo,* and THREE ROWS is required for the association of PIMPLES with SEPARASE (Leismann *et al.* 2000, Jager *et al.* 2001). By genomic and structural studies, THREE ROWS has been demonstrated to correspond to the N-terminal regulatory domains of other eukaryotic SEPARASES and, given Drosophila SEPARASE is significantly smaller than other eukaryotic SEPARASES, it seems that the SEPARASE enzyme has broken into two genes in the evolution of Drosophila (Leismann *et al.* 2000, Jager *et al.* 2001, Jager *et al.* 2004). This likely indicates, therefore, that SEPARASE and THREE ROWS together form the active protease enzyme.

Two interesting mechanisms for regulation of SEPARASE have emerged from studies in Drosophila. First, sister chromatids are not separated in mutants of *pimples*, a phenotype also seen in *cut2* mutants in *S. pombe*, suggesting that *pimples* is both an inhibitor and activator of sister-chromatid separation (Funabiki *et al.* 1996a, Stratmann and Lehner 1996). Analysis of SEPARASE protein levels in *pim* mutant extracts has demonstrated that *pim* is not required for stability of SEPARASE; it has been proposed, therefore, that binding of SEPARASE, THREE ROWS and PIMPLES prior to the initiation of anaphase is necessary for activation of the SEPARASE protease or that PIMPLES might be required for SEPARASE localization (Jager *et al.* 2001). Second, THREE ROWS is cleaved at the metaphase-anaphase transition and this cleavage only occurs in complexes with active SEPARASE (Herzig *et al.* 2002). Phenotypes caused by expression of noncleavable THREE ROWS are relieved by reduction of *separase* gene copy number, indicating that cleavage of THREE ROWS acts to negatively regulate SEPARASE (Herzig *et al.* 2002). Human SEPARASE has also been observed to self-cleave in anaphase and

this cleavage is not required for activation of SEPARASE, but may assist in inactivating the enzyme (Waizenegger *et al.* 2000, Stemmann *et al.* 2001, Waizenegger *et al.* 2002).

Dissociation of cohesin from sister-chromatids is also linked to the activity of the mitotic kinase POLO/CDC5 (see Figure 3). POLO-like kinases have been implicated in many processes in mitosis including entry into mitosis, centrosome regulation, APC/C activation and cytokinesis (for reviews see Glover *et al.* 1998 and Nigg 1998). Phosphorylation of SCC1 has been observed in many systems and enhances SCC1 cleavage by SEPARASE (Tomonaga *et al.* 2000, Uhlmann *et al.* 2000, Alexandru *et al.* 2001, Hoque and Ishikawa 2001, Sumara *et al.* 2002, Hornig and Uhlmann 2004, Hauf *et al.* 2005). In *S. cerevisiae*, mutation of SCC1 phosphorylation sites leads to a delay in SCC1 cleavage and dissociation of SCC1 from chromatids *in vivo* (Alexandru *et al.* 2001, Hornig and Uhlmann 2004). Induction of *cdc5* in G1 induces the appearance of hyperphosphorylated SCC1, and mutants in *cdc5* are less efficient in SCC1 cleavage and dissociation of SCC1 from sister chromatids at the metaphase-anaphase transition, implicating this kinase in SCC1 phosphorylation and regulation of sister-chromatid cohesion (Alexandru *et al.* 2001). In *pds1 cdc5* double mutants, limited SCC1 cleavage, dissociation from chromatids and sister-chromatid separation are observed, revealing the mechanism for SCC1 cleavage in *pds1* mutants (Alexandru *et al.* 2001). Vertebrate Polo-like kinase 1, PLK1, has been also demonstrated to phosphorylate SCC1 *in vitro*, enhancing its cleavage by SEPARASE as well (Sumara *et al.* 2002, Hauf *et al.* 2005).

Polo-like kinases have also been implicated in the prophase dissociation of the cohesin complex in vertebrates. In Xenopus extracts, PLK1 was shown to be required for loss of cohesin in prophase *in vitro*, as immunodepletion of PLK1 blocked dissociation of cohesin from chromatin (Sumara *et al.* 2002). Addition of PLX1 back to these extracts or to interphase

extracts induced loss of cohesin, suggesting that PLK1 regulates the dissociation of cohesin in prophase (Sumara *et al.* 2002). A connection between PLK1 and cohesin was demonstrated by the fact that in PLK1-depleted extracts, cohesin was not phosphorylated, but addition of PLK1 restored cohesin phosphorylation (Sumara *et al.* 2002). Finally, it was demonstrated that phosphorylation of cohesin directly by PLK1 reduced the ability of cohesin to bind chromatin *in vitro*, indicating a link between cohesin phosphorylation and dissociation of cohesin from chromatin (Sumara *et al.* 2002).

Interestingly, in addition to phosphorylation of SCC1, Sumara *et al.* observed phosphorylation of SA2, the SCC3 homolog, as well (Sumara *et al.* 2002). A recent paper has analyzed the contribution of both SCC1 and SA2 phosphorylation to cohesin dissociation in human cells (Hauf *et al.* 2005). By mutating the phosphorylation sites on both SCC1 and SA2 and analyzing the *in vivo* phenotypes, Hauf *et al.* determined that phosphorylation of SCC1 is dispensable for cohesin dissociation in prophase, but, as previously reported, does enhance the cleavability of SCC1 by SEPARASE at the onset of anaphase (Hauf *et al.* 2005). Phosphorylation of SA2, however, is essential for the prophase dissociation of cohesin, but is not required for cohesin cleavage by SEPARASE (Hauf *et al.* 2005). As the *in vivo* phenotype of non-phosphorylatable SA2 is similar to that seen with depletion of PLK1, the authors conclude that SA2 is the important PLK1 target in the dissociation of cohesin (Hauf *et al.* 2005). Whether SCC3/SA2 phosphorylation assists in loss of cohesin in other systems remains to be shown and the physiological relevance of the prophase dissociation pathway is still unclear, although it has been proposed that this pathway is required for sister-chromatid resolution as the timing of cohesin loss correlates with chromosome condensation (Waizenegger *et al.* 2002, Losada *et al.* 2002, Sumara *et al.* 2002, Hauf *et al.* 2005).

Drosophila *polo* was identified by the presence of abnormal metaphase and anaphase chromosome configurations in mutant larval neuroblast cells (Sunkel and Glover 1988). Aberrant spindles have been observed in mutant embryos, spermatocytes and eggs, and POLO was demonstrated to associate with the spindle pole, indicating a role for POLO in the function and/or organization of the centrosome (Sunkel and Glover 1988, Riparbelli *et al.* 2000). The identification and characterization of two stronger *polo* alleles revealed that the majority of larval neuroblast cells arrest in metaphase without separation of the sister chromatids, suggesting that POLO may regulate separation of sister chromatids in Drosophila as well (Donaldson *et al.* 2001). A recent study revealed a new role for POLO in the regulation of sister-chromatid cohesion. POLO was demonstrated to phosphorylate MEI-S332, a regulator of cohesion, *in vitro* and POLO activity is required to remove MEI-S332 from sister chromatids at anaphase in mitosis and meiosis II (Clarke *et al.* 2005). Although it remains to be determined whether POLO assists in the dissociation of cohesin in prophase or in the modification of SCC1/MCD1/RAD21 to promote its cleavage at anaphase, POLO clearly contributes to the regulation of sister-chromatid cohesion in Drosophila.

In the last ten years, our knowledge and understanding of the proteins responsible for establishing and maintaining sister-chromatid cohesion has greatly increased. The conserved subunits of the cohesin complex have been identified in many organisms and basic models for how cohesin creates cohesion and how the cohesin complex is dissociated from chromosomes have been developed. Despite the wealth of details in various systems, there is still much to be learned about the mechanism of sister-chromatid cohesion and its regulation in different cell cycles.

Variants of the mitotic cell cycle utilize regulators of the canonical cell cycle

Although the canonical, mitotic cell cycle is sufficient for most cell divisions, variant cell cycles have evolved to serve particular developmental requirements. For example, meiosis is a cell cycle variant that produces haploid products for sexual reproduction, as two rounds of chromosome segregation occur without an intervening round of DNA replication. Insects, amphibians and marine invertebrates proceed through rapid cell divisions in early embryogenesis; to facilitate these divisions, these cells make use of a cell cycle without gap phases, consisting of alternating S and M phases. Finally, there are many examples throughout nature of cells that utilize a cell cycle consisting of alternating synthesis and gap phases known as the endocycle (for review see Edgar and Orr-Weaver 2001). Endocycling cells lack an intervening mitosis and thus produce polyploid cells, cells that contain multiple copies of the genome. Endocycles enable an organism to increase cell size, as an increase in genomic DNA is correlated with an increase in cell size. Additionally, endocycling enables a cell to increase its metabolic activity and highly active metabolic cells are often polyploid. Finally, the increased gene copy in polyploid cells also promotes survival of environmental stresses that damage DNA.

A diverse group of organisms and tissue types employ the endocycle to achieve these goals. In plants, a number of tissues become polyploid during development such as hair tricomes, leaf epidermal cells, root tip cells and cells in the hypocotyl in Arabidopsis (Galbraith *et al.* 1991, Melaragno *et al.* 1993). Endocycling has been noted in insect tissues, particularly in Drosophila, in the follicle and nurse cells during oogenesis and in the majority of larval tissues (see below and Lilly and Duronio 2005 for review). Polyploidy resulting from the endocycle has also been observed in mammalian tissues. Mammalian megakaryocytes, specialized blood cells that produce platelets, become polyploid during their differentiation (for review see Ravid *et al.*

2002). This results in a size increase that is related to the ability of megakaryocytes to bud off a sufficient number of platelets. The trophoblast giant cells of the mammalian placenta and hepatocyte cells of the liver also endocycle; polyploidy may assist these cells in meeting a demand for high metabolic activity (for review see Zybina and Zybina 1996 and Gupta 2000). These diverse examples illustrate the utility of the endocycle and resulting polyploidy and demonstrate the importance of this cell cycle in nature.

Studies of Drosophila polyploid tissues demonstrated that the endocycle is not a single, continuous round of DNA replication, but rather consists of a period of DNA replication (S phase) followed by a period in which DNA is not replicated and growth and gene expression occur (G phase) (Rudkin 1973, Pearson 1974, Mahowald *et al.* 1979, Hammond and Laird 1985b, Hammond and Laird 1985a, Smith and Orr-Weaver 1991). Similar studies demonstrated that the DNA content in these tissues fall into discrete categories differing by a factor of two, suggesting a distinct period of DNA replication in the endocycle (Hammond and Laird 1985b, Hammond and Laird 1985a, Smith and Orr-Weaver 1991, Lilly and Spradling 1996). These observations implied that the endocycle itself is a regulated, modified mitotic cycle and suggested a period during which replication origins are reset.

In the canonical cell cycle, the DNA is replicated once in S phase of each cycle. At the end of mitosis, when mitotic cyclins have been degraded, there is a period of low CDK activity. This lack of CDK activity is essential to reset the origins of replication for the next S phase (for review see Bell and Dutta 2002). The pre-replication complex (pre-RCs) is formed on the origins, consisting of the origin recognition complex (ORC), CDC6, CDT1/DUP and mini-chromosome maintenance proteins (MCMs) (Bell and Dutta 2002). The DNA is then considered "licensed" for replication. In S phase, the levels of the S-phase CDK, CYCLIN

E/CDK2, increase. Ectopic expression of CYCLIN E is sufficient to induce cells to enter S phase in Drosophila embryos and eye imaginal discs (Knoblich *et al.* 1994, Richardson *et al.* 1995). DNA replication is initiated and origins are then blocked from re-licensing due to the high level of CDK activity, which continues through mitosis.

CYCLIN E is a regulator of endocycles

In endocycles, it becomes necessary to prevent re-replication in a single S-phase and to reset the pre-RCs without going through mitosis. As all endocycles have gap phases, a period of low CDK activity must exist. This period appears to be generated through regulation of CYCLIN E and evidence for the role and regulation of CYCLIN E in the endocycle is multifold. First, mutation of *cyclin E* disrupts endocycling tissues (Knoblich *et al.* 1994, Lilly and Spradling 1996). Second, CYCLIN E can downregulate its own expression, resulting in oscillations: periods of high CDK activity for DNA replication and periods of low CDK activity for the licensing of origins (Sauer *et al.* 1995). Finally, continuous CYCLIN E/CDK2 activity inhibits the endocycle and growth of endocycling tissues (Lilly and Spradling 1996, Follette *et al.* 1998, Su and O'Farrell 1998, Weiss *et al.* 1998, Royzman *et al.* 2002, Weng *et al.* 2003, Doronkin *et al.* 2003, Shcherbata *et al.* 2004). CYCLIN E, therefore, appears to be a key molecule that regulates the endocycle in Drosophila. CYCLIN E is also required for endocycles in mammalian tissues; studies of mice knockouts for CYCLIN E1 and CYCLIN E2 show a disruption in the endocycles and marked reduction in DNA content in both trophoblasts and megakaryocytes (Geng *et al.* 2003, Parisi *et al.* 2003). Additionally, oscillations in CYCLIN E protein levels were observed in rat trophoblast giant cells, indicating that CYCLIN E may be a conserved regulator of endocycles in diverse tissues and organisms (MacAuley *et al.* 1998).

Several mechanisms likely contribute to CYCLIN E oscillations in Drosophila, although the importance of each of these mechanisms in different tissues may vary. First, transcription of a number of S phase genes, including *cyclin E*, is controlled by the heterodimeric transcription factor, E2F1/DP (Duronio and O'Farrell 1995, Royzman *et al.* 1997). CYCLIN E negatively regulates its own transcription through this transcription factor in endocycles, and E2F1 is required for normal larval endocycles (Duronio and O'Farrell 1995, Sauer *et al.* 1995, Royzman *et al.* 1997). Additionally, E2F2/DP can act negatively on transcription as a repressor. It has been shown that loss of E2F2 repressor function in larval endocycles leads to continuous CYCLIN E and a disruption of endocycles (Weng *et al.* 2003). Furthermore, E2F1/DP appear to have functions in the endocycle that are not directly related to *cyclin E*; *dp* or *e2f1* mutants do not display defects in nurse cell endocycling, but do affect underreplication in this tissue without altering oscillations of CYCLIN E (Royzman *et al.* 2002). In follicle cells, both *rbf* and *dp* are required to shut off endocycles as mutations in these genes result in increased ploidy, but the *dp* mutant does not affect *cyclin E* mRNA and protein levels or activity (Royzman *et al.* 1999, Bosco *et al.* 2001). It seems, therefore, that E2F1/DP contribute to endocycles, although it is not likely that this occurs solely through control of transcription of *cyclin E* and it is unclear whether this contribution is the same in each endocycling tissue.

Regulation of E2F, DP and RB for proper endocycles appears to be a conserved mechanism, as overexpression or deletion of these genes disrupts development of mammalian endocycling tissues. Overexpression of E2F-1 in megakaryocytes leads to increased numbers of megakaryocytes, but blocks terminal differentiation (Guy *et al.* 1996). *Dp1*$^{-/-}$ mice are embryonic lethal, displaying extra-embryonic defects that correlate to defects in the proliferation of trophoblast precursors and in the endocycle of the existing trophoblast cells (Kohn *et al.* 2003,

Kohn *et al.* 2004). Finally, inactivation of *Rb* in mice also leads to embryonic lethality with overproliferation of the trophoblasts (Wu *et al.* 2003). E2F, DP and RB, therefore, play critical roles to ensure proper development in endocycling tissues.

Second, CYCLIN E protein levels are also regulated by degradation, as they are targeted for destruction by a particular E3 ubiquitin ligase, the SCF (Koepp *et al.* 2001, Moberg *et al.* 2001, Strohmaier *et al.* 2001). Mutations in SCF components disrupt the endocycle in nurse and follicle cells and allow accumulation of high levels of CYCLIN E, providing an explanation for oscillations of CYCLIN E levels in the absence of changes in transcript levels (Doronkin *et al.* 2003, Shcherbata *et al.* 2004). Finally, the activity of CYCLIN E/CDK2 is inhibited by the $p27^{CIP/KIP}$ cyclin kinase inhibitor, DACAPO (de Nooij *et al.* 1996, Lane *et al.* 1996, de Nooij *et al.* 2000). DACAPO binds to and inhibits CYCLIN E/CDK2 activity and oscillations of DACAPO have been observed to follow CYCLIN E oscillations in nurse cells (Lane *et al.* 1996, de Nooij *et al.* 2000). Intriguingly, levels of DACAPO itself are regulated by CYCLIN E; transcript levels of *dacapo* are reduced in *cyclin E* mutants and overexpression of *cyclin E* leads to increases in DACAPO protein levels (de Nooij *et al.* 2000). This mechanism may be conserved in other endocycles as well; mammalian trophoblast cells express and show oscillations in protein levels of p57, a CIP/KIP cyclin kinase inhibitor, and introduction of a stabilized form of p57 blocks the endocycle (Hattori *et al.* 2000). In mammalian megakaryocytes, overexpression of p21, another CIP/KIP CKI, blocks endocycling in this tissue (Baccini *et al.* 2001). Thus, several mechanisms likely contribute to the restriction of CYCLIN E/CDK2 activity and the generation of oscillations in CDK activity in the endocycle. CYCLIN E, therefore, is a key molecule that regulates the endocycle: its presence drives DNA replication in S phase and its absence allows origins to reset in G phase.

Mitotic activity is repressed in endocycles

While CYCLIN E is a critical regulator of endocycles, mitotic cyclins do not appear to play a role in many endocycles. As mitosis is absent in the endocycle, mitotic activity must be downregulated in the transition from mitosis to the endocycle and this is achieved in several ways. For example, in the larval tissues, Drosophila mitotic cyclins A and B are not expressed in endocycling larval tissues and are not required for the endocycle in these cells. Thus in these tissues there is an absence of mitotic activity, and the CDK activity associated with these cyclins does not contribute to the endocycle (Lehner and O'Farrell 1989, Lehner and O'Farrell 1990, Whitfield *et al.* 1990, Stern *et al.* 1993, Lilly and Spradling 1996, Jacobs *et al.* 1998, Schaeffer *et al.* 2004). The timing of a switch from mitotic cycles to endocycles in the Drosophila larval tissues was demonstrated to be specific and reproducible for each tissue, indicating that initiation of the endocycle was a developmentally programmed event (Smith and Orr-Weaver 1991). This switch to the endocycle has been shown to require the activity of FZR to downregulate levels of mitotic cyclins, as *fzr* mutant embryos and mutant follicle cells do not initiate endocycles (Sigrist and Lehner 1997, Schaeffer *et al.* 2004). The activity of the APC/C does not appear to be required for larval endocycles once they have initiated endocycling, further suggesting that mitotic cyclins are not present in these endocycles (Reed and Orr-Weaver 1997, Kashevsky *et al.* 2002). A FZR homolog in plants, CCS52, has also been shown to be required for the endocycle in plant tissues, as knock down of *ccs52* transcripts reduces the ploidy of endocycling cells (Cebolla *et al.* 1999, Vinardell *et al.* 2003). Recent studies have also begun to elucidate the integration of environmental signals and inducers of the endocycle in Drosophila; the Notch/Delta signaling pathway has been connected to the downstream events of the mitotic/endocycle switch in follicle cells and endocycles have been demonstrated to be linked to growth regulatory pathways like the

insulin pathway and the growth stimulator Myc (reviewed in Lilly and Duronio 2005, Edgar and Orr-Weaver 2001).

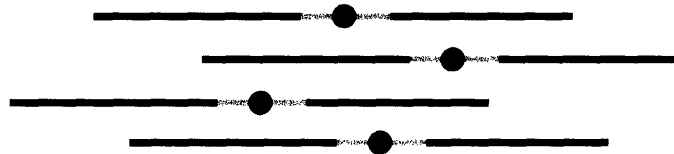
The endocycle produces polyploid cells with varying chromosome structures

The chromosomes of polyploid cells generated by the endocycle show great diversity in their structure. At one extreme the chromosomes are completely detached and separate from one other; these are referred to as polyploid chromosomes (Figure 4A). These chromosomes are frequently found in mammalian cells like megakaryocytes that produce platelets and vascular smooth muscle cells and plants (Nagl 1990, Nagata *et al.* 2005). At the other extreme, each newly replicated sister chromatid is held tightly in register with the parental strand, producing polytene chromosomes (Figure 4B). The best known example of polytene chromosomes are those in the salivary glands of Drosophila where up to 2048 copies of each chromosome are held in parallel (see below, Urata *et al.* 1995). Intermediate structures also exist where certain regions of the chromosome are dispersed and separate while other regions are polytene (Figure 4C). Examples of polyteny are not limited to insects and regions of polyteny in chromosomes are found in ciliates, mammalian cells and plants (Nagl 1990). Rat giant trophoblast cells can contain up to 1000 copies of each chromosome and all the chromosomes in the nucleus appear to form thick, short bundles (Zybina 1961). It is currently not understood how the endocycle can produce these different chromosomal structures or the proteins that differentiate polyploid chromosomes from polytene chromosomes.

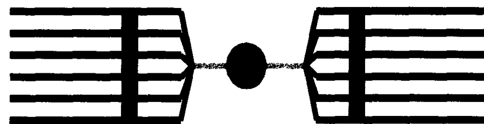
Figure 4: Endocycles produce polyploid cells with varying chromosome structures.

A. A schematic of polyploid chromosomes in which the chromosomes are separate and distinct within the nucleus. In this example, a tetraploid cell with polyploid chromosome structure is depicted. In each schematic, the centromere is represented by a green dot, centric heterochromatin is represented by the gray lines and the euchromatic arms are represented by the black lines. B. A schematic of a polytene chromosome in which each newly replicated sister chromatid is held tightly in parallel with its sister (represented by the purple band). In addition, the centric heterochromatin is underreplicated in this example. These chromosomes are common in insects. C. A schematic representing chromosomes in which certain regions are polyploid and dispersed, but other regions are polytene (purple band). This is the structure observed in mammalian trophoblast chromosomes.

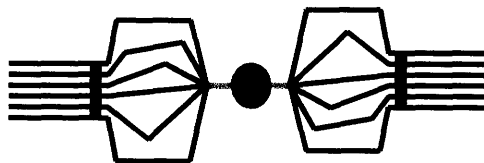
A. Polyploid



B. Polytene



C. Intermediate

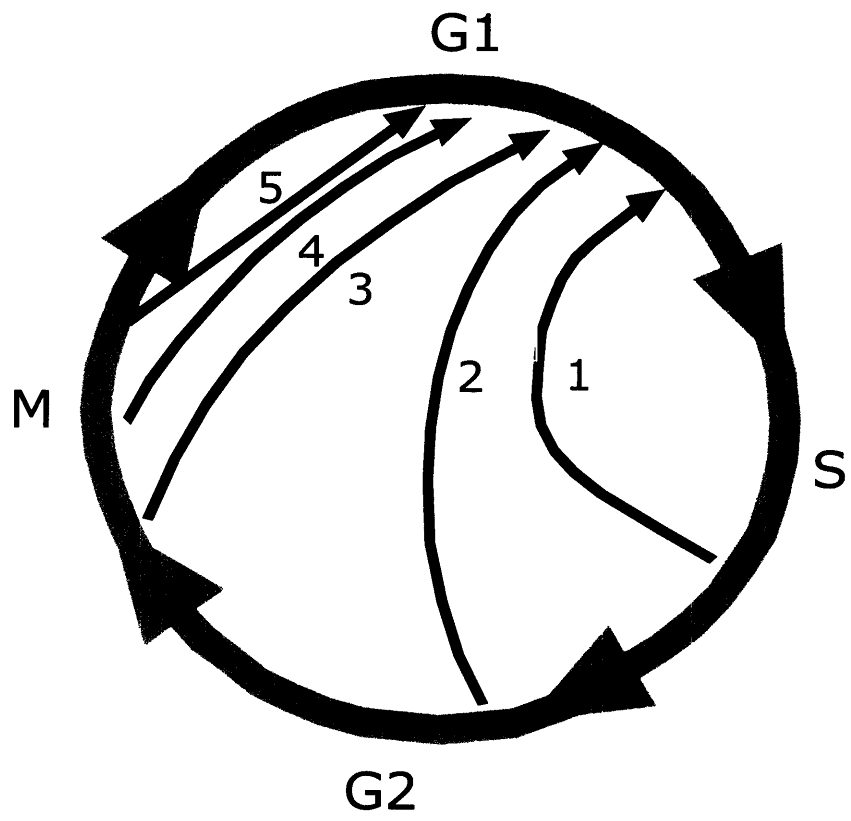


Polyploid chromosome structure may reflect variations in the endocycle

Differences in the endocycle itself are likely responsible for the differences observed in chromosome structures in polyploid cells; these variations in the endocycles are presented in Figure 5. One significant difference results from the truncation of S phase, a variant that is seen in all the polytene larval tissues and in late nurse cells in Drosophila (#1 in Figure 5, Edgar and Orr-Weaver 2001). This truncation results in underrepresentation of certain sequences in these cells, particularly in late-replicating heterochromatin, but in certain euchromatic regions as well (Edgar and Orr-Weaver 2001). Truncation of S phase appears to be linked to oscillation of CYCLIN E levels, as mutations in *cyclin E* that lead to continuous CYCLIN E demonstrate replication of normally underreplicated regions in the nurse cells (Lilly and Spradling 1996, Doronkin *et al.* 2003). Mutants in E2F1 and DP, however, show replication of heterochromatin in nurse cells that is independent of *cyclin E* disruption, suggesting that other factors can alter the control of S phase timing, or that E2F1 and DP play direct roles in affecting heterochromatin replication in this tissue (Royzman *et al.* 2002). It has been speculated that underreplication of these regions may be an energy-saving measure, as the gene-poor regions may not be necessary for the biology of these tissues. The identification of a protein that blocks replication at these sites, Suppressor of Underreplication (SuUR), indicates that underreplication is an active process although it remains to be demonstrated that underreplication serves a biological purpose (Belyaeva *et al.* 1998). Additionally, endocycles vary in the amount of mitotic character they have. Although the majority of endocycles consist of only alternating S and G phases, without any vestiges of mitosis, exceptions do exist and suggest that not every endocycle is the same. The giant trophoblast cells contain chromosomes up to 1000C and observations of these

Figure 5: Variations in the nature of the endocycle used in nature.

While a strict endocycle consists of a full synthesis phase (S) followed by a gap phase (G), many variations of this cycle are seen in various polyploidy tissues. 1. In some endocycles, S phase is truncated, leading to the underreplication of late-replicating sequences. This type of endocycle is seen in the Drosophila larval tissues and late nurse cells in the ovary (after stage 6). 2. A conventional endocycle with a complete S phase. This cycle is used by early nurse cells (stages 1-4). 3. Examples of mitotic character in the endocycle have been described; these endocycles are also referred to as endomitosis. Chromosomes in mammalian giant trophoblasts condense and bundle and then decondense upon DNA replication. 4. Sister separation, but not segregation, is observed in cycling mammalian megakaryocytes. As nuclear division and cytokinesis do not occur in these cells, this results in a polyploid nucleus. 5. Finally, mammalian hepatocytes proceed through separation of their sisters and nuclear division, but cytokinesis is not observed. This figure was adapted from Edgar and Orr-Weaver 2001.


G1
5
4
3
2
1
M
S
G2

chromosomes revealed that regions along the chromosome arms are polytene (Varmuza *et al.* 1988, reviewed in Zybina and Zybina 1996). During their endocycle, these chromosomes were observed to condense into chromosomal bundles without dissolution of the nuclear membrane, suggestive of entry into a mitotic-like prophase before resetting the cycle for another round of DNA replication (#3 in Figure 5, Varmuza *et al.* 1988).

Mammalian megakaryocytes cycle through endomitosis, a term used to describe cycles that proceed through anaphase but lack nuclear division and cytokinesis (#4 in Figure 5, reviewed in Zimmet and Ravid 2000). Megakaryocytes demonstrate nuclear envelope breakdown and the appearance of condensed chromosomes and multipolar spindles, but not features of late mitosis (Nagata *et al.* 1997, Vitrat *et al.* 1998, Roy *et al.* 2001). The sister chromatids have been observed to separate, but do not segregate (Roy *et al.* 2001). The presence of mitotic cell cycle regulators has been observed as well; endomitosis appears to occur with decreased levels of CYCLIN B/CDK1 (Zhang *et al.* 1996, Datta *et al.* 1996). Additionally, CYCLIN B is degraded in these cells, and the onset of degradation appears to be analogous to that of a canonical mitosis (Roy *et al.* 2001). Both CDC20/FZY and CDH1/FZR are expressed in megakaryocytes, although it remains to be shown whether they serve a function (Roy *et al.* 2001). Similar studies have observed enhanced degradation of CYCLIN B in these cells, suggesting that this may account for a premature mitotic exit without cytokinesis (Zhang *et al.* 1998).

Finally, cells can proceed through an endocycle with nuclear division, but no observable cytokinesis (#5 in Figure 5). This variant of the endocycle results in cells with multiple nuclei as are seen in mammalian hepatocytes (Brodsky and Uryvaeva 1977). Cytological observation of these cells revealed that cytokinesis does not occur and that the absence of cytokinesis results in polyploid cells, but in this case with multiple, separate nuclei (Guidotti *et al.* 2003). The

endocycle, therefore, can produce several different types of polyploid cells, and these differences in features of the endocycle likely generate the diversity of chromosome structure.

Drosophila tissues endocycle during development

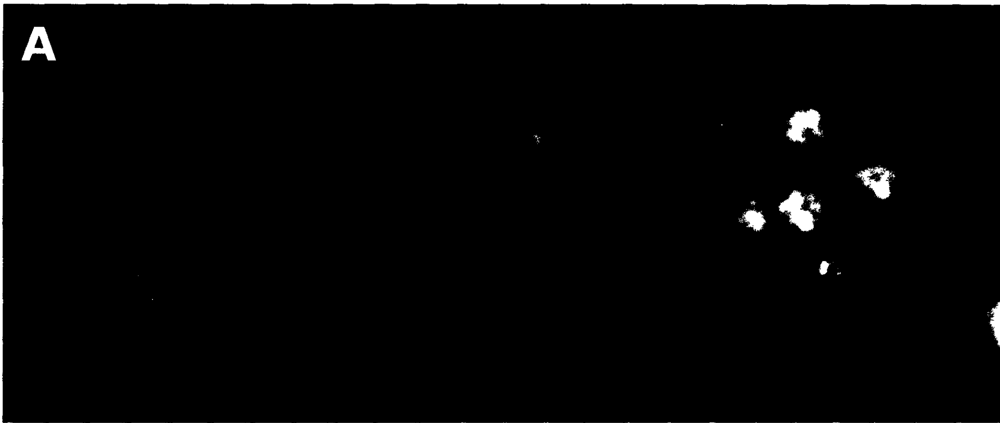
Drosophila utilize the endocycle at several times during their development, in particular in the larval-specific tissues and the nurse and follicle cells during oogenesis. This has allowed characterization of the endocycle at different stages in development and in different tissues, as these tissues show differences in the endocycle itself and in the resulting chromosomes. Following the rapid S-M cycles of Drosophila embryogenesis, the larval-specific tissues initiate their endocycles utilizing a truncated S phase (Smith and Orr-Weaver 1991). These endocycles produce polytene chromosomes in the gut, epidermis, fat body, malpighian tubules, trachea and salivary glands of the larvae. This high degree of DNA replication permits rapid cell and organismal growth during larval development and enables these tissues to metabolically support the growth and development of the imaginal discs which give rise to the adult tissues (Lilly and Duronio 2005). These tissues remain polytene throughout their duration until apoptosis at the larval-pupal stage. Similarly, the nurse cells of the growing Drosophila egg chamber use the endocycle, enabling them to fill the oocyte with enough mRNAs and protein stockpiles for the first fourteen mitotic cycles of embryogenesis (Spradling 1993). Finally, the somatically-derived follicle cells of Drosophila egg chambers go through four endocycles before entering a period of gene amplification, facilitating the high production of proteins required for the eggshell (for review see Claycomb and Orr-Weaver 2005).

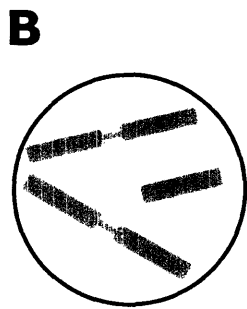
Drosophila nurse cells progress through a polyteny-polyploidy transition

The fifteen nurse cells in the Drosophila ovary are formed by four mitotic divisions of germline cells, known as the cystoblast divisions. Due to an incomplete cytokinesis, these fifteen cells and the sixteenth, which will become the oocyte, remain connected by cytoplasmic bridges. Following these divisions, the nurse cells begin endocycling and go through 10-12 endocycles, corresponding to egg chamber stages 1-10. Morphological studies revealed a significant and programmed change in nurse cell chromosome structure during their growth that has been shown to correlate directly with the endocycle (Figure 6, King 1970 and Dej and Spradling 1999). Chromosomes in early egg chambers (stages 1-4) are polytene and undergo complete DNA replication (Figure 6A, B). The chromosomes then condense to a "bulbous" appearance in the following stage (Figure 6A, C). In the unique fifth endocycle, an incomplete S phase is followed by the dissociation of the chromosomes into chromatid pairs that are held together by their unreplicated regions. This period has been proposed to include a transient mitotic-like state to allow separation of the sister chromatids, an idea that is supported by the identification of a mitotic regulator, *morula*, that is required for this transition in nurse cells (Reed and Orr-Weaver 1997, Dej and Spradling 1999). After stage 6, the chromosomes are "dispersed," and these endocycles lack late S phase (Figure 6A, D). It is important to note that nurse cells cycle asynchronously, such that the polyteny-polyploidy transition does not take place uniformly within nurse cells of a stage 5 egg chamber (Dej and Spradling 1999). This transition thus provides a unique environment in which to examine the molecular requirements for both polytene and polyploid chromosomes.

Figure 6: Drosophila nurse cell chromosomes progress through a polyteny-polyploidy transition during their development.

A. Drosophila ovaries consist of lines of developing egg chambers (ovarioles) in which egg chambers are in a row according to their developmental age (known as stages). In this image of a single ovariole, several early egg chambers are evident and the DNA has been stained with a fluorescent dye. Nurse cell chromosomes, in the interior of each egg chamber, go through a programmed structural transition. In early egg chambers, the endocycle produces nurse cell polytene chromosomes (green arrow, schematic in B). The arms of the two somatic chromosomes, the *2nd* and *3rd* chromosomes, are held together by their centric heterochromatin and the single-armed *X* chromosome is represented. The small, heterochromatic 4th chromosome is not pictured here. After the fifth endocycle, the nurse cell chromosomes condense and after this stage the polyteny character of the chromosomes is lost (blue arrow, schematic in C). The condensed arms of the *2nd*, *3rd*, and *X* chromosomes appear as five balls, thus this transition stage is also referred to as the "blob" stage. In stage 6 egg chambers, the nurse cell chromosomes now have a dispersed structure and the DNA loosens to fill the nucleus (purple arrow, schematic in D). The nurse cell chromosomes retain this structure until their demise at the end of oogenesis.


A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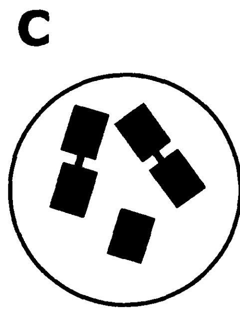
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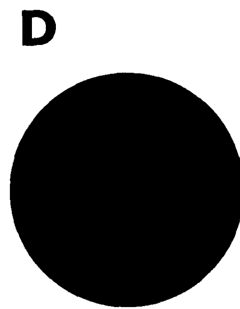
D

Characteristics and studies of Drosophila polytene chromosomes

Drosophila salivary gland cells undergo approximately ten endocycles producing chromosomes with up to 2048 copies of the euchromatic genome (Figure 7, Urata *et al.* 1995). These chromosomes are highly polytene, as both the homologs and chromatids are held in parallel. The size and extensive polyteny of these chromosomes have made them a favorite of cytologists for quite some time; the first description of insect larval salivary glands came by Balbiani in 1881, and we still utilize the detailed banding pattern maps of these chromosomes made by C.B. Bridges (Balbiani 1881, Bridges 1935). Salivary gland polytene chromosomes have greatly facilitated studies of the Drosophila genome and genomic organization and have provided a useful tool for studies of many chromosomal processes (for review see Zhimulev *et al.* 2004). These chromosomes contain several characteristic features that have been extensively described cytologically but whose molecular details still remain generally unknown.

The most apparent cytological detail of these chromosomes is the highly reproducible banding pattern, a characteristic not unique to Drosophila that is found in polytene chromosomes of other insects as well. The chromosomes consist of bands of varying widths, which stain darkly with visible dyes, and interbands that vary in width as well, which stain lightly with such dyes (Figure 7C). Bands were cytologically determined to constitute 95% of the total genomic DNA with interbands constituting 5% (Paul and Mateyko 1970). With the sequencing of the Drosophila genome, it has been possible to revisit the cytological map of the genome with the newly determined molecular map and studies have begun to analyze the molecular characteristics of the bands. In situ hybridizations of a BAC from the tip of the *X* chromosome to polytene chromosomes demonstrated that 102 bands were included by 2.6 megabases of DNA, with an average of 26.2 kb per band, similar to previous estimates

Figure 7: Drosophila salivary gland chromosomes are highly polytene and have several characteristics.

A. Drosophila salivary gland cells undergo ten endocycles producing large chromosomes that are highly polytene. In addition to the tight association of the sister chromatids, homologs of each four chromosomes in Drosophila are held together as well (see drawing in B). This single nucleus squash, stained with a visible DNA dye, reveals several of the key characteristics of these chromosomes. First, the underreplicated centric heterochromatin of each chromosome forms a diffuse, netlike structure known as the chromocenter (blue arrow in A, grey circle in B). Second, differences in compaction along the length of the chromosome arms produce bands and interbands (arrowhead in C denotes interband, arrow marks band). The width of each band and interband varies, but the pattern itself is highly reproducible.

B

3R

4

2L

2R

3L

X

(Benos *et al.* 2000, Sorsa 1988). Evidence against the "one gene/one band" hypothesis came from the genome project as well; the genome is predicted to contain ~13,600 genes in 3000-5000 bands or 2.7-4.5 genes per band (Zhimulev *et al.* 2004, Adams *et al.* 2000, Lefevre 1976). Electron microscope studies have demonstrated that polytene chromosomes consist of many chromatids that are differentially condensed along their lengths; bands contain condensed DNA, while interbands contain decondensed DNA (Zhimulev *et al.* 2004). Importantly, it was shown that the DNA in bands and interbands was replicated to the same extent, further suggesting that these structures resulted from differences in compaction (Spierer and Spierer 1984).

Several non-mutually exclusive models have been proposed to account for the presence of interbands and their diverse functional purposes. First, it has been hypothesized that interbands contain "housekeeping genes" that are transcriptionally active, as localization of DNA/RNA hybrids and RNA polymerase II to interbands suggest that these regions are involved in transcription (Zhimulev *et al.* 2004). Puffs, a localized loosening of chromatid packing, are induced by heavy transcription, implying that active transcription is correlated with less condensed DNA in these chromosomes (Zhimulev *et al.* 2004). Second, adjacent bands and interbands can act as a functional unit with regulatory regions being located in interbands as appears to be the case at the *Notch* locus (Rykowski *et al.* 1988, Zhimulev *et al.* 2004). Finally, based on the interband-specific localization of insulator proteins, it has been proposed that interbands may represent boundary elements that define chromosomal domains (Zhimulev *et al.* 2004). Intriguingly, two of these factors, a histone H3 kinase JIL-1 and a protein of unknown function, Z4, not only localize to interbands, but are required for the establishment or maintenance of the band/interband structure (Jin *et al.* 1999, Wang *et al.* 2001, Eggert *et al.* 2004).

The mechanism of banding and function of interband/band organization, therefore, remain exciting questions to be addressed on molecular and mechanistic levels.

A number of chromosome features result from underreplication of certain regions in these chromosomes. The chromocenter is diffuse, nonbanded region that consists of the underreplicated, centromeric heterochromatin of the four Drosophila chromosomes (blue arrow in Figure 7A, reviewed in Zhimulev *et al.* 2004). Non-homologous DNA contacts, termed ectopic pairings, can be made in this region, linking the chromosome arms through their centric regions. The chromocenter consists of two types of heterochromatin; α-heterochromatin, highly repetitive pericentric DNA that is dense and compact and β-heterochromatin that is less repetitive and more diffuse (Zhimulev *et al.* 2004). β-heterochromatin forms the majority of the chromocenter; the middle of the chromocenter, however, contains α-heterochromatin (Zhimulev *et al.* 2004). This region, therefore, plays an important structural role in these polytene chromosomes, but whether the association of the chromosomes arms via the chromocenter serves a specific purpose remains to be determined. Specific regions dispersed in the euchromatin are underreplicated as well, termed intercalary heterochromatin (IH). Like the pericentric heterochromatin, IH regions make ectopic contacts and the reproducible nature of these contacts has allowed them to be detailed (Zhimulev *et al.* 2004). The identification of these regions will assist in determining whether euchromatic underreplication serves a particular structural role for these chromosomes as well.

Nurse cell polytene chromosomes are significantly smaller than their larval counterparts; these chromosomes consist of 32 chromatids before proceeding through the polyteny-polyploidy transition (Dej and Spradling 1999). Additionally, these chromosomes lack a chromocenter and remain separate in nurse cells (Dej and Spradling 1999). By hybridization of fluorescent single-

copy probes to pericentric heterochromatin, Dej and Spradling observed that more than 25% of nurse cell chromosome length corresponded to centromeric heterochromatin, suggesting that in early nurse cells, S phase is not truncated and, subsequently, a chromocenter is not formed (Dej and Spradling 1999). Furthermore, nurse cell polytene chromosomes lack the distinct banding pattern seen in other polytene chromosomes (Dej and Spradling 1999). Finally, in these studies it was observed that nurse cell chromosomes are shorter and wider than larval, somatic polytene chromosomes, implying that nurse cell polytene chromosomes are more compact (Dej and Spradling 1999). To explain these differences, the authors propose that the presence of a full S phase allows more time for chromosome condensation, a hypothesis supported by their observation of condensation of these chromosomes during late S phase (Dej and Spradling 1999). As the polytene larval tissues and polytene nurse cells undergo different versions of the endocycle, it seems likely that these differences in polytene chromosome structure are linked to differences in the endocycle, as suggested by Dej and Spradling. The direct link between the endocycle and these chromosome characteristics, however, remains to be established, and it is not clear if and how these differences affect the biological activity of these tissues. As we identify and characterize the factors that contribute to the structure of polytene chromosomes, the biological significance of each of these features in promoting the goals of the endocycle in development will be elucidated.

Summary of Thesis

Although we are beginning to understand the regulators responsible for the cycling aspect of the endocycle, we know surprisingly little about how mitosis is curtailed in the endocycle and about how varying chromosome structures are generated by multiple rounds of DNA replication.

Previous studies of the Drosophila mutant *morula* revealed a requirement for this regulator to block the accumulation of mitotic cyclins at a specific stage in Drosophila nurse cell development. In Chapter 2, the cloning of this mutant and further phenotype characterization are described. In addition, the discovery that *mr* encodes a mitotic regulator provoked experiments addressing the role of mitotic regulators in nurse cell development. These experiments are described in Chapters 2, 3 and Appendix 1. Finally, demonstration of a requirement for the cohesin complex in polytene chromosome structure is presented in Chapter 3. These results increase our understanding of the endocycle and its modification to produce variation in chromosome structure throughout development.

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Chapter Two

The anaphase promoting complex/cyclosome is required during development for modified cell cycles

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Summary

Animals and plants use modified cell cycles to achieve particular developmental strategies. In one common example, most animals and plants have tissues in which the cells become polyploid or polytene by means of an S-G cycle, but the mechanism by which mitosis is inhibited in the endocycle is not understood. The Drosophila *morula* (*mr*) gene regulates variant cell cycles, because in addition to disrupting the archetypal cycle (G1-S-G2-M), *mr* mutations affect the rapid embryonic (S-M) divisions as well as the endocycle (S-G) that produces polyploid cells. In dividing cells *mr* mutations cause a metaphase arrest, and endocycling nurse cells inappropriately reenter mitosis in *mr* mutants. We show *mr* encodes the APC2 subunit of the anaphase promoting complex/cyclosome. This finding demonstrates that anaphase promoting complex/cyclosome is required not only in proliferating cells but also to block mitosis in some endocycles. The *mr* mutants further indicate that transient mitotic functions in endocycles change chromosome morphology from polytene to polyploid.

Introduction

The regulation of variant cell cycles is a crucial aspect of developmental control, yet many of these cycles are poorly understood. This observation is true for the endocycle, a modified cell cycle used throughout the plant and animal kingdoms to produce polyploid or polytene cells (for review see ref. 1). In this cycle, DNA replication cycles with a gap phase, but mitosis does not occur. There is, however, variability in endocycling tissues in the extent to which mitotic functions are repressed. In polytene cells, in which the replicated sister chromatids remain in tight association, it appears that no aspects of mitosis occur. In contrast, in mammalian megakaryocytes sister-chromatid separation and anaphase A movements occur, but anaphase B and cytokinesis are lacking (for review see ref. 2). Oscillations in the levels and activity of CYCLIN E/cyclin-dependent kinase (CDK) complexes are crucial for endocycles (for review see ref. 1), but the mechanism by which mitotic functions are inhibited remains to be defined. Somehow, expression of mitotic cyclin proteins is shut off, and they may be destroyed in a regulated fashion. Variation in the control of the destruction of mitotic cyclins and other mitotic activators could explain the differences to which mitotic functions persist in distinct endocycling cell types.

A pathway for inactivation of mitotic regulators by targeted proteolysis has been delineated (for reviews see refs. 3-5). Polyubiquitination of substrate proteins by a ubiquitin ligase, the anaphase promoting complex/cyclosome (APC/C), targets them for destruction by the 26S proteosome. The APC/C is composed of at least 11 subunits. In the yeast *Saccharomyces cerevisiae* mutations in the APC subunits *cdc16*, *cdc23*, and *cdc27* were identified because they block cyclin ubiquitination and destruction. They cause a failure of release of sister-chromatid cohesion, block the metaphase/anaphase transition, and prevent exit from mitosis. The APC/C is

regulated in part by two associated proteins, CDC20 (FIZZY in Drosophila) and CDH1 (FIZZY-RELATED in Drosophila), and these proteins both activate the APC/C with proper timing and provide substrate specificity. The APC/C is activated at the metaphase/anaphase transition by the CDC20 protein and later in telophase and G1 by the CDH1 protein. Mutations in the Drosophila *fizzy* and *fizzy-related* APC/C regulators have been characterized (6-9). Embryos mutant for *fizzy* arrest in metaphase of mitosis, whereas embryos lacking *fizzy-related* fail to cease proliferation at the appropriate stage. Recently, mutations have been described in the Drosophila *APC5* subunit gene and shown to affect mitotic divisions during larval stages (10).

The failure of mitosis to progress beyond metaphase in mutants for APC/C subunits is caused by the failure to degrade substrates whose sequential destruction is needed for steps through mitosis (for reviews see refs. 3-5). At the metaphase/anaphase transition the securin protein family members are ubiquitinated and proteolyzed. Members of this family include the Pds1 protein in *S. cerevisiae*, Cut2 in *Schizosaccharomyces pombe,* and PIMPLES in Drosophila (11-13). The securin proteins regulate the separase protease that targets the cohesin complex (for review see ref. 14), and in yeast the Slk19 protein needed for mitotic spindle function (15). Thus, by indirectly activating separase, the APC/C causes the release of sister-chromatid cohesion and events needed for the completion of mitosis. Mitotic cyclins are also targeted for degradation by the APC/C; this shuts off the mitotic cyclin/CDK1 complex to inactivate mitosis-promoting functions and to also permit resetting of the replication origins for another round of DNA synthesis. Additional direct substrates of the APC/C as well as indirect substrates that are cleaved by separase are likely to be involved in the exit from mitosis.

The Drosophila *morula* (*mr*) gene is critical for the inactivation of mitotic functions throughout development in a variety of developmentally-modified cell cycles (16). The initial *mr*

alleles, described in 1919 and 1937 by Bridges, are female sterile (17). In these mr^1 and mr^2 mutants, the endo cell cycle of the polyploid ovarian nurse cells is affected (16). The nurse cells initiate the endocycle, but after several cycles return to mitosis, condensing their chromosomes, assembling mitotic spindles, and arresting in a metaphase-like state. Stronger alleles of *mr* cause lethality late in larval development (16). In these mutant animals, there is a failure to inactivate mitotic functions in proliferating cells. Dividing cells in the larval brain arrest in metaphase. The *mr* phenotypes indicate that *mr* is required to prevent mitosis in some endocycling cells, but also for the inactivation of mitotic functions and exit from mitosis in dividing cells. These intriguing phenotypes made it important to define the molecular mechanism by which *mr* inhibits mitotic activities.

Here we describe a molecular analysis of *mr*. We find that it encodes the APC2 subunit of the APC/C, thus explaining the dual role that *mr* plays in inhibiting mitotic functions in the endocycle and in promoting mitotic exit. These results uncover a surprising requirement for the APC/C in controlling chromosome morphology in polyploid cells.

Materials and Methods

Southern and Northern Blots

Quantitative Southern blots to map deficiency breakpoints from heterozygous flies were done as described in Bickel *et al.* (18). cDNAs obtained from the Berkeley Drosophila Genome Project were sequenced by Research Genetics (Huntsville, AL). RNA from different developmental stages was isolated, Northern blots were prepared, and these were hybridized to the purified insert fragment from the LD21042 cDNA that was labeled by random priming (18). The expression pattern of the *mr* transcript was analyzed during oogenesis by *in situ* hybridization to whole mount ovaries as described (19).

cDNA Rescue Experiments

The cDNA insert in clone LD24965 was excised with *Eco*R 1/*Xho* I and subcloned into the pCS2+ vector to acquire desired sites. The fragment was then cut out with *Bam*H I/*Xba* I and subcloned into the same sites of the pUASp vector, which was obtained from P. Rørth (European Molecular Biology Laboratory, Heidelberg). This transposon is called *P[w+ UAS- mr]*. Embryo injections and the establishment of transgenic lines was as described by Spradling (20). In two of the lines used for rescue experiments (A1, 6D), *P[w+ UAS- mr]* was inserted on the third chromosome, and in line C5 the transposon was inserted on the X chromosome. Two GAL-4 driver lines were used to induce expression of the *mr* cDNA: the *actin*-GAL-4 line was from the Bloomington Stock Center (Bloomington, IN), and the *nanos*-GAL-4:VP16 line was obtained from P. Rørth (21).

DNA Sequencing of *mr* Mutations

To sequence the *mr* mutations DNA was prepared from homozygous animals, the ORF was PCR amplified, and the PCR products were sequenced directly by Research Genetics. For mr^{1} or mr^{2}, homozygous adult females were used. For the lethal alleles mr^{3} and mr^{5}, homozygous mutant larvae were identified from stocks in which the *mr* mutants were in trans to the *CyO* balancer chromosome containing *P[w+, Act-GFP]*. Homozygous mr^{4} mutant larvae were collected from stocks containing the *TSTL* chromosome 2;3 translocation that is marked with the dominant *Tubby* marker, which can be scored in larvae or adults. mr^{4} mutant larvae that were non-Tubby were collected. The DNA sequence was determined for both strands, and the isogenic chromosome on which the mr^{3}, mr^{4}, and mr^{5} mutations were induced was sequenced as a control.

Immunostaining of Ovaries and Embryos

Strains containing extra copies of the *cyclin B* gene were provided by C. Lehner (Univ. of Beyreuth, Beyreuth, Germany). The chromosome morphology of nurse cells was examined after 4',6-diamidino-2-phenylindole (DAPI) or propidium iodide staining as described (16). Mitotic spindles were examined in embryos after staining with rat anti-tubulin antibodies from Accurate Chemical and Accurate Scientific (Westbury, NY) as described by Tang *et al.* (22), except that the embryos were fixed in methanol. Anti-cyclin B staining of egg chambers was done as previously described (16). The monoclonal antibody developed by P. O'Farrell (Univ. of California, San Francisco) was obtained from the Developmental Studies Hybridoma Bank. Microscopy was done on a Zeiss LSM510 confocal laser system mounted on a Zeiss Axiovert 100M microscope with a x40/1.2 W Korr C-APOCHROMAT water objective. Optical sections were taken and projected onto a single plane.

Results

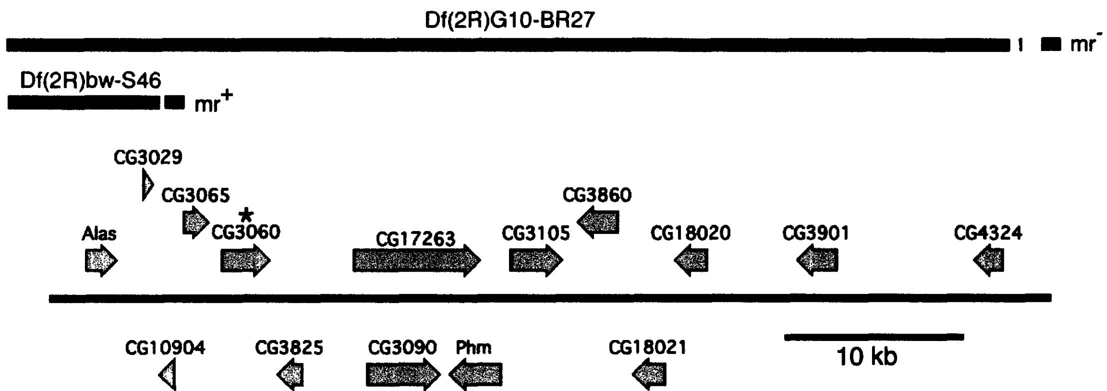
Identification of the *mr* gene

We used a positional cloning strategy to recover the *mr* gene. The gene is removed by the deficiency *Df(2R)G10-BR27*, but it is present in *Df(2R)bw-S46* (16). Quantitative Southern blots were used to map the position of the breakpoints of these two deficiencies (data not shown), defining a minimal region of 40 kb that contained the gene (Fig. 1*A*). Within this region, the CG3060 was an ideal candidate for *mr,* because it contains a cullin domain (http://www.fruitfly.org/), and cullin-domain proteins are involved in protein degradation during the cell cycle (3). We sequenced the longest cDNA corresponding to this ORF, LD24965. The sequence analysis confirmed the intron/exon structure predicted by the genome project, with a transcription unit spread over 2.96 kb of genomic DNA producing a processed transcript of 2.53 kb with eight exons. We tested the ability of this cDNA to rescue the *mr* mutant phenotypes. The insert was cloned into the pUASp expression vector to generate transposon *P[w+ UAS- mr]* (Fig. 1*B*). Transformant lines were generated, crossed to *mr* mutants, and expression of the cDNA was induced and examined for phenotypic rescue. To exclude phenotypes from potential background mutations on the *mr* chromosomes, complementation by the transgenes was scored in transheterozygotes with two different *mr* mutant chromosomes. To test for rescue of the female-sterile *mr* alleles, the GAL-4 activator was expressed in the female germ line under the control of the *nanos* regulatory elements. Three independent cDNA transformant lines restored female fertility to mr^1/mr^2 transheterozygotes when GAL-4 was induced but not in uninduced controls (Table 1). Induction of GAL-4 by the ubiquitously expressed *actin* promoter also rescued fertility in these flies (Table 1). The *actin*-GAL-4 driver was able to restore viability to

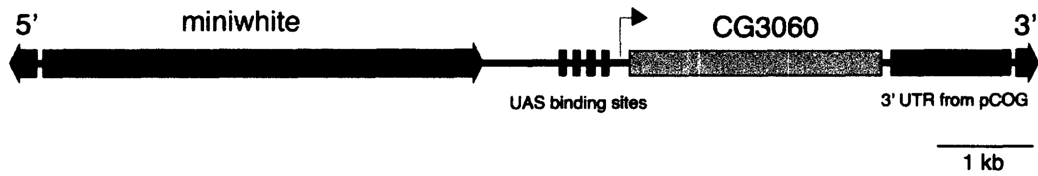
Figure 1: Isolation and expression of the *mr* gene

(*A*) The genomic region of 60A containing the *mr* gene. The DNA intervals removed by the two crucial deficiencies that are *mr*- or *mr*+ were determined by quantitative Southern blots and are shown by solid lines. The restriction fragments within which each deficiency breaks are drawn as dotted lines to denote that the exact position of the breakpoint within each fragment is not known. The two known genes, *Alas* and *Phm*, and the predicted ORFs are shown by filled arrows whose length is proportional to the size. The arrowheads indicate the 3' end of each gene. The CG3060 ORF (asterisk) is *mr*. (*B*) The structure of *P[w*+ UAS-*mr*]. The LD24965 cDNA was used. The black arrows at the end of the transposon denote P element sequences. (*C*) Developmental Northern blot of *mr* expression. Poly(A)+ mRNA was isolated from each of the indicated developmental periods, and the Northern filter was probed with the labeled cDNA fragment from LD21042. Three *mr* transcript forms are detected, and these vary in expression level at different developmental stages. (*D*) The same Northern filter stained with Ponceau-S to detect loading levels. The size standards are the 1-kb ladder from GIBCO/BRL.

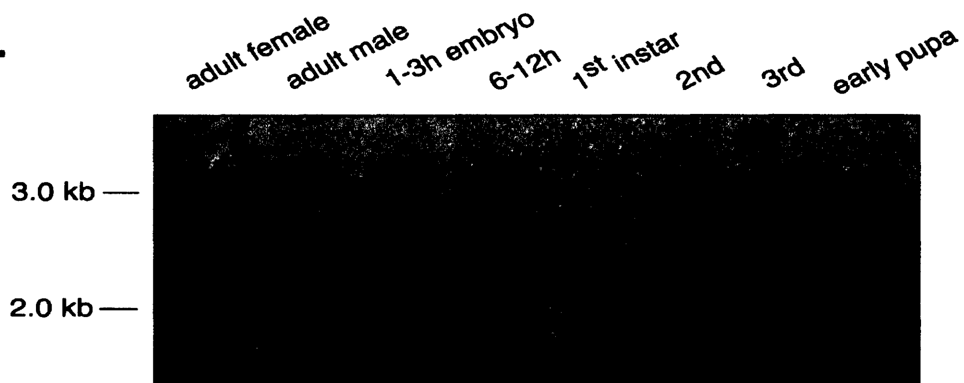
A.



B.



C.



D.

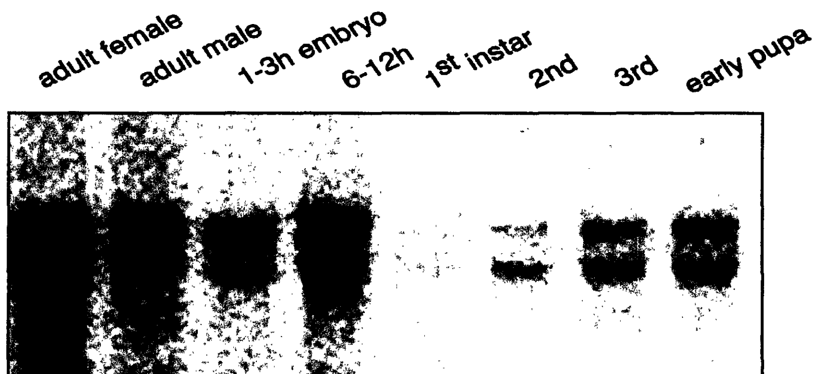


Table 1: Rescue of *mr* phenotypes by ectopic expression of transgenes

mr genotype	*actin*-GAL4 Induction				*nanos*-GAL4 Induction			
	Line A1	Line 6D	Line C5	Control	Line A1	Line 6D	Line C5	Control
mr^3/mr^4	Viable & Semi-fertile	Viable & Sterile	Viable & Fertile	Dead				
mr^1/mr^2	Fertile	Fertile	Fertile	Sterile	Fertile	Fertile	Fertile	Sterile

transheterozygotes of two lethal alleles, *mr*^{3} and *mr*^{4}. These experiments revealed differences in each of the transgenic lines, presumably reflecting different levels of expression, in that the *P[w+ UAS- mr]*-C5 line complemented fully to restore both viability and fertility, the *P[w+ UAS- mr]*-A1 line restored viability and partial fertility, whereas the *P[w+ UAS- mr]*-6D line solely rescued viability. The ability of the LD24965 cDNA to rescue both strong *mr* lethal alleles and weaker female-sterile alleles demonstrates that it encodes the structural gene for *mr*.

The phenotypes of the *mr* mutations indicated that the gene is required for cell cycle regulation throughout development: during adult oogenesis, in the early S-M embryonic cycles, in larval endocycles, and in mitotically dividing larval tissues. We examined the expression pattern of the gene by hybridizing the insert from a *mr* cDNA (LD21042) to a Northern blot with RNA isolated from different developmental stages (Fig. 1*C*). This experiment showed that the *mr* gene is expressed throughout development, but, interestingly, three different transcript forms are present, and these show different developmental regulation. There is an abundant transcript of 2.5 kb present in adult females and early embryos, most likely the form expressed during oogenesis and deposited into the developing oocyte. In larval development, transcripts of 2.9 and 3.2 kb become more prevalent, and in adult males solely the 3.2-kb transcript is detectable. The cDNAs recovered by the genome project from embryonic libraries all encode one protein form and are likely to represent the transcript that experimentally measures 2.5 kb. Additional analyses will be required to determine whether the three transcript forms arise from distinct promoters or alternative processing, and whether these result in alternative forms of the protein.

The MORULA protein is the ortholog of APC2

BLAST searches of the predicted ORF of the LD24965 cDNA showed that the protein is

closely related to the APC2 subunit of the APC/C (23, 24). APC2 contains a cullin domain, but MR shows sequence conservation throughout the protein sequence, not solely within the cullin domain. Overall, MR is 36% identical to human APC2 and shares 56% homology (Fig. 2). MR is more distantly related to the APC2 subunit from *S. cerevisiae* (Fig. 2). To understand the basis of the lethal and sterile phenotypes in *mr* mutants, we sequenced the five *mr* mutations. The mr^{3} mutant has the most severe phenotype in larval brains, and the molecular analysis confirms that this is the strongest allele. The mr^{3} strain contains a nucleotide substitution that is predicted to change Trp-282 to a stop codon, truncating the protein to approximately one-third of its length and removing the cullin domain (Fig. 2). The mr^{4} and mr^{5} alleles were phenotypically characterized as strong alleles because they cause lethality, and these too have pronounced molecular changes. Both alleles share the same nucleotide substitution that would alter a splice acceptor site after the sixth intron (Fig. 2). If the intron were not spliced, the protein would be expected to be missing the C terminus, including part of the cullin domain. The mr^{4} and mr^{5} were recovered from the same ethyl methanesulfonate screen and likely represent repeat isolates from the same premeiotic mutation event. The mr^{1} and mr^{2} alleles were isolated from natural populations about 20 years apart, and thus could contain the same mutation (17). Indeed, both have a single nucleotide change predicted to cause a Trp to Arg amino acid substitution (Fig. 2). This change is C-terminal to the cullin domain. This Trp is conserved in mammalian APC2 subunits, but not in the budding yeast protein. This flexibility in amino acid sequence may explain why these are the weakest of the *mr* mutations.

The APC/C is required for the repression of mitotic functions in some endocycles

The identification of MR as APC2 readily explains the metaphase arrest observed in

Figure 2: The Drosophila *mr* gene is an ortholog of APC2

The translated *mr* cDNA sequence (Dm) is aligned with the APC2 coding region from human (Hs) and *S. cerevisiae* (Sc). The cullin domain is underlined and indicated by brackets. Residues conserved in all three species are highlighted by asterisks. Double dots indicate that one of the following groups is fully conserved: STA, NEQK, NHQK, NDEQ, QHRK, MILV, HY, or FYW. A single dot represents conservation of groups with less similarity: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, or HFY. The dashed lines show where the alignment program introduced gaps to maximize homologous alignment. The changes present in the *mr* mutants are also indicated. In the mr^3 mutant Trp-282 is changed to a stop codon. The mr^4 and mr^5 mutants have a nucleotide substitution at a splice acceptor site that would cause remove the C terminus of the protein from Glu-657 on. The sole change found in mr^1 and mr^2 strains was a substitution of Trp-739 to Arg.

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                 .      .   . .. *       :  :  .
DmAPC2 MCDFDALWADVLRIFP---VLGGANAVSNDPVEEN--VFQNV--RQHLMVLGSIDTFAAV   53
HsAPC2 MAAAVVVAEGDSDSRPGQELLVAMNTVSTCLVPPA--ALCLVSSRTSCAVPPKEEELRAA   58
ScAPC2 -MSFQITPTRDLKVITDELQTLSSYIFHTNIVDDLNSLLTWMSPNDAKSNHQLRPPSLRI   59

        ::     ...     :      .      .      .     :  .        :
DmAPC2 VDLEIQSTIRNVLVPKFWKHIHDGLVSSKLQGWINDQALAIEALPDKNGLFTQFIDAINE  113
HsAPC2 VEVLRGHGLHSVLEEWFVEVLQNDLQANISPEFWN-AISQCENSADEPQCLLLLLDAFGL  117
ScAPC2 KNIIKVLFPNNATTSPYSMINTSQANNSIVNEGNTNKELQLQLFSTLKEFYIFQVRYHFF  119

       *.     ::      :                     :      . **   *:       . :
DmAPC2 LHDSFQSLMQVKPRLIQLVGAETKPT-AKTVELMQEEGIKNCIRDCLLAQLPPNFSVVVG  172
HsAPC2 LESRLDPYLRSLELLEKWTRLGLLMG-TGAQGLR--EEVHTMLRGVLFFSTPRTFQEMIQ  174
ScAPC2 LHFNNINYLKDIQRWENYYEFPLRYVPIFDVNVNDWALELNSIRHYLLNRNIKFKNNLRT  179

        :     :     .       ..   .                    :   .   . ::
DmAPC2 AFYWVHFKLFTKASNLALTTVDSDVFDEL----------------LCVGCNFEAEQCCCQ  216
HsAPC2 RLYGCFLRVYMQSKRKGEGGTDPELEGELDSRYARRRYYRLLQSPLCAGCSSDKQQCWCR  234
ScAPC2 RLDKLIMDDDFDLADNLIQWLKSANGSLSS------------TELIVNALYSKINKFCED  227

        .     ::    :  :. :  .   . .:  :       :           :  :.  :.. :
DmAPC2 RLTDMVNKTNMKLFEMNLIDRLTGSALTALIKLKIKEHISDTCMGIFDRSHLKQLETWLS  276
HsAPC2 QALEQFHQLSQVLHRLSLLERVSAEAVTTTLHQVTRERMEDRCRGEYERSFLREFHKWIE  294
ScAPC2 NMSRVWNKRFMIMETFNKFINQYWSQFSKLVGCPEDD--------HELTTTVFNCFESNFL  280

             mr^3 W->STOP
        :    |: .:     .     :  *   : :: ::    ::   :    :  .  :   .: :
DmAPC2 DVIMSWLTNIFTEWKSKDSISDIEVPESVKSFK---VKLTYFMYETFAQSVIGQFFSIIID  334
HsAPC2 RVVG-WLGKVFLQDGPARPAS-PEAGNTLRRWR---CHVQRFFYRIYASLRIEELFSIVRD  350
ScAPC2 RIRTNEIFDICVLAYPDSKVTLLELRKIMKDFKDYTNIVTTFLSDFKKYILNPSVTTVDA  340

           :*. :                :    ::   :: *     .*    ::      :*      :
DmAPC2 YPDSIPAIDDLKICMEKIDMRVYLTESLRNSLEARILHPGVNTMDILTGYVAAIKAIRHL  394
HsAPC2 FPDSRPAIEDLKYCLERTDQRQQLLVSLKAALETRLLHPGVNTCDIITLYISAIKALRVL  410
ScAPC2 LLRYVKTIKAFLVLDPTGRCLHSITTFVKPYFQERKHLVNVLLYAMLDLPEEELKEKINF  400

        :     *      .::    .:: .: :     .  .         .:  :      .:.
DmAPC2 DSTGVILEMVTAPIKDYLRKRNDTVRRVVTGLTEE--GPTDLSEELAKG--ETIKECKDS  450
HsAPC2 DPSMVILEVACEPIRRYLRTREDTVRQIVAGLTGDSDGTGDLAVELSKTDPASLETGQDS  470
ScAPC2 NVDMKALLSLVDTLHDSDINQDTNITKRDKNKKSPFLWNLKVKGKREINKDLPIRHAMLY  460

          .   .      * *:*  .   .       :  .  .  .::::.:::.::  *:::*:.*:*.*::*
DmAPC2 GTDEFSNWENWQPDPFGIDASIMQYNSSRKMRSADIISMVVDIYGSKELFMTEYRNLMAD  510
HsAPC2 -EDDSGEPEDWVPDPVDADP----GKSSSKRRSSDIISLLVSIYGSKDLFINEYRSLLAD  525
ScAPC2 -EHILNYYIAWVPEPNDMIP----GNIKSSYIKTNLFEVLLDILFESREFFISEFRNLLTD  515

       **:      :.    *:    *  ::*::  ::                                :: ::
DmAPC2 RLLAQLDFNS-EKEIRNLELLKIRFG--------------------------------ESLLH  540
HsAPC2 RLLHQFSFSP-EREIRNVELLKLRFG--------------------------------EAPMH  555
ScAPC2 RLFTLKFYTLDEKWTRCLKLIREKIVKFTETSHSNYITNGILGLLETTAPAADADQSNLN  575

         :*** *:    *..:    :::.                :.:          ..:        :.     :.:* ::
DmAPC2 SCEVMLKDVTDSKRINAHIHSDGDR---TENQLFDISSLIVSAQFWPSFNKESLQLPEEI  597
HsAPC2 FCEVMLKDMADSRRINANIREEDEKRPAEEQPPFGVYAVILSSEFWPPFKDEKLEVPEDI  615
ScAPC2 SIDVMLWDIKCSEELCRKMHEVAGLDPIIFPKFISLLYWKYNCDTQ-GSNDLAFHLPIDL  634

        .   :: *  . *.   *   *.*.        * *  :::  : * *.:  : *:      . ::   *:
DmAPC2 ENEFKKYTKAYEAYKGNRTLNWRTVTGRVNIEIEIGD-RTMEMVVSPILAVIIYHFQTKN  656
HsAPC2 RAALEAYCKKYEQLKAMRTLSWKHTLGLVTMDVELAD-RTLSVAVTPVQAVILLYFQDQA  674
ScAPC2 ERELQKYSDIYSQLKPGRKLQLCKDKGKVEIQLAFKDGRKLVLDVSLEQCSVINQFDSPN  694

        mr^4,mr^5 splice site mutation
       .|      ::*:**.   ::.  .  *  :  :..*  ::*::  :      *  ::::*.  *       :  :.
DmAPC2 E----WTIEDLSSITKVPASALRRRLSFWQNHGLISETTPGIFTLLEK-ESEKSQYEDMS  711
HsAPC2 S----WTLEELSKAVKMPVALLRRRMSVWLQQGVLREEPPGTFSVIEE-ERPQ-DRDNMV  728
ScAPC2 DEPICLSLEQLSESLNIAPPRLTHLLDFWIQKGVLLKEN-GTYSVIEHSEMDFDQAQKTA  753

                                    mr^1,mr^2 W->R
        :  ::.   :        *    :::  *    *    |.:*  .*****  ::   :.:*:.:*::   .    .   .
DmAPC2 LAEADEEDLESAMASASDQR-EEELQVFWSYIVGMLTNLDSMPIDRIHQMLKLFASHS-G  769
HsAPC2 LIDSDDE-SDSGMASQADQK-EEELLLFWTYIQAMLTNLESLSLDRIYNMLRMFVVTGPA  786
ScAPC2 PMEIENSNYELHNDSEIERKYELTLQRSLPFIEGMLTNLGAMKLHKIHSFLKITVPKDWG  813

         .:   ::*: :*:   .  :  :*  :  .  *  *.:  *
DmAPC2 GVEFTQDELKHFLQRKVREHKLIFSG-GVYQLAK------  802
HsAPC2 LAEIDLQELQGYLQKKVRDQQLVYSA-GVYRLPKNCS---  822
ScAPC2 YNRITLQQLEGYLNTIADEGRLKYIANGSYEIVKNGHKNS  853
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Cullin Domain

proliferating tissues from *mr* mutants and establishes that APC2 is essential for APC/C activity. This identification is significant also for demonstrating that the APC/C is necessary during endocycles to inhibit mitotic functions and is consistent with the previous observation that levels of CYCLIN B are inappropriately high in *mr* mutant nurse cells (16). Our finding that APC/C is required for endocycles raised the question of whether increased levels of CYCLIN B were responsible, at least in part, for the larval *mr* mutant phenotypes. To address this question, we tested whether increased levels of CYCLIN B could enhance *mr* phenotypes. The transheterozygous combination of the mr^1/mr^3 mutant alleles provided a sensitized test because these transheterozygotes produce viable adults, though at only 50% the number predicted for a fully viable combination (Table 2). We increased the copy number of wild-type *cyclin B* genes by two, thereby increasing the level of CYCLIN B protein (25, 26). We found that the increased CYCLIN B enhanced the lethal phenotype such that in the presence of extra copies of the *cyclin B* gene, no viable mr^1/mr^3 adults were recovered (Table 2). These results provide *in vivo* confirmation that levels of CYCLIN B affect the *mr* phenotype and contribute to the lethality of strong *mr* mutants.

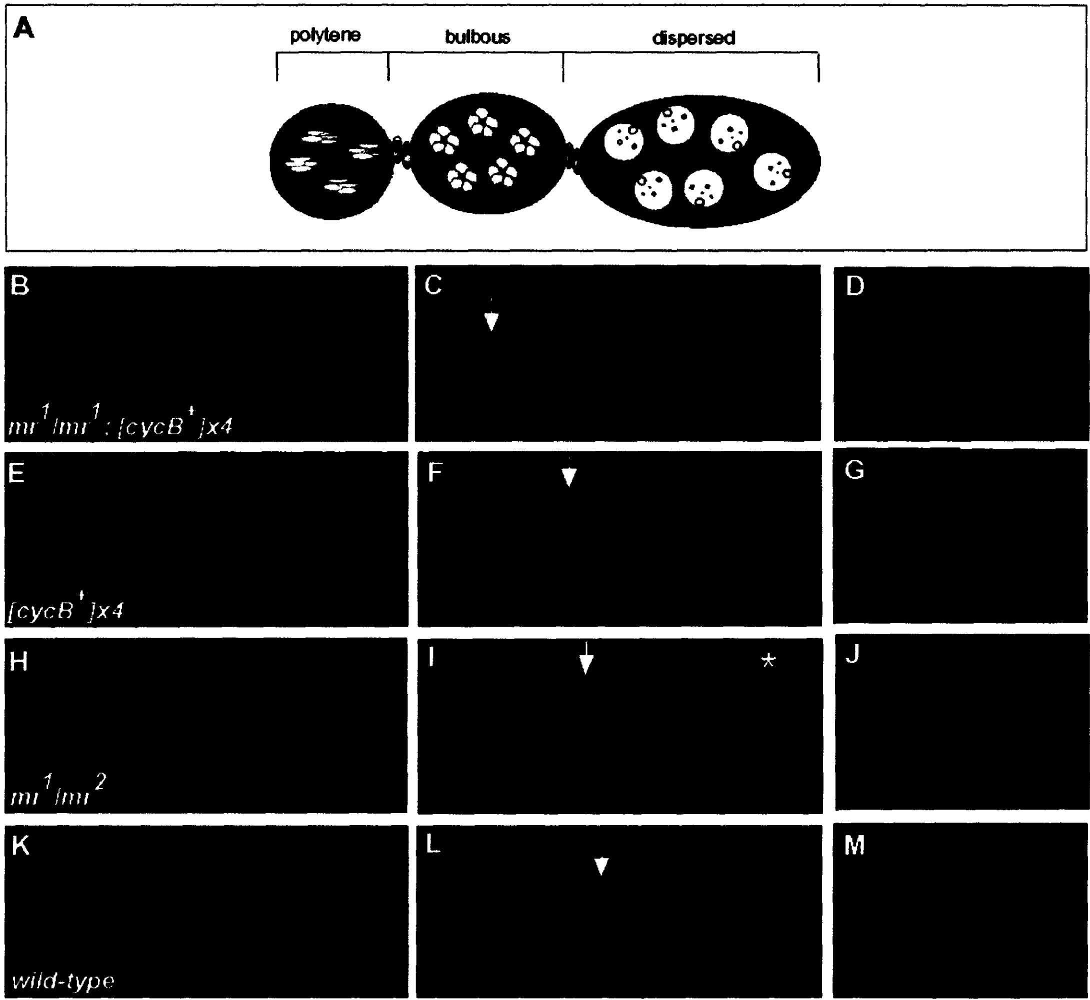
We tested also for enhancement of the female-sterile phenotype of the mr^1/mr^2 alleles by increased levels of CYCLIN B to examine the requirements for APC/C function during specific differentiation aspects of the nurse cell endocycle (see schematic in Fig. 3*A*). The five initial endocycles of the nurse cells produce polytene chromosomes in which the replicated sister chromatids remain in tight association. After cycle 5, the chromosomes condense, and then the replicated copies partially disperse so that in subsequent endocycles the chromosomes appear polyploid rather than polytene (27). A striking feature of the mr^1/mr^2 phenotype is that the first five nurse cell endocycles appear normal (16). The *mr* defect is not manifested until the

Table 2: Enhancement of *mr* lethality by increased *cyclin B*⁺ genes

mr genotype in progeny	Cross 1: mr^3/SM6a x mr^1sp/SM6a	Cross 2: mr^3/SM6a x mr^1sp/SM6a;[$CycB^+$]x2
mr/SM6a	210	104
mr^1/mr^3	39	0

Figure 3: The *mr* mutant nurse cell phenotype and the effect of increased CYCLIN B protein

(*A*) Schematic diagram of the changes in nurse cell chromosomes during stages 4-6 egg chamber development. The nurse cell chromosomes are polytene through stage 4; they then condense and take on a bulbous appearance before dispersing to be polyploid. (*B-M*). The effect of *mr* mutations and increased CYCLIN B as visualized by propidium iodide staining of the DNA (red; *C*, *D*, *F*, *G*, *I*, *J*, *L*, and *M*) and immunolabeling of CYCLIN B (green; *B*, *E*, *H*, and *K*). In *mr* mutants the nurse cells revert to mitosis at stage 5, shown by an arrow in *C*, *F*, *I*, and *L* and enlarged in *D*, *G*, *J*, and *M*. The onset of mitosis in mutant stage-5 egg chambers is evidenced by the appearance of condensed chromosomes (*D* and *J*) compared with the interphase appearance of wild type (*G* and *M*). Increased CYCLIN B did not cause the onset of mitosis to occur earlier in nurse cell development. Increased CYCLIN B did not result in the onset of mitosis in wild-type nurse cells, even when levels were higher than in *mr* mutants. In *mr* mutants the nurse cells in egg chambers after stage 7 frequently became pycnotic, as shown by the egg chamber with the asterisk in *I*.


A
polytene
bulbous
dispersed
B
C
D
mr^1/mr^1; $[cycB^+]x4$
E
F
G
$[cycB^+]x4$
H
I
J
mr^1/mr^2
K
L
M
wild-type

polytene/polyploid transition, when in *mr* mutant nurse cells the chromosomes condense more fully than in wild type, spindles are formed, and the condensed chromosomes remain arrested in a metaphase-like state. This phenotype showed the same time of onset in nurse cells mutant for the lethal mr^5 mutation, generated by germline clones (16). This finding raised the possibility that the polyteny/polyploidy transition involves a cell cycle change to a transient mitotic state and that, at this point, *mr* mutant nurse cells are vulnerable to reenter mitosis fully.

Consistent with the proposal that the onset of the *mr* phenotype reflects cell cycle changes in the nurse cells at the polytene/polyploid transition, we found that increased levels of CYCLIN B protein did not cause an earlier appearance of mitosis in the *mr* mutant nurse cells (Fig. 3 *B*, *C*, *H*, and *I*). We did observe an increase in the number of later stage egg chambers with pycnotic or degenerating nurse cells in the presence of increased CYCLIN B (data not shown). We also found that elevation of CYCLIN B protein in a wild-type background was insufficient to cause nurse cells to revert to mitosis (Fig. 3 *E* and *F*). It remains possible that increasing the levels of other APC/C substrates would cause an earlier endocycle defect.

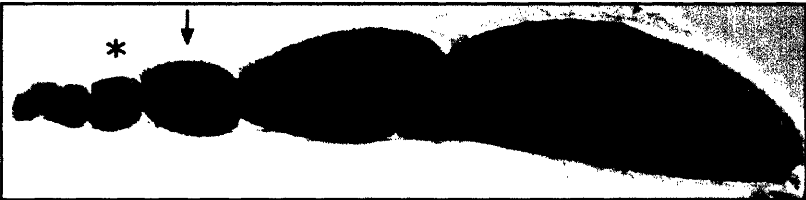
We examined the levels of *mr* transcript during egg chamber development by *in situ* hybridization and found that the transcript was present in the nurse cells throughout oogenesis (see Fig. 4). There was not a detectable induction of *mr* transcript at the polyteny-polyploidy transition (black arrow in Fig. 4), as expected given that APC/C activity is controlled posttranscriptionally (5, 28). The *mr* transcript levels were increased in stage-10 egg chambers, a time when nurse cells undergo maximal gene expression.

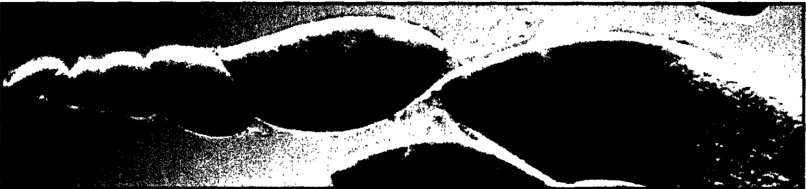
APC/C function is necessary for S-M cycles and centrosome attachment

The *mr* mutants permitted us to analyze the requirements for APC/C function in two other

Figure 4: The expression pattern of *mr* during oogenesis

Sense (control) and antisense (*mr* transcript) labeled probes were made from a cDNA fragment from LD21042. The *mr* transcript is present in the nurse cells (see black asterisk) throughout oogenesis and increases to particularly high levels in stage 10 egg chambers. Additionally, there is not a detectable induction of *mr* transcript at the polyteny-polyploidy transition (black arrow).


mr transcript

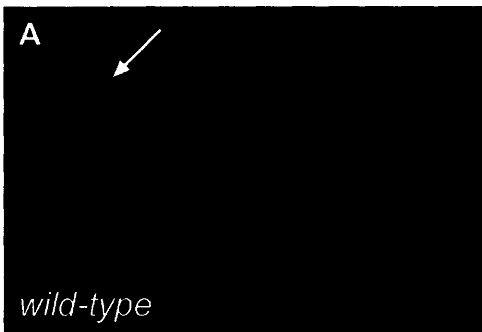

control

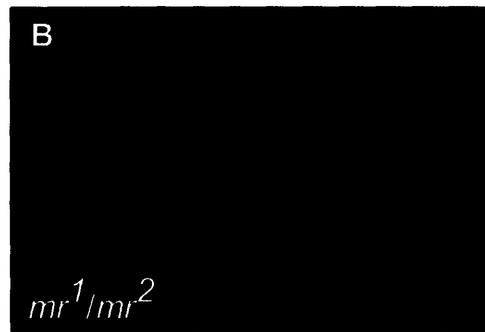
variant cell cycles, meiosis and the embryonic S-M cycles. Although many egg chambers degenerate in the female-sterile mr^1 and mr^2 alleles after stage 7 because of attempted mitosis in the nurse cells (Fig. 3 *H* and *I*), some egg chambers complete oogenesis. This number is affected by genetic background (16). We previously observed that, in embryos produced by mr^1/mr^2 mutant mothers, the zygotic nuclei were arrested in metaphase. We reexamined mature oocytes and embryos from these mothers in more detail to determine whether meiosis was completed, whether pronuclear fusion occurred, and whether spindle structure was affected. Mature Drosophila oocytes were arrested in metaphase I, and the metaphase I arrest was properly maintained in all of the mature oocytes examined from mr^1/mr^2 mutant females ($n = 169$). We examined embryos to test whether meiosis was completed in mr^1/mr^2 mutants. Thirty-three embryos from mr^1/mr^2 mutant females that had been stained with antibodies against tubulin and a DNA stain were analyzed by confocal microscopy. Meiosis was completed in all of these embryos (data not shown). There was not a meiosis I or a meiosis II spindle present, and this can be readily seen in mutants blocked in the meiotic divisions (29).

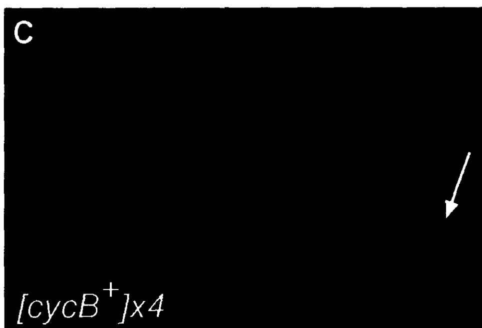
Although meiosis is completed in these *mr* mutants, two striking features were that all of the zygotic nuclei, and frequently the polar bodies, were arrested on metaphase spindles that were anastral and had broad poles (Fig. 5*B* and *D*). An additional phenotype was that the chromosomes were hypercondensed in the embryos from *mr* mutant mothers (compare Fig. 5*A* with *B*). This phenotype was observed previously in metaphase-arrested neuroblasts, cells that are undergoing the canonical cell cycle (16). The excessive condensation seen in metaphase-arrested embryonic nuclei indicates that during the S-M cycles as well as the normal cell cycle the chromosomes continue to undergo condensation if they remain arrested in metaphase. To determine whether these phenotypes were the consequence of increased levels of CYCLIN B

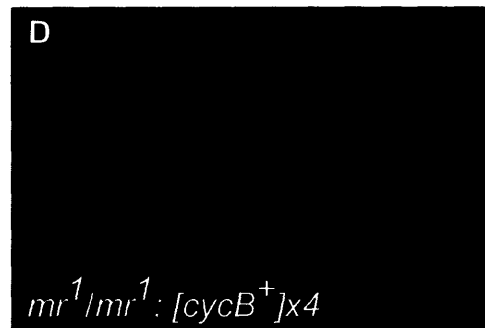
Figure 5: Spindle and chromosome morphology in *mr* mutant embryos

Embryos were collected from mothers that were wild type, mr^1/mr^2, wild type with four extra copies of *cyclin B+*, or mr^1/mr^2 with four extra copies of *cyclin B+*. The fixed embryos were stained with propidium iodide to visualize DNA (red) or anti-tubulin antibodies to visualize the spindle (green). (*A*) A metaphase nucleus in an embryo from a wild-type mother has asters of microtubules at each spindle pole, revealing functional centrosomes (arrows). (*B*) An example of a metaphase figure from an embryo from mr^1/mr^2 mutant mothers. In these mutant embryos the spindles are wide, with broad poles, they lack asters, and the chromosomes are hypercondensed. (*C*) Increased CYCLIN B in an embryo from a wild-type mother does not result in broad, anastral spindles or increased chromosome condensation. (*D*) Increased CYCLIN B in embryos from mr^1/mr^2 mutant mothers does not alter the mutant spindle and chromosome morphology.


A
wild-type


B
mr1/mr2


C
[cycB+]x4


D
mr1/mr1; [cycB+]x4

protein, we attempted to phenocopy these affects by increasing CYCLIN B levels using strains with four extra copies of the *cyclin B* gene in a wild-type background. Embryos produced from these mothers did not exhibit the *mr* phenotypes (Fig. 5*C*). These observations complement those of Wakefield *et al.* (30), who showed that increasing levels of CYCLIN B protein did not cause centrosomes to dissociate from the mitotic spindles. Increased levels of CYCLIN B did not worsen phenotypes in embryos from mr^1/mr^2 mutant mothers (Fig. 5*D*), suggesting these defects may be caused by increased levels of other APC/C targets.

Discussion

The identification of the Drosophila *mr* gene as APC2 demonstrates the essential role of the APC/C in developmentally modified cell cycles as well as the archetypal mitotic cycle. In particular, it is striking that APC/C function is crucial for endocycles in which it appears that mitosis does not occur. The *mr* phenotypes reveal an unexpected and intriguing role for the APC/C in setting the parameters of the endocycle that affect the chromosome structure of the replicated sister chromatids. These results are significant also in establishing an essential role for the APC2 subunit in metazoans.

The roles of APC/C in endocycles

The endocycle can produce polytene or polyploid chromosomes. In the former case, the replicated sister chromatids remain tightly associated, whereas they are dispersed in polyploid cells (for review see ref. 1). The *mr* results provide clues into possible cell cycle differences in endocycles leading to polyteny versus polyploidy. In Drosophila, most cells are polytene, and the nurse cells are rare in becoming polyploid. We did not observe an endocycle failure in any larval polytene tissue in *mr* mutants except the ring gland, which begins the endocycle late in development (16). In polytene cells, APC/C activity may be required only at the initial transition from the mitotic cycle to the endocycle to remove any remaining mitotic regulators. The majority of larval tissues undergo the transition to the endocycle late in embryogenesis (31). Once entrenched in the endocycle with expression of mitotic cyclin genes shut off, the APC/C would be dispensable. Consistent with this hypothesis, in embryos homozyous for a deletion that removes the *fzr* gene, the onset of the first S phase of the endocycle is inhibited in several tissues (9). These observations indicate that APC/C is required during embryogenesis, but it is likely

that maternal stockpiles of MR protein are present to permit the onset of the endocycle. It remains possible that *mr* is essential not only for the onset but also for the maintenance of polytene endocycles throughout development and that the maternal pools persist during larval development and into adult stages. The molecular identification of MR permits the generation of reagents to distinguish whether this is the case.

Even though the APC/C does not appear to be required for the maintenance of polytene endocycles, it plays a critical role in the parameters of endocycles that produce polyploid chromosomes. In polytene cells, APC/C may need to be inactive so that securin remains constitutively active and that the cohesin complex and sister-chromatid cohesion contribute to the tight alignment of replicated sister chromatids. In polyploid cells, degradation of securin by the APC/C could lead to the separation of sister chromatids as a result of separase activity. This activity would explain why the APC/C becomes crucial in the nurse cells when the transition from polyteny to polyploidy occurs. In addition, a low level of transient induction of CYCLIN B/CDK1 activity, so far undetectable by immunolabeling methods, could account for the chromosome condensation observed at this transition. This hypothesis is supported by the presence of CYCLIN B protein in *mr* mutant nurse cells at this time. Overexpression of CYCLIN B does not, however, induce the change from polyteny to polyploidy at an earlier developmental stage, and this would be consistent with other mitotic activities such as the separase protease being necessary. Elimination of securin and separase activity in the nurse cells by making mutant clones might permit a test of this hypothesis.

The requirement of APC/C activity for the endocycle leading to polyploid chromosomes that we observe in Drosophila may be a characteristic feature of endocycles in many organisms. In alfalfa the expression of a Cdh1-like gene is increased in nodules that have cells undergoing

endocycles (32). Overexpression of an antisense RNA reduced the ploidy of polyploid cells in the petioles, hypocotyls, and roots (32). These results are consistent with a role for the APC/C in the maintenance of the endocycle in polyploid plant cells, though effects on the onset of the endocycle were not addressed by these analyses. Elimination of mitotic cyclin protein is necessary for endocycles in plants, because ectopic expression of *cyclin B1;2* in Arabidopsis trichome cells causes these cells to undergo mitosis rather than endocycles (33).

Functions for APC/C in archetypal, S-M, and meiotic cycles

The *mr* mutant effects on the canonical G1-S-G2-M cycles are consistent with mutant phenotypes described for the budding yeast *S. cerevisiae*. An increased number of mitotic cells is seen in brains from mutant larvae, and the majority of these are arrested in metaphase (16). Interestingly, many of these are polyploid, revealing that the metaphase arrest is not indefinite and the cells re-replicate. It appears that sister-chromatid separation is occurring before this replication, because the extra chromosome copies are separate and not attached at their centromeres as in the *pimples* securin mutant (34). Thus, either sufficient *mr* function is present even in the lethal alleles (possibly from maternal pools) to allow eventual exit from mitosis, or an APC/C independent pathway for sister separation and resetting of replication origins may exist.

The regulation of mitotic exit during the syncytial S-M cycles of early Drosophila embryogenesis requires localized degradation of mitotic cyclins in the vicinity of each nucleus (35). In *mr* mutant embryos the initial S-M cycles arrest in metaphase; this observation combined with the metaphase arrest seen in maternal-effect *fzy* alleles (6) demonstrates that APC/C function is required for mitotic exit during the S-M cycles.

Mutations in APC/C subunits in *Caenorhabditis elegans* have been demonstrated to block the metaphase I/anaphase I transition and completion of meiosis (36–38). In contrast in Xenopus oocytes, inactivation of the APC by injection of antibodies to either the CDC27 APC subunit or the FZR activator or injection of inhibitory peptides does not affect the completion of meiosis I but causes a metaphase II block (39). Both meiotic divisions are completed in the *mr* mutant eggs. This does not exclude a role for APC/C either in the separation of homologs in meiosis I or sister chromatids in meiosis II, because the *mr* mutations that produce eggs are weak alleles and residual activity may be sufficient for the completion of meiosis.

Analysis of APC function during metazoan development, here exemplified by the phenotypes of Drosophila *mr* mutants, defines the role of this ubiquitin ligase in cells undergoing an archetypal cell cycle but also illustrates its use in modified cell cycles. The role of the APC in meiosis requires further investigation, but its activity in the embryonic S-M cycles is clear. In addition to demonstrating a critical role for APC/C in endocycles, the *mr* mutants uncover an intriguing use of mitotic activities to alter chromosome morphology in polytene and polyploid cells.

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Chapter Three

Mitotic cohesin is required for polytene chromosome structure and differentiates polytene and polyploid chromosomes

Julie A. Wallace and Terry L. Orr-Weaver

* J.A.W. performed all of the experiments presented in this chapter.

Summary

Many organisms produce polyploid cells, cells with multiple copies of the haploid genome, normally in the course of development or in highly metabolic differentiated tissues. Polyploid cells are frequently generated through a programmed variant of the mitotic cell cycle, called an endocycle that consists of a synthesis phase followed by a gap phase without an intervening mitosis. These polyploid cells demonstrate two different chromosome structures: the chromosomes can be polyploid where the sister chromatids are separate or polytene where the sisters are held together. To elucidate the molecular mechanism for these two chromosome states and how the endocycle can produce both, we studied the contribution of mitotic regulators to these structures in the salivary gland cells and nurse cells of Drosophila. We show that the mitotic kinase POLO is required for a proper transition between polyteny and polyploidy in the nurse cells, implicating the loss of cohesion pathway in this change. We also demonstrate that the cohesin complex localizes to salivary gland chromosomes and that both *rad21* and *smc1* are required for proper polytene chromosome structure in the salivary glands. The results presented here reveal a new function for the cohesin complex in maintaining polytene chromosome structure.

Introduction

Variants of the mitotic cell cycle are commonly used in nature to achieve different developmental goals. In one such variant, the endocycle, a synthesis (S) phase is followed by a gap (G) phase without an intervening mitosis, producing cells that contain multiple copies of the genome (polyploid cells) (reviewed in Edgar and Orr-Weaver 2001). As cell size is correlated to the amount of DNA, the endocycle is often used by a cell to rapidly increase its size. Additionally, highly metabolic cells often utilize the endocycle to boost their protein and mRNA production. The chromosomes in polyploid cells show great diversity in structure. At one extreme, chromosomes are completely detached and separated, referred to as polyploid chromosome structure, seen in mammalian megakaryocytes and hepatocytes (for review see Ravid *et al.* 2002). At the other extreme, each newly replicated sister chromatid is held tightly together with the parental strand, producing polytene chromosomes. Polytene chromosome structure is often found in insects, most familiarly in the salivary glands of *Drosophila melanogaster*. Additionally, there are examples where these distinctions are not absolute and one region of the chromosome may be polytene while other regions are polyploid, as in mammalian trophoblasts (for review see Zybina and Zybina 1996). It is not currently understood how the endocycle can produce these different chromosomal structures or what are the proteins that differentiate polyploid chromosomes from polytene chromosomes.

Programmed differences in the endocycle itself may play a role in determining the chromosome structure in polyploid cells. For example, in the larval-specific tissues of Drosophila, S phase is truncated, resulting in underreplicated regions that frequently correlate to late-replicating heterochromatin (Edgar and Orr-Weaver 2001). Although a strict endocycle consists of only S-G cycles, there are endocycling tissues that demonstrate the presence of

mitotic character in each cycle. Mammalian trophoblast chromosomes condense following their replication in a manner suggestive of mitosis and mammalian megakaryocyte chromosomes condense and separate their sister chromatids, but do not proceed through nuclear division. Finally, mammalian hepatocytes separate their sisters and divide their nuclei, but do not undergo cell division. It seems likely, therefore, that differences in chromosome structure in polyploid cells may reflect the extent to which mitotic character is present in each endocycle.

To directly address this hypothesis, we focused on the two extremes in chromosome structure- polyploidy and polyteny. We utilized *Drosophila melanogaster* as a model system, taking advantage of the range of genetic techniques and the cytology of diverse chromosome structures in Drosophila endocycling tissues. Drosophila use the endocycle several times during their development. First, endocycles are used in the larval-specific tissues through the three larval stages, called instars. The majority of larval tissues are highly polytene, including the giant chromosomes from the salivary gland, which can contain up to 2000 copies of the genome (Urata *et al.* 1995). Second, during oogenesis in the adult fly, the germline-derived nurse cells and somatically-derived follicle cells go through endocycles. Within the ovary, a germline stem cell undergoes four incomplete mitotic divisions to generate 16 cells that are interconnected by cytoplasmic bridges (Spradling 1993). One cell becomes the oocyte, while the other 15 differentiate into the nurse cells and begin endocycling. The nurse cells produce large quantities of maternal mRNAs and proteins that are transported into the oocyte for use during embryogenesis (Spradling 1993). During their development, the nurse cell chromosomes proceed through a developmentally programmed change in their structure, progressing from polytene chromosome structure to polyploid structure. The molecular mechanism for this polyteny to polyploidy transition is unknown.

Studies of mammalian megakaryocytes have demonstrated that mitotic regulators are present in these cells and likely contribute to the mitotic character in these endocycles (Zimmet and Ravid 2000, Roy *et al.* 2001). In mitosis, the two major physical activities of mitosis are the separation of the sisters and the extension of the mitotic spindle, which segregates the sisters to opposite poles. These events are controlled by multiple cell cycle regulators to ensure that they occur in the proper order. Many of these events are initiated by the kinase activity of CDK1, a CDK that can be bound by different mitotic cyclin types (reviewed in Murray 2004). CDKs are activated by their association with a specific cyclin; CYCLIN/CDK activity is then inactivated at a precise time in mitosis by destruction of the associated cyclin via an ubiquitin-dependent pathway (Murray 2004). Mitotic cyclins and other mitotic substrates are targeted for degradation via a multi-subunit E3 ubiquitin-ligase called the anaphase promoting complex/cyclosome (APC/C) (Murray 2004). Degradation of these regulators by the APC/C in a precise temporal pattern allows each step to take place at the right time.

In mitotically dividing diploid cells, the proper segregation of sister chromatids is essential for the production of genetically identical sister cells. To ensure this, the sisters remain attached following replication in S phase to facilitate the attachment of each sister kinetochore to microtubules from a different pole. Sister-chromatid cohesion is then dramatically lost at the metaphase-anaphase transition, allowing the sisters to separate and segregate to opposite poles. The proteins that facilitate this are components of the loss of cohesion pathway. Many of the components required for sister-chromatid cohesion have been identified in various organisms, several of which have been demonstrated to interact together in a complex known as the cohesin complex (for review see Uhlmann 2003). The cohesin complex is evolutionarily conserved and consists of four subunits, two SMC family members, SMC1 and SMC3, and two non-SMC

family members, SCC1/MCD1/RAD21 and SCC3 (Uhlmann 2003). At the initiation of anaphase, a subunit of this complex, SCC1/MCD1/RAD21, is cleaved by the protease enzyme SEPARASE, releasing cohesin from sister chromatids (Uhlmann 2003). Prior to the metaphase-anaphase transition, SEPARASE is held inactive by binding to SECURIN, ensuring that the sister chromatids remain joined until bipolar attachment is achieved (Uhlmann 2003). CDC20/FZY activates the APC/C at the metaphase-anaphase transition, targeting SECURIN for degradation via ubiquitin-mediated proteolysis. This then releases SEPARASE to act on its targets (Uhlmann 2003). In addition, the mitotic kinase POLO has been shown to facilitate the loss of cohesin from chromosomes at the metaphase-anaphase transition and in prophase (Alexandru *et al.* 2001, Sumara *et al.* 2002, Losada *et al.* 2002, Hornig and Uhlmann 2004 and Hauf *et al.* 2005).

Studies of the Drosophila APC/C mutant, *morula* (*mr*), revealed a specific requirement for the APC/C at the polyteny-polyploidy transition in nurse cells and suggested that mitotic regulators play a special role following the fifth endocycle in oogenesis (Reed and Orr-Weaver 1997, Kashevsky *et al.* 2002). *mr* mutants display a striking oogenesis phenotype: at the time when nurse cell chromosomes should be transitioning from polyteny to polyploidy, they inappropriately enter mitosis and do not progress to the dispersed state. The chromosomes condense and become associated with spindle-like microtubules, arrest with this phenotype and do not form the polyploid chromosomes that normally follow the transition stage (Reed and Orr-Weaver 1997). We previously identified *mr* as encoding dAPC2. a subunit of the APC/C (Kashevsky *et al.* 2002). Intriguingly, increasing *cyclin B* gene copy number did not alter the timing of the transition or phenocopy the *mr* mutant, suggesting that the APC/C must have additional targets at the polyteny-polyploidy transition (Kashevsky *et al.* 2002). As SECURIN

and the loss of cohesion pathway are also targets of the APC/C in mitosis, we hypothesized that the removal of cohesin from polytene chromosomes is the key molecular event at the polyteny-polyploidy transition and that the cohesin complex is required for polytene chromosome structure.

Here we report our studies of the loss of cohesion pathway in the polyteny-polyploidy transition in Drosophila nurse cells. We reveal that a hypomorphic allele of the mitotic kinase POLO has a block in the transition and maintains condensed polytene chromosomes in late stage egg chambers. We also show that a subunit of the cohesin complex, RAD21, is localized onto polytene chromosomes from the salivary gland. Finally, we demonstrate that the presence of the cohesin complex is essential for polytene chromosome structure. Mutants in two cohesin subunits, *rad21* and *smc1*, show aberrant polytene chromosome structure in which the sister chromatids are unable to maintain their polyteny. These results suggest a critical role for the cohesin complex in the endocycle and implicate the loss of cohesion pathway in a novel developmental context.

Results

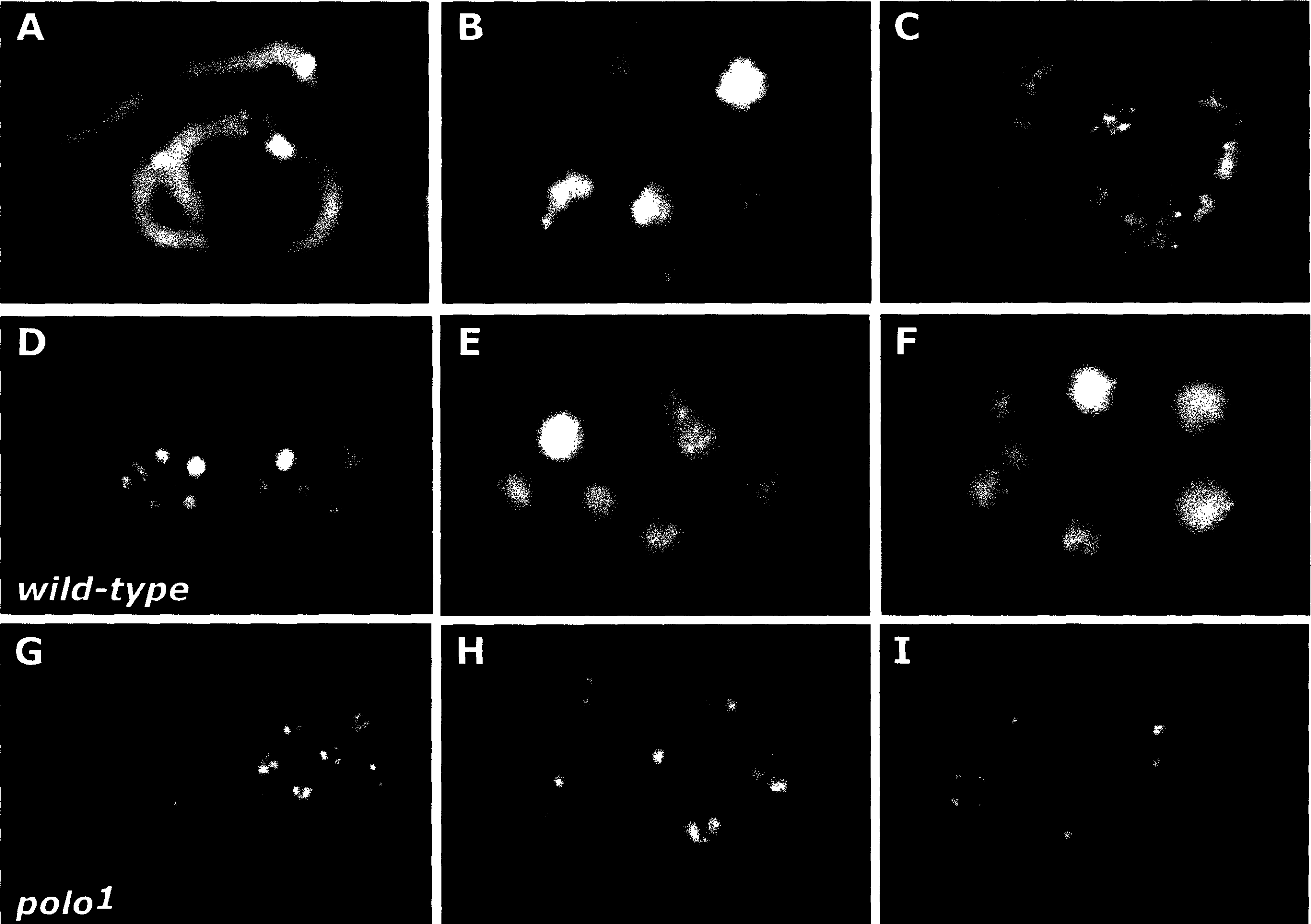
POLO is required for the polyteny-polyploidy transition

In *Drosophila melanogaster* the fifteen germline-derived nurse cells proceed through five endocycles that produce polytene chromosomes where the sister chromatids are held together tightly (King 1970, Dej and Spradling 1999, Figure 1A). Following the fifth endocycle, the nurse cell chromosomes undergo a striking transition in structure. First they become highly condensed (Figure 1B) and then the chromosomes disperse into a polyploid structure in later egg chambers (Figure 1C). Studies of the mutant *mr* revealed a specific requirement for APC/C at the polyteny-polyploidy transition in nurse cells and suggested that mitotic regulators may play a unique role following the fifth endocycle in oogenesis (Kashevsky *et al.* 2002). In addition, these studies indicated that CYCLIN B is not the only target for APC/C at this transition, suggesting that additional mitotic regulators may be critical for the dissociation of polytene chromosomes to polyploid (Kashevsky *et al.* 2002). Given that a transition from polytene to polyploid chromosome structure includes the separation of sister chromatids, it seemed likely that mitotic regulators involved in the loss-of-cohesion pathway are active at the transition (Dej and Spradling 1999).

To test a role for the loss-of-cohesion pathway in the polyteny-polyploidy transition in nurse cells, we generated germline mutant clones for *separase* (sse^{13m}), *pimples* (pim^{1}) and *three rows* (thr^{1B}) using previously characterized mutants (Jager *et al.* 2001, Stratmann and Lehner 1996 and D'Andrea *et al.* 1993). We failed to observe any mutant egg chambers in the germline for *pimples* and *three rows*, suggesting an absolute requirement for these regulators in the four mitotic cycles that generate the nurse cells. This did not allow us to determine whether these regulators are involved in the transition. We did observe mutant clones for sse^{13m} and analysis of

Figure One: ***polo***^{1} **mutant nurse cells do not pass through the polyteny-polyploidy transition properly.**

(A-C) Nurse cell chromosome squashes stained with a fluorescent DNA dye demonstrate the programmed change in nurse cell chromosomes during oogenesis. The first four endocycles (egg chamber stages 1-4) generate polytene nurse cell chromosomes (A). After the fifth endocycle (approximately stage 5), nurse cell chromosomes condense (B) before dispersing to a polyploid state (C, egg chamber stages 6-12). (D-F) *polo*^{1}*/TM6* ovaries stained with a fluorescent DNA dye. Egg chambers after the transition show dispersed chromosomes (red arrow). (G-I) *polo*^{1} ovaries stained with a fluorescent DNA dye. Nurse cell chromosomes remain condensed in post-transition egg chambers (red arrow).


A
B
C
D
E
F
wild-type
G
H
I
polo1

the chromosome structure revealed that the mutant clones were able to disperse nurse cell chromosomes with the proper timing. However, the *sse*^{13m} allele is lethal at the larval/pupal boundary suggesting that maternal SSE perdures and that SSE protein may remain in the mutant clones despite their genotype.

We were able to address a role for POLO kinase in the polyteny-polyploidy transition. *polo*^{1} is an allele that shows a high frequency of aberrant metaphase and anaphase figures in mitotic larval neuroblasts and escapers are female sterile (Sunkel and Glover 1988). Distinct egg chamber morphology and the deposition of yolk into the oocyte allowed us to identify stage 8 egg chambers, a stage after the transition to polyploidy, in both *polo*^{1} mutant ovaries and heterozygous control ovaries (Spradling 1993). We compared the nurse cell chromosome structure of these egg chambers in both samples. In the control, the nurse cell chromosomes are polyploid and the dispersed DNA fills the nucleus (Figure 1D-F, red arrow in E). In *polo*^{1} mutants, however, the nurse cell chromosomes often maintain their condensed structure, indicative of a block in the transition (Figure 1G-I, red arrow in H). Quantification of this phenotype revealed that 31% (n=94) of *polo*^{1} stage 8 egg chambers display these undispersed chromosomes, as compared to 0% (n=81) of heterozygous control egg chambers. Additionally, 38% (n=94) of *polo*^{1} stage 8 egg chambers have nurse cell chromosomes that are not fully dispersed, maintaining some degree of polyteny, while only 10% of heterozygous control egg chambers show similar nurse cell chromosome structures.

Although POLO plays multiple cell cycle roles, the most likely explanation is that these effects on chromosome structure result from the function of POLO in controlling sister-chromatid cohesion. Currently no role for POLO kinase in the endocycle has been identified. Additionally, the polytene nurse cell chromosomes show no alteration of their structure, and the

timing of chromosome condensation in nurse cells is not altered in *polo*^{1} mutants. Therefore, it appears that the maintenance of condensed, undispersed chromosomes in *polo*^{1} mutant egg chambers reveals a specific role for POLO kinase at the polyteny to polyploidy transition. As previous studies have demonstrated a role for POLO in the loss of sister-chromatid cohesion (Alexandru *et al.* 2001, Sumara *et al.* 2002, Losada *et al.* 2002, Hornig and Uhlmann 2004 and Hauf *et al.* 2005), we suggest that the *polo*^{1} mutant phenotype implicates loss of sister-chromatid cohesion as the key step in the polyteny to polyploidy transition.

RAD21 is present on polytene chromosomes

The multi-subunit cohesin complex has been demonstrated to have a role in sister-chromatid cohesion (Guacci *et al.* 1997, Michaelis *et al.* 1997, Losada *et al.* 1998). Two members of the SMC family, SMC1 and SMC3, have been identified as components of the cohesin complex with SCC1/MCD1 and SCC3 (Losada *et al.* 1998, Toth *et al.* 1999, Sumara *et al.* 2000, Losada *et al.* 2000). In Drosophila, DRAD21 is the SCC1/MCD1 homolog (Warren *et al.* 2000a). RAD21 has been localized in mitotic Drosophila S2 tissue culture cells by DRAD21 antibodies that have been reported to recognize a single band on immunoblots (Warren *et al.* 2000b). The RAD21 protein is present on condensing chromosomes in prophase, whereas it localizes to discrete chromosomal regions in prometaphase and to centromeric regions in metaphase (Warren *et al.* 2000b). As expected, DRAD21 is not detectable on chromosomes at anaphase. Additionally, monitoring localization of a DRAD21-GFP fusion protein revealed a similar pattern of detection in syncytial and cellularized embryos (Warren *et al.* 2000b). Intriguingly, *in situ* hybridizations of developing embryos revealed that *rad21* transcript is

present in the endocycling tissues of the midgut and hindgut in stage 12 embryos (Warren *et al.* 2000a).

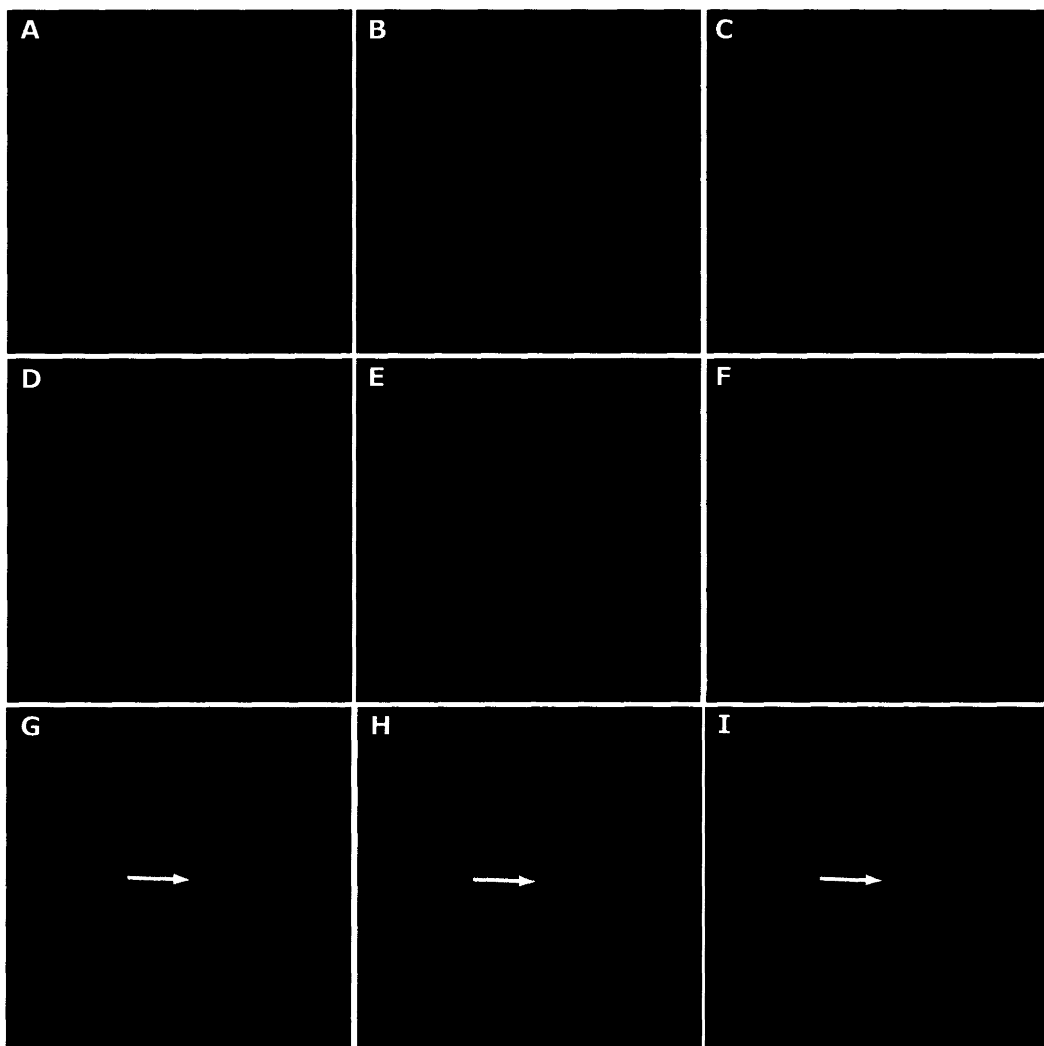
We hypothesized that if the cohesin complex is involved in maintaining polytene chromosome structure, the complex should be found on polytene chromosomes. We utilized the giant polytene chromosomes of the Drosophila salivary gland to look for the presence of DRAD21. Cells of the salivary gland endocycle, producing chromosomes that can contain up to 2000 copies of the genome. Salivary gland polytene chromosomes were squashed and bound to antibodies against DRAD21. The protein was present on salivary gland polytene chromosomes in a discrete banding pattern (Figure 2). Additionally, many of the RAD21 bands correspond to interbands, regions of the salivary gland chromosomes that do not darkly stain with a visible dye (white arrow in 2G-I). This difference between interband and band staining is thought to correspond to the less compact nature of the DNA in interbands (reviewed in Zhimulev *et al.* 2004). These observations are in agreement with the RAD21 staining pattern seen on salivary gland chromosomes in other studies (Markov *et al.* 2003). Currently, the cytological position of the RAD21 bands has not been detailed. However, the presence of a cohesin subunit on polytene chromosomes further suggests that the cohesin complex may be important for the structure of polytene chromosomes.

Loss of cohesin subunits disrupts polytene chromosome structure

To address whether DRAD21 was required for polytene chromosome structure, we examined the chromosomes in the absence of DRAD21. A mutant for *rad21* has not been identified, therefore, we took advantage of RNA interference to reduce levels of *rad21* transcript in an inducible manner. We generated a construct in which 600 bp of *rad21* sequence and its

Figure Two: RAD21 is localized on salivary gland polytene chromosomes.

RAD21 is found on wild-type salivary gland polytene chromosomes squashes in discrete bands as visualized by propidium iodide staining of the DNA (red; *A, C, D, E, F, G, I*) and immunolabeling of RAD21 (green; *B, C, D, E, F, H, I*). (A-C) All four chromosomes of a single cell are shown; (D-I) Portions of single chromosomes are magnified to highlight the bands of RAD21. (G-I) RAD21 is often found in interbands along the chromosome arms (white arrow).


A
B
C
D
E
F
G
H
I

inverted repeat were separated by 300 bp of spacer sequence, a design that had proven successful in previous studies (Kennerdell and Carthew 2000, Piccin *et al.* 2001). This construct was introduced into a P-element vector, pUASP, which was then used to generate transgenic lines by standard techniques (Spradling 1986). Lines were screened for the presence of an intact construct insertion by PCR and for an effect on viability by crossing the lines to an *ubiquitin*-GAL4 driver. Because RAD21 is required for proper chromosome segregation in other organisms, we expected that lines in which expression of *rad21* led to a decrease of DRAD21 protein would show decreased organismal viability (Guacci *et al.* 1997, Michaelis *et al.* 1997, Sonoda *et al.* 2001, Toyoda *et al.* 2002, Mito *et al.* 2003). Surprisingly, although 37 of 40 lines had an intact insertion, only 1 line showed a significant decrease in viability. This line, designated as *P{w+ UAS-rad21 RNAi}*-C4-2, showed a 30% decrease in viability. Phenotypes associated with significant cell death in imaginal discs, resulting from mitotic defects, such as rough eyes, deleted wing parts, and missing bristles were not observed (Lindsley *et al.* 1972).

Western blot analysis was performed to determine whether expression of *rad21* RNAi resulted in a decrease of DRAD21 protein. Protein extracts were made from 3rd instar whole larvae in which *P{w+ UAS-rad21 RNAi}*-C4-2 was expressed by *ubiquitin*-GAL4 or from larvae with the *P{w+ UAS-rad21 RNAi}*-C4-2 transgene alone. Additionally, protein extract made from mitotic S2 cells served as a positive control for the presence of DRAD21 (Lee *et al.* 2004). Protein levels of RAD21 were reduced in larvae expressing *rad21* RNAi (Figure 3). Probing with an antibody to α-tubulin, as a loading control, revealed that much more protein had been loaded from the *rad21* RNAi protein extract. Using Image J software to quantify the band intensity revealed that there is 6 times more tubulin in the *rad21* RNAi sample loaded than in the control sample, yet only twice the amount of RAD21 protein in the *rad21* RNAi sample. This

Figure Three: Expression of an RNAi construct to *rad21* decreases RAD21 protein levels. Protein extract was generated from 20 3rd instar larvae of the designated genotypes: *P{w+UAS-rad21}*-C4-2 transgene alone (lane 1), *P{w+UAS-rad21 RNAi}*-C4-2 in the presence of an *ubiquitin*-GAL4 driver (lane 2) and from a pool of S2 cells (lane 3). Equal volumes of the larval samples were analyzed by SDS-PAGE followed by Western blotting and probing for RAD21. The same blot was stripped and probed for TUBULIN as a loading control. By Image J quantification, there is twice the amount of RAD21 protein in lane 2 as there is in lane 1. Quantificiation of the TUBULIN band revealed that there was six times as much TUBULIN in lane 2 as in lane 1. Thus, more protein has been loaded in lane 2 than in lane 1. This shows that reduced levels of RAD21 protein are present in extracts from the transgenic *rad21* RNAi animals.

rad21 RNAi transgene alone
rad21 RNAi transgene + *gal4* driver
S2 cells

DRAD21

TUBULIN

confirms that DRAD21 protein is reduced in the sample in which *rad21* RNAi is being expressed.

Salivary glands dissected from wandering 3rd instar larvae expressing *P{w+ UAS-rad21 RNAi}*-C4-2 from an *ubiquitin*-GAL4 driver show a decrease in the size of the tissue compared to glands from larvae with the transgene alone (compare Figure 4A and 4B). To determine whether the salivary gland size phenotype was specifically due to loss of DRAD21 in the salivary gland tissue itself, we crossed the *P{w+ UAS-rad21 RNAi}*-C4-2 transgene line to a salivary gland specific driver, *forkhead*-GAL4. The salivary gland tissue in these larvae was also reduced in size. To analyze the morphology of the chromosomes, we squashed and stained these chromosomes with orcein dye. In the presence of either driver there was a dramatic alteration of polytene chromosome structure. In chromosomes from larvae not expressing the *P{w+ UAS-rad21 RNAi}*-C4-2 transgene, the polytene chromosomes are thick and the distinct banding pattern is discernable (Figure 4C). In the absence of DRAD21, the size and thickness of the polytene chromosomes is greatly reduced. The polytene banding pattern can be discerned in some regions, while in others the sister chromatids are no longer polytene (note red arrow in Figure 4D).

We quantified this phenotype by designating chromosomes from a single nucleus into one of the following categories: 1) wild-type (chromsomes are thick with the distinct banding pattern); 2) RNAi phenotype (chromosomes are small with regions where the DNA is dispersed) or 3) intermediate (chromosomes in which less than 50% of the bands were discernable, but were still wild-type and did not display regions in which the DNA was dispersed). Each sample counted contains a single pair of salivary glands stained with orcein and squashed (Table 1). We found that in salivary glands with the transgene alone the majority of chromosomes were either

Figure Four: Loss of RAD21 effects salivary gland tissue size and disrupts polytene chromosome structure.

(A, B) Whole salivary glands were stained with a fluorescent DNA dye to demonstrate the dramatic decrease in cell and tissue size of salivary glands lacking RAD21. 3rd instar salivary gland nuclei from larvae with *P{w+UAS-rad21 RNAi}*-C4-2 transgene in the absence of a GAL4 driver (A) stain brightly (red arrow). 3rd instar salivary gland nuclei from larvae expressing *rad21* RNAi from the *P{w+UAS-rad21 RNAi}*-C4-2 transgene by an *ubiquitin-* GAL4 driver (B) are significantly smaller (red arrow). The white arrowhead in each picture marks the fat body tissue attached to the salivary glands.

(C, D) Salivary gland polytene chromosome squashes stained with orcein dye reveal disruption of polytene chromosome structure in the absence of RAD21. Chromosomes from control salivary glands (C) are thick and the distinct banding pattern is visible. Chromosomes from salivary glands lacking RAD21 (D) are small and display regions where the sister chromatids are clearly not polytene (red arrow).

(E, F) Salivary gland polytene chromosome squashes stained with a fluorescent DNA dye reveal the separation of sister chromatids in the absence of RAD21. Chromosomes from control salivary glands (E) demonstrate the tight association of the sister chromatids in polytene chromosomes. Chromosomes from salivary glands lacking RAD21 (F) demonstrate the separation of sister chromatids (red arrow). Scale bars in each image correspond to 10 μm.

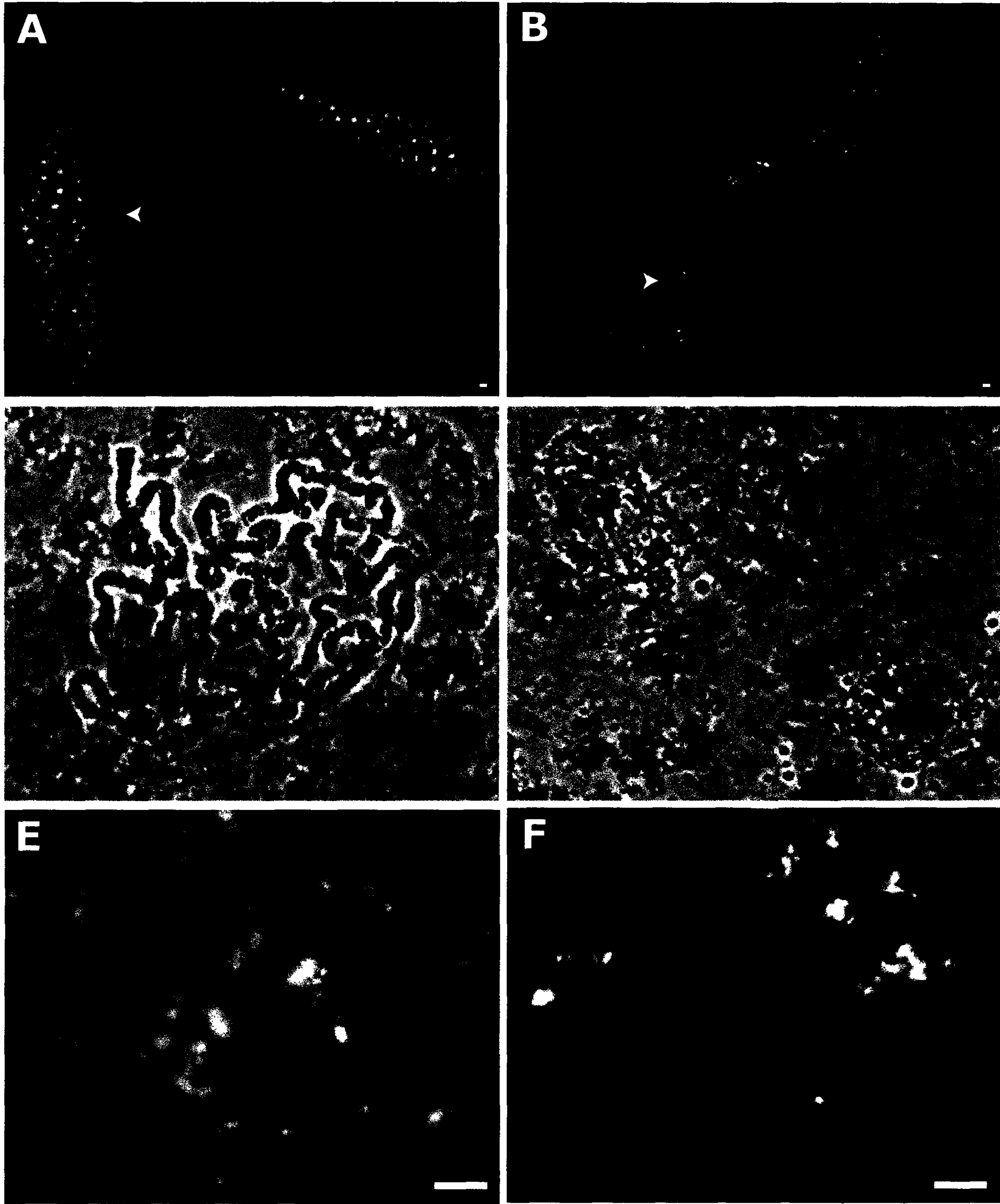

A
B
E
F

Table 1: Loss of RAD21 alters salivary gland polytene chromosome structure

Larval Type[a]	# wild-type[b]	# intermediate	# RNAi phenotype	% RNAi phenotype[c]
Control	196	17	11	5% (n=224)
Control	80	184	38	13% (n=302)
Control	52	198	12	5% (n=262)
Transgene Expressed	0	0	56	100% (n=56)
Transgene Expressed	0	2	42	95% (n=44)
Transgene Expressed	0	0	16	100% (n=16)
Transgene Expressed	0	0	17	100% (n=17)
Transgene Expressed	0	2	109	98% (n=111)
Transgene Expressed	0	4	86	96% (n=90)

a: Each row represents a single salivary gland pair from the indicated larval type, fixed and squashed in orecin dye for quantification. Control larvae are from a single bottle of genotype *P{w+ UAS-rad21 RNAi}*-C4-2. Transgene expressing larvae are from a single bottle generated from crossing *P{w+ UAS-rad21 RNAi}*-C4-2 to *P{GAL4}1032.hx*.

b: Chromosomes were counted as wild-type if they were large and demonstrated the characteristic banding pattern. Chromosomes were counted as ambiguous if <50% of bands were discernable, but were large and lacking regions where DNA was distinctly dispersed. Chromosomes were counted as having the RNAi phenotype if they were small and clearly displayed regions in which the DNA was not polytene (red arrow in Figure 4D).

c: The number of total chromosomes counted varies because chromosomes were only counted if they could unambiguously be identified as DNA and differentiated from cellular debris in the background.

in the first or third category. Few chromosomes appeared disrupted, less than 15% in more than 200 nuclei in each of 3 samples. In nuclei from salivary glands lacking DRAD21 (*P{w+ UAS-rad21 RNAi}*-C4-2 driven by an *ubiquitin*-GAL4 driver), we did not observe salivary glands with wild-type chromosomes. Total nuclei counted were fewer in these lines, as chromosomes were only tallied if they could be unambiguously differentiated from the cellular background. In all of the samples quantified, greater than 95% of the nuclei had chromosomes with the altered phenotype.

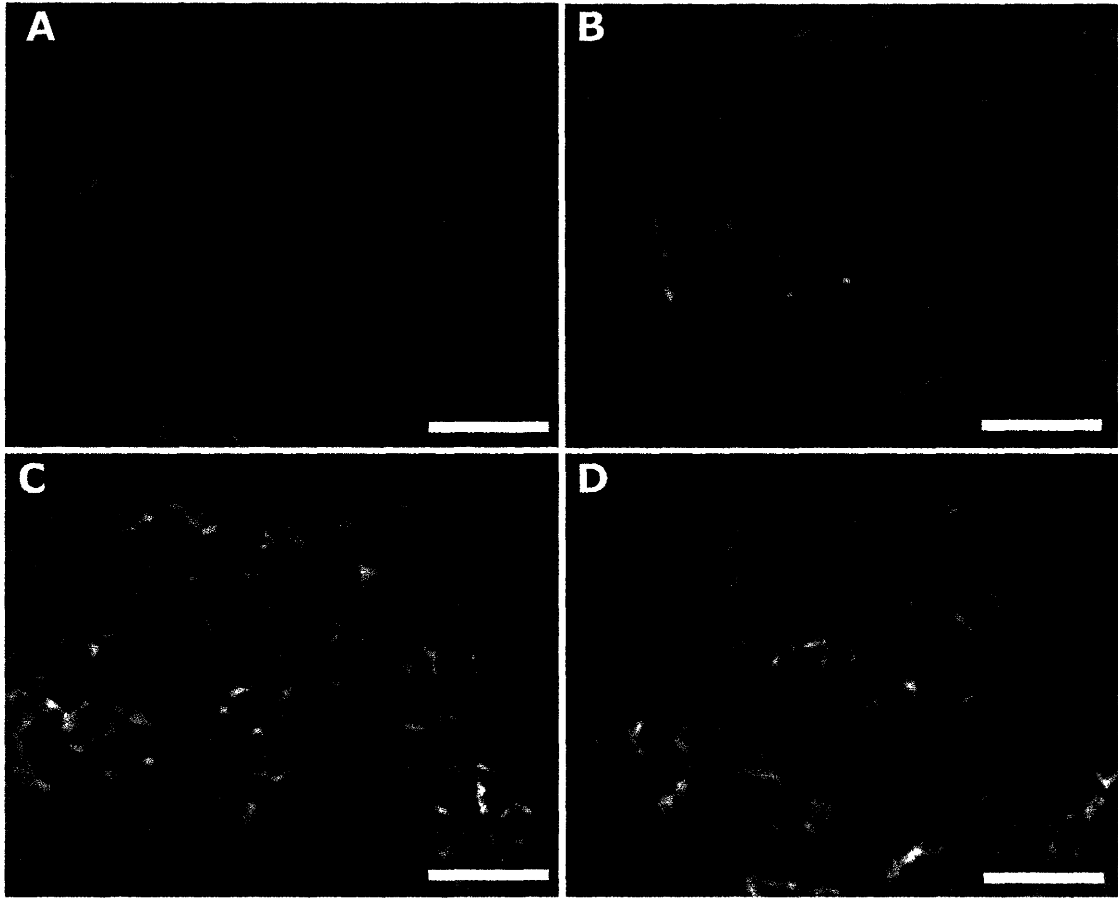
To further characterize the mutant phenotype, we stained these chromosomes with a fluorescent DNA dye, which increases the contrast between the chromatids and the background and ensures visualization of decondensed chromatin. In chromosomes in which *P{w+ UAS-rad21 RNAi}*-C4-2 is not expressed, the sister chromatids are held together and the banding pattern is visible (Figure 4E). However, in the absence of DRAD21, regions where the sister chromatids have separated were visible (red arrow in Figure 4F). These studies demonstrate a requirement for DRAD21 in the maintenance of proper polytene chromosome structure.

Although the studies of the *P{w+ UAS-rad21 RNAi}*-C4-2 transgenic line revealed a requirement for DRAD21 in polytene chromosome structure, we used additional cohesin mutants to establish a role for the cohesin complex in polytene chromosome structure. Drosophila SMC1 was identified based on its homology to SMC family members (Cobbe and Heck 2000). A mutant in *smc1* was generated by imprecise excision of a P-element (S. Page and S. Hawley, personal communication). To address whether DSMC1 was also required for polytene chromosome structure, we dissected salivary glands from the mutant and squashed the chromosomes in the presence of orcein dye. We compared late 2nd instar chromosomes from a control and the *smc1* mutant, because the *smc1* mutants die as 2nd instar larvae. In the control,

these chromosomes are considerably smaller than those of 3rd instar larvae and do not squash well (Figure 5A and 5B). The sister chromatids of these chromosomes are held together in the polytene structure and bands are visible. However, in the *smc1* mutant the salivary gland chromosomes are thicker and less dense suggesting that the sister chromatids are not held together as tightly as in the control (Figure 5C and 5D). In addition, the banding pattern is disrupted suggesting that the chromatids have dispersed. From these observations we conclude that both DRAD21 and DSMC1 are required for proper polytene chromosome structure, implicating the cohesin complex in a crucial role for interphase chromosomes and in a new developmental context.

Figure Five: Loss of SMC1 also disrupts polytene chromosome structure.

Salivary gland polytene chromosome squashes stained with orecin dye reveal a requirement for SMC1 in polytene chromosome structure. Chromosomes from *yw* 2nd instar salivary glands (A, B) are small reflecting the tight association of sister chromatids. Chromosomes from *smc1* mutant salivary glands (C, D) are thicker and have lost the distinct banding pattern suggesting that the association of the sisters has weakened and the sister chromatids are more dispersed. Scale bars in each image correspond to 10 μm.


A
B
C
D

Discussion

Here we present a previously unidentified role of the cohesin complex in chromosome structure in the endocycles of the Drosophil*a* germline-derived nurse cells and somatic salivary gland cells. As chromosomes in both these tissues are polytene, we have taken advantage of tools available for each of these tissues to address the requirements for polytene chromosome structure in two complimentary tissues. We describe a requirement for the loss-of-cohesin pathway in a transition from polyteny to polyploidy in the nurse cells, implicating the cohesin complex as the key determinant between polytene and polyploid chromosome structure. We also show that the cohesin complex is localized to the giant polytene chromosomes of salivary gland cells and that cohesin is required to maintain polytene chromosome structure in these cells. From these data we conclude that the cohesin complex plays an integral role in a modified cell cycle lacking mitosis and that the role of the cohesin complex can be adapted for different developmental goals.

Nurse cell chromosomes from a weak mutation in the mitotic regulator *polo* are defective in the polyteny to polyploidy transition. In $polo^1$ ovaries, the nurse cell chromosomes remain condensed and do not disperse in late egg chambers that should have progressed through the transition (Figure 1). This phenotype specifically indicates a role for POLO in the polyteny-polyploidy transition, as $polo^1$nurse cell chromosomes do not show defects in polytene structure and begin the transition with proper timing. In this mutant, the nurse cell chromosomes are affected by disruption of POLO only at the transition. As this mitotic kinase has been demonstrated to act in the removal of cohesin from chromatids in mitosis, requirement for POLO and APC/C both suggest that the loss-of-cohesin pathway acts in the polyteny-polyploidy transition and implicates the cohesin complex in polytene chromosome structure in nurse cells

(Alexandru *et al.* 2001, Sumara *et al.* 2002, Losada *et al.* 2002, Hornig and Uhlmann 2004, Hauf *et al.* 2005, Reed and Orr-Weaver 1997, Kashevsky *et al.* 2002). Surprisingly, we were unable to localize the cohesin complex onto polytene nurse cell chromosomes and have not been able to confirm this model. In addition, we do not see an effect on nurse cell chromosome structure with expression of *rad21* RNAi (J.A. Wallace and T.L. Orr-Weaver, unpublished observation). We suggest that these results are due to technical differences between working with nurse cell and salivary gland chromosomes, although it may be that the nature and/or degree of association of the cohesin complex with nurse cell and salivary gland chromosomes differs. We also attempted to generate germline clones with the *smc1* mutant, but did not observe any mutant egg chambers (J.A. Wallace and T.L. Orr-Weaver, unpublished observation). We speculate that this reflects an essential function for SMC1 in the four mitotic cycles that generate the nurse cells.

As mutants in *rad21* have not been identified, the generation of an RNAi line to *rad21* provides a valuable resource with which to address RAD21 function in an *in vivo* context. In a previous study, transgenic, inducible RNAi lines were created for *rad21* and *sa/scc3* and crossed to a GAL4 driver to ubiquitously express the RNAi in Drosophila (Rollins *et al.* 2004). Three insertions were compared for each RNAi construct and these lines showed varying degrees of reduction in viability, ranging from 25% to 100% viability for RAD21. Interestingly, this loss of viability was reported to occur as a result of a small reduction in mRNA levels, but the reduction in protein levels were not determined and PSCS was not observed in mitotic neuroblasts, as might be expected with a severe loss of cohesin (Rollins *et al.* 2004). We screened transgenic lines for an effect on viability and found that our strongest line only resulted in a 30% decrease in viability. No phenotypes associated with mitotic defects were observed in the eyes, wings or bristles as well. Additionally, viable female progeny expressing *rad21* RNAi are fertile (J.A.

Wallace and T.L. Orr-Weaver, unpublished observation). We do, however, observe a significant decrease in RAD21 protein levels by Western analysis (Figure 3). Why does this decrease in RAD21 protein levels not result in mitotic defects? It seems unlikely that Drosophila RAD21 does not have a function in mitosis. In embryos, the localization pattern of an ectopic RAD21-GFP fusion suggests that RAD21 acts as a member of the cohesin complex and in embryo extracts, RAD21 physically interacts with SMC1, SMC3 and SA/SCC3, demonstrating that RAD21 acts as a member of the cohesin complex *in vivo* (Warren *et al.* 2000b, Vass *et al.* 2003). Additionally, in insect cell culture, RNAi to *rad21* leads to the premature separation of sister chromatids and localization of RAD21 during mitosis is also consistent with a role in the cohesin complex (Warren *et al.* 2000b, Vass *et al.* 2003). We suggest, therefore, that the lack of mitotic defects in our *rad21* RNAi line is due to the persistence of a small level of RAD21 protein and that this decreased level is sufficient for the mitotic functions of the cohesin complex.

It is intriguing, then, that the decrease in RAD21 protein levels is not sufficient to affect the mitotic function of RAD21but has such a dramatic effect on the salivary gland polytene chromosomes (Figure 4). Does this reflect a stronger dependence on the presence of RAD21 and/or a need for higher levels of RAD21 in this tissue? A requirement for higher protein levels of the cohesin complex in salivary gland cells seems likely. The chromosome arms in these cells are highly decondensed and can contain up to 2000 sister chromatids, more than any other tissue in Drosophila. Indeed these chromosomes were originally calculated to be 70-110 times longer than Drosophila metaphase chromosomes (Bridges 1935). Both of these characteristics could result in a higher demand for protein levels of the cohesin complex than on diploid, condensed mitotic chromosomes. It is also possible that this difference in protein level demand explains why nurse cell polytene chromosomes, which are decondensed, but only contain 32 chromatids,

do not appear to be disrupted in our *rad21* RNAi line (J.A. Wallace and T.L. Orr-Weaver, unpublished observation).

In addition to the observed separation of chromatids, the size of the salivary gland tissue itself is affected in organisms expressing RNAi to *rad21* (Figure 4). Salivary gland cells expressing RNAi to *rad21* are smaller than their control counterparts, suggesting that the small tissue size is not due to a reduction in cell number, but to a reduction in cell size (J.A. Wallace and T.L. Orr-Weaver, unpublished observation). As salivary gland cells endocycle and increase in ploidy, they rapidly enlarge their cell size, implying a correlation between cell size and nuclear DNA content. We suggest that the reduced cell and tissue size resulting from loss of RAD21 in the salivary gland also reflects a reduction in ploidy. This implies, therefore, that RAD21 is required for DNA replication in the endocycle. Although this may reflect an unidentified, direct role for RAD21 in DNA replication, we favor the possibility this reflects the importance of proper polytene chromosome structure for DNA replication. In the absence of tight polyteny and organization of the sister chromatids, DNA replication is dramatically affected.

The disruption of salivary gland polytene chromosomes in the *smc1* mutant supports a requirement for the cohesin complex and not RAD21 alone (Figure 5). We note, however, that there are differences between polytene chromosomes perturbed by the expression of *rad21* RNAi and those perturbed in the *smc1* mutant. By orcein staining, the phenotype resulting from lack of RAD21 appears more severe than that resulting from lack of SMC1. Polytene chromosomes from the *rad21* RNAi experiment are greatly reduced in size in comparison to their control siblings. In addition, the majority of polytene chromosomes from 2nd instar larvae expressing *rad21* RNAi do not look like the 2nd instar *smc1* polytene chromosomes. Instead, these

chromosomes look like those from 3rd instar larvae expressing *rad21* RNAi (J.A. Wallace and T.L. Orr-Weaver, unpublished observation). We interpret this difference as a reflection of the two different genetic techniques used in this study. Salivary glands expressing RNAi to *rad21* likely contain RAD21 protein in their initial endocycles and the phenotype results as the demand for RAD21 becomes higher than the supply that is diminished by the RNAi. The *smc1* mutants, however, behave as genetic nulls and likely have no SMC1 present once the maternal supply runs out (S. Page and S. Hawley, personal communication). The difference in these phenotypes may reflect distinctions in having some cohesin present in early salivary gland endocycles versus a complete absence of cohesin. It may be possible that in the complete absence of the cohesin complex, DNA replication can proceed and that replication is disrupted in the *rad21* RNAi salivary glands due to constrictions resulting from the presence of some, but not enough, cohesin. These two distinct mutants, therefore, may allow us to speculate on the temporal requirement for the cohesin complex. As loss of cohesin does not appear to affect replication in the 2nd instar *smc1* mutants, the demand for proper polytene chromosome structure to facilitate DNA replication must occur in the 3rd instar larvae.

Disruption of RAD21 and SMC1 clearly affect the structure of salivary gland polytene chromosomes, revealing that organization of polytene chromosomes is an active process in these cells. Does the lethality associated with these mutants reflect the importance of polytene chromosome structure specifically in the salivary gland? Although the salivary gland phenotypes are severe in the *rad21* RNAi-expressing organisms, they are not strictly correlated with lethality, as larvae expected to have small glands survive to adulthood (J.A. Wallace and T. L. Orr-Weaver, unpublished observation). Salivary glands do not seem to be required for larval growth and survival, as mutants in *eyegone* lack salivary glands but are able to survive to

pupation and, in some cases, adulthood (Jones *et al.* 1998). The *smc1* mutant larvae, however, die as 2nd instars. This likely reflects a requirement for SMC1 during the larval endocycles, as defects in mitotic regulators are lethal later in development, specifically at the larval-pupal boundary (S. Page and S. Hawley, personal communication; Gatti and Baker 1989). As the majority of larval tissues are endocycling, the requirement for the cohesin complex in the larvae may extend to other polytene tissues as well. We infer that this requirement is for polytene chromosome structure in the larval endocycling tissues, suggesting the significance of proper chromosome structure for viability of the larvae. Given that polytene chromosomes in salivary gland cells are organized at many levels-by holding the sister chromatids in tight register, by condensing specific regions into bands, and by blocking replication of gene sparse heterochromatin- it is hard to believe that polytene structure is not necessary for viability of the organism.

This study provides insight into polytene chromosome structure and suggests that further characterization of the cohesin complex on polytene chromosomes will provide insight into the nature of the cohesin complex itself. Although the subunits of the cohesin complex have been demonstrated to form a ring, it is still unknown how this ring interacts with the sister chromatids in the canonical cell cycle (Gruber *et al.* 2003). The cohesin complex has been suggested to be loaded onto chromatids during G1 phase and cohesion is then activated with the replication of the sister chromatids in S phase (Toth *et al.* 1999). Given the 50 nm size of the ring, it remains to be shown that the replication machinery will be able to pass through the ring, complicating this model (Gruber *et al.* 2003). Currently, we do not know whether the cohesin complex remains on the polytene chromosomes during replication in the endocycle or whether cohesin is transiently removed to allow replication to proceed. Determining whether the cohesin band

pattern changes during DNA replication will be an important first step, and further cytological studies using polytene chromosomes may help to elucidate the relationship between cohesin and DNA replication. It is hoped, therefore, that this exciting, initial investigation into the requirement for the cohesin complex in polytene chromosome structure will provoke further investigations of these chromosomes and of the cohesin complex.

Materials and Methods

Fly Stocks

Flies were maintained on standard cornmeal-based medium supplemented with dry yeast. *mr*^{2} flies are described in Reed and Orr-Weaver 1997 and Kashevsky *et al.* 2002. *polo*^{1} flies (*yBS; ru st polo[1] ec/TM6B, Hu e Tb*) were obtained from the Bloomington Stock Center (Bloomington, IN) and are described in Sunkel and Glover, 1998. *forkhead*-GAL4 flies were a gift of Ilaria Rebay (Zhou *et al.* 2001) and *ubiquitin*- GAL4 flies (P{GAL4}1032.hx) were a gift of Frank Lyko (Zink and Paro 1995). The *yw/+;smc1[exc46]/TM6B, Ubi-GFP* stock was a gift from Scott Hawley.

Creation of *rad21* RNAi transgenic line

600bp of *rad21* cDNA LD16422 was amplified by PCR, using primers JAW3 (CGGGATCCCGAACCAGCCCTTTTGAAG) and JAW4 (GGGGTACCCCGTGCAAGAATTTCCATTG) that put *BamH* I and *Kpn* I restriction sites on the ends of the PCR product. This insert was ligated into pUC19 digested with *BamH* I and *Kpn* I (pUC19 + RAD21M). 300bp of *GFP* was amplified from *UAS-mGFP6* from Andrea Brand. Primers used were JAW1 (GGGGTACCCCGTTACCCTGATCATATGAAG) and JAW2 (GGAATTCCGAGTTGCACGCCGCCGTC) that put *Kpn* I and *EcoR* I sites on the ends of the PCR product. This insert was ligated into pUC19 + RAD21M digested with *Kpn* I and *EcoR* I. The inverted 600bp of *rad21* was amplified from cDNA LD16422 using primers JAW5 (GGAATTCCGTGCAAGAATTTCCATTG) and JAW6 (GCTCTAGAGCAACCAGCCCTTTTGAAG) that put *EcoR* I and *Xba* I sites onto the ends of the PCR product. This insert was ligated into pCS2+ digested with *EcoR* I and *Xba* I. RAD21-

GFP was digested out of the pUC19 vector by *BamH* I and *EcoR* I and ligated into pCS2+ digested with *BamH* I and *EcoR* I. The RAD21-GFP-RAD21 IR insert was sequenced in the pCS2+ vector before digesting out the fragment with *BamH* I and *Xba* I. The insert was ligated into pUASP digested with *BamH* I and *Xba* I and this transposon is called *P{w+ UAS-rad21 RNAi}*-C4-2. All restriction enzymes used were from New England Biolabs (Beverly, MA).

Plasmid DNA was purified by centrifugation in CsCl (Sambrook *et al.* 1989) and verified by restriction mapping. Embryos injections and the establishment of transgenic lines was as described by Spradling (Spradling 1986). Insertions were mapped onto a single chromosome and stable stocks were generated for 40 transgenic lines by balancing the insert over either *CyO GFP* or *TM3 GFP*. These lines were screened by PCR for the presence of an intact RAD21 RNAi construct and for an effect on viability by crossing the lines to an *ubiquitin-* GAL4 driver.

Western Analysis

Protein extracts were generated by grinding 20 3rd instar larvae in sample buffer on ice. Samples were separated on SDS-PAGE gel using standard techniques. Guinea pig anti-DRAD21 was used at 1:10,000 (Lee *et al.* 2004) and rat anti-tubulin YOL1/34 was used at 1:500 (Axyll, Westbury, NY). Secondary antibodies used were alkaline phosphatase-conjugated anti-rabbit (Promega, Madison, WI) and HRP-conjugated anti-rat (Jackson ImmunoResearch Laboratories, West Grove, PA).

Cytology and Microscopy

Females were fattened on wet yeast for one to two days and ovaries were dissected out in Grace's solution. Ovaries were fixed in 8% formaldehyde (Ted Pella, Inc., Redding, CA) in PBS

for ten minutes and stained with 4', 6-diamidino-2-phenylindole (DAPI). Larvae were grown on wet yeast at 18°C until wandering 3rd instar larvae appeared along sides of bottle. Larvae were dissected in Grace's solution. Both *smc1* homozygous larvae and larvae expressing *rad21* RNAi were identified by the absence of a balancer chromosome containing *GFP* with a Leica fluorescent dissecting microscope. The developmental stage of larvae was determined by examining the larval mouth hooks that are distinct for each of the three instars (described in Roberts 1986).

For orcein chromosome squashes, salivary glands were fixed for one minute in 45% acetic acid, transferred for three minutes to a solution of 3% synthetic orcein in 60% acetic acid and then squashed. For immunofluorescence staining of RNAi-induced salivary gland chromosomes, glands were fixed for one minute in 45% acetic acid, transferred for 3' to a 1:2:3 solution of lactic acid:ddH2O:acetic acid and then squashed. Slides were washed in 1xPBS and stained with DAPI. Whole-mount salivary glands were fixed in 8% formaldehyde in 1xPBS for ten minutes and stained with DAPI.

For RAD21 detection, larval dissections and salivary gland processing were done following an adaption from Zink and Paro 1995 by G. Cavalli (www.igh.cnrs.fr/equip/cavalli). Briefly, larvae were dissected in 0.1% Triton X-100 in 1xPBS, fixed for 30 seconds in 1% Triton X-100, 3.7% paraformaldehyde in PBS and transferred to 3.7% paraformaldehyde, 50% acetic acid on a siliconized coverslip for two minutes. Slides were blocked in 3% BSA, 0.2% NP40, 0.2% Tween 20, 10% non-fat dry milk and 1 mg/mL RNAse A in PBS. Following antibody incubations, slides were washed in 1xPBS, 300 mM NaCl, 0.2% NP40, 0.2% Tween20 and in 1xPBS, 400 mM NaCl, 0.2% NP40, 0.2% Tween-20.

Antibodies used in this study were rabbit anti-RAD21 (a gift from Margarete Heck and Claudio Sunkel, Warren *et al.* 2000b) at 1:500. All secondary antibodies were fluorescently-conjugated and used at 1:200 (Jackson ImmunoResearch Laboratories). Samples were mounted in Vectashield.

Imaging of *polo*^{1} ovaries, *rad21* RNAi and *smc1* polytene chromosomes was performed using a Zeiss Axiophot microscope and Spot CCD camera and imaging software. Imaging of polytene chromosomes stained with anti-RAD21 antibodies was performed using a Zeiss microscope with LSM 510 confocal imaging software (Keck Imaging Facility). All images were processed using Adobe Photoshop.

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Chapter Four

Conclusions and Perspectives

The importance of polytene chromosome structure

Polyploidy supports many objectives in nature: rapid cell growth, high metabolic activity and resistance to genetic damage. What contribution do polytene chromosomes provide in achieving these goals? It seems most likely that polyteny aids in the high level of metabolic activity, as the production of mRNAs and proteins appear to be the primary function for these tissues. The salivary gland is the largest secretory organ in Drosophila and, in response to steroid hormones, particularly ecdysone, transcription of specific genes is upregulated to meet the high organismal demand of these proteins. These genes encode proteins that are required by the larva for each molt and for pupation, as with the glue genes that encode glycoproteins that enable to pupa to adhere to a substrate during metamorphosis (Beckendorf and Kafatos 1976, Korge 1977). Interestingly, the polytene chromosomes alter their structure in response to hormone treatment. In early analysis of these chromosomes, swellings were noted at specific regions along the chromosomes; these were later recognized as localized decondensation of the DNA and were termed "puffs" (for review see Zhimulev *et al.* 2004). These puffs are highly transcriptionally active and are activated in response to developmental hormones (Zhimulev *et al.* 2004). The high production of these proteins, therefore, aids in the developmental progression of the larvae and is an important function of the salivary gland. Could polytene chromosome structure facilitate the elevated transcription of these genes? Though there is no direct evidence to support this hypothesis, it is an enticing possibility. Polytene structure could enable the puff sterically, allowing a region of decondensed and less organized DNA in the middle of a more rigid structure. Additionally, polytene structure could support high levels of transcription by concentrating the transcriptional machinery to the puff. Further potential

implications of polytene structure in chromosome organization and gene regulation are discussed below.

The nurse cell polyteny-polyploidy transition

Nurse cell chromosome reorganization is not unique to Drosophila, but appears to be conserved in several other insects as well (Dej and Spradling 1999). While the mechanism of the transition is becoming more evident, it is not clear what developmental signals initiate this transition or what biological purpose it might serve. As the nurse cells produce high levels of mRNA and proteins for the developing oocyte, one possibility is that chromosome reorganization might facilitate this high metabolic activity. Previous studies have observed that the polyteny-polyploidy transition coincides with a reorganization of the nucleolus, suggesting that these events may be linked (Dapples and King 1970, Dej and Spradling 1999). It has been hypothesized that changes in nurse cell chromosome organization aid the production of high levels of ribosomes and other factors required for rapid oocyte formation and growth (Spradling 1993). Decisive demonstration of differences in metabolic activity between polytene and polyploid nuclei in nurse cells remains lacking, yet these observations make it an enticing question worthy of future study.

Several mutants that appear unrelated to the endocycle also block the polyteny-polyploidy transition but allow nurse cells to continue their growth, resulting in large polytene chromosomes in late egg chambers. This is particularly evident in mutants of *otu*, whose giant polytene chromosomes display a banding pattern similar to that of salivary gland polytene chromosomes (reviewed in Koryakov *et al.* 2004). *otu* plays a critical role in proper localization of patterning factors in the oocyte and does so by interactions with two RNA-binding proteins,

HRB27C and SQD (Goodrich *et al.* 2004). *otu* itself appears to be regulated by *half pint*, which affects splicing of *otu* and by the translational regulator *cup* (Van Buskirk and Schupbach 2002, Keyes and Spradling 1997). Mutants in *hrb27c*, *sqd*, *half pint* and *cup* all show defects in the polyteny-polyploidy transition indicating that they function to regulate this transition likely through OTU (Keyes and Spradling 1997, Van Buskirk and Schupbach 2002, Nakamura *et al.* 2004, Nelson *et al.* 2004, Goodrich *et al.* 2004). It is currently unclear how these proteins affect the polyteny-polypoidy transition although it seems likely that this may be indirect and that disruption of the developmental program in oogenesis may block the transition. This suggests, therefore, that the polyteny-polyploidy transition is linked to developmental progression in the egg chamber. Interestingly, several mutants with defects in the polyteny-polyploidy transition also show defects in the development of the oocyte, indicating that the developmental progression of these tissues may be associated (Morris *et al.* 2003). This apparent dependence between the nurse cell and oocyte development indicates the importance of the nurse cell support for the oocyte during oogenesis.

Studies of the mutant *mr* revealed high levels of CYCLIN B protein at the polyteny-polyploidy transition in the nurse cells (Reed and Orr-Weaver 1997). Importantly, inappropriate levels of CYCLIN B do not appear in early endocycling *mr* mutant nurse cells and CYCLIN B is not detected in wild-type nurse cells, suggesting that *cyclin B* may be specifically transcribed or translated at low levels at the transition. It is not currently known how expression of mitotic cyclins is turned off during endocycles and analysis of the transcriptional regulation of mitotic cyclins may prove insightful. Studies of transcriptional regulation of both *cyclin E* and the mitotic inducer *string* revealed large and complex cis-regulatory regions with tissue and stage-specific elements (Edgar *et al.* 1994, Lehman *et al.* 1999, Jones *et al.* 2000). It is possible that

other cell cycle regulators in Drosophila may have equally complex regulatory elements and these may regulate specific expression of mitotic regulators at the polyteny-polyploidy transition. CYCLIN B translation can also be repressed in the course of Drosophila development suggesting that relief of CYCLIN B translational repression at the polyteny-polyploidy transition could regulate the transient mitosis (Dalby and Glover 1993). Further studies of the mitotic character of the polyteny-polyploidy transition will reveal other mitotic regulators required for this transient mitosis and will elucidate the upstream pathways controlling this specific reorganization of the nurse cell chromosomes.

Cohesin and polytene chromosome structure

We show here that RAD21 localizes in bands on salivary gland polytene chromosomes, a result consistent with a previous study (Markov *et al.* 2003). In our *rad21* depletion and *smc1* mutant studies, the effects on polytene chromosome structure do not appear to be limited to certain regions along the chromosomes, but rather result in a global disruption of polytene chromosome structure. We suggest, therefore, that undetectable levels of cohesin may be found along the polytene chromosome arms, but that there are particular regions with high levels of the cohesin complex. Future studies determining whether the cohesin complex consistently localizes to specific cytological positions on the chromosomes will likely prove interesting. If the cohesin complex is consistently found at the same locations, it will be useful to determine the underlying characteristics of these regions and to begin to analyze the potential structural or gene regulatory roles for cohesin at these sites.

We did observe, however, that most of our RAD21 bands correlate with interband regions where the DNA is less condensed. Interbands have been suggested to serve several purposes in polytene chromosomes; some contain highly transcriptionally active "housekeeping"

genes, others contain the cis-regulatory elements for genes found in adjacent bands, while others contain elements that assist in organizing the chromosomes into specific domains (reviewed in Zhimulev *et al.* 2004). Is the localization of cohesin to interbands in order to serve a particular purpose at these sites or merely a consequence of another activity that restricts it to these sites? In *S. cerevisiae*, the location of the cohesin complex along chromosome arms appears to be the consequence of transcriptional activity with cohesin being situated in regions that are not undergoing transcription, as has been suggested by genome-wide mapping of cohesin localization (Glynn *et al.* 2004, Lengronne *et al.* 2004). A similar effect, however, may be unlikely if cohesin on salivary gland polytene chromosomes is mapped to interbands that are actively transcribed. Another possible explanation for the localization of cohesin to interbands may involve the highly condensed nature of bands. In metazoans, the reorganization of mitotic chromosomes at prophase into their tightly condensed mitotic structure is associated with the loss of the cohesin complex from chromosome arms, although removal of cohesin is not required for condensation *in vitro* (Losada *et al.* 2002). Cohesin localization to polytene chromosomes may be increased, therefore, in chromosomal regions that are less condensed. Finally, it is intriguing that cohesin is found in interbands that can contain boundary elements that define independent domains of genetic activity. Components of the cohesin complex and the cohesin loading complex appear to play critical roles in enhancer-promoter interactions (Rollins *et al.* 2004, Cuvier *et al.* 1998). In *S. cerevisiae*, mutations in *smc1* and *smc3* suggest that the cohesin complex may act in defining boundaries as these mutants show an inability to maintain a silencing boundary at the HMR silent mating-type locus (Donze *et al.* 1999). Although it remains to be shown how direct these relationships are, it is enticing to speculate that cohesin may participate in higher order chromosome structure and that its localization to interbands on

polytene chromosomes serves a biological function in the regulation of transcription and chromosomal architecture.

Cohesin localization to polytene chromosomes may also provide a useful cytological tool to study the regulation of the cohesin complex in G and S phases and in the absence of mitosis. In mitosis, cleavage of SCC1/MCD1/RAD21 at the metaphase-anaphase transition leads to the loss of cohesin from chromosomes, allowing the sister chromatids to separate. Cohesin reassociates with chromosomes in G1 and cohesion is then reestablished in S phase with the synthesis of a new sister chromatid. In the endocycle, however, the absence of mitosis suggests that the cohesin complex may not be removed from chromosomes prior to another round of S phase. Localization of the cohesin complex on polytene chromosomes combined with an S phase marker should demonstrate whether the cohesin complex remains on these chromosomes during S phase. If cohesin is not removed, how does DNA replication occur while the sister chromatids remain attached? The answer to this question will require a better understanding of how the cohesin complex generates cohesion between two sister chromatids. It is possible, though seems unlikely, that the replication fork could pass through the cohesin ring if the ring is shown to enclose the sister chromatids. Physical models of sister-chromatid cohesion involving multimers of the cohesin complex may allow more room for the replication machinery. Alternatively, could cohesin be altered, but not removed completely to allow DNA replication? The requirement for an acetyltransferase, ECO1, in the establishment of cohesion has led to the hypothesis that the cohesin subunits might be posttranslationally modified in S phase and that this modification could result in a change in cohesin structure to establish cohesion. If this hypothesis stands experimental examination, reversal of such modifications may alter cohesin structure sufficiently to allow replication.

If cohesin is removed to allow each round of DNA replication, how is this removal of cohesin regulated in the absence of M phase? Recent studies have suggested that the cohesin complex can be removed locally from chromosomes in interphase in *S. pombe*. Although the mechanism of this remains to be detailed, Nagao *et al.* have reported a requirement for *securin* and *separase* in the local repair of damaged DNA in interphase (Nagao *et al.* 2004). They demonstrate that mutants with uncleavable cohesin or protease-dead SEPARASE are impaired in DNA repair, implying that this repair occurs through the separase-mediated cleavage of cohesin (Nagao *et al.* 2004). We have not examined polytene chromosomes from *separase* mutants to determine whether this protease is required in polytene chromosomes. *mr* mutants, however, do not show defects in salivary gland polytene chromosomes suggesting that if SEPARASE does act in larval endocycles, it is regulated independently of APC/C, a mechanism that seems unlikely. Clearly, increasing our knowledge of the cohesin complex and its regulation will be necessary to refine these preliminary speculations. Studies of cohesin on salivary gland polytene chromosomes may assist in answering these questions and could provide a valuable system to reveal new mechanisms in the regulation of cohesion.

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Appendix One

Studies of the nurse cell polyteny-polyploidy transition

Julie A. Wallace and Terry L. Orr-Weaver

* J.A.W. performed all the experiments described in this appendix.

Introduction

The experiments described in this section further detail attempts to understand the nature of the mitotic-like state in the polyteny-polyploidy transition in the nurse cells. First, we continued our characterization of the role of MORULA in the nurse cells by overexpressing *morula* (*mr*) and also examined the levels of mitotic cyclin transcripts in wild-type and *mr* mutant ovaries. Second, we sought to determine the presence of mitotic kinase activity (CDK1) at the transition by using two established mitotic markers, phosphorylated histone H1 and phosphorylated histone H3 as assays for kinase activity. We also examined the effects of depleting CDK1 activity on the polyteny-polyploidy transition. Third, we describe two additional mutants that show defects in nurse cell chromosome structure following the transition: the Drosophila *cdc27* homolog, *mákos*, and a member of the CDC20/FIZZY family, *cortex*. Finally, we demonstrate that proteins can be localized to squashed polytene nurse cell chromosomes and present preliminary characterization of the localization patterns of heterochromatin protein 1 (HP1) and a putative transcription factor, PIPSQUEAK, on these chromosomes.

Results

Overexpression of *mr* does not lead to a defect in the nurse cells

The generation of a transgene containing GAL4-inducible *mr* allowed us to determine whether overexpression of *mr* had any effect on the polyteny-polyploidy transition. Although mitotic APC/C activity is controlled by its association with activators and by phosphorylation, we wanted to determine whether high levels of MR protein had any effect on APC/C activity in the endocycles. The A1, the C5 or the 6D trangenes have been shown to rescue the lethality of the larval mutant alleles of *mr* and the sterility of the female sterile alleles of *mr* (Kashevsky *et al.* 2002). These three lines were crossed to the *nanos*-GAL4 driver to overexpress *mr* in the germline. Ovaries dissected from female progeny were fixed and stained with a DNA dye. Examination of the polyteny-polyploidy transition and nurse cell chromosome structure in each of these cases revealed no defects, suggesting that excess MR does not affect these processes (data not shown). As it is unlikely that increasing MR (APC2) levels alone are able to increase APC/C activity, these findings are not surprising.

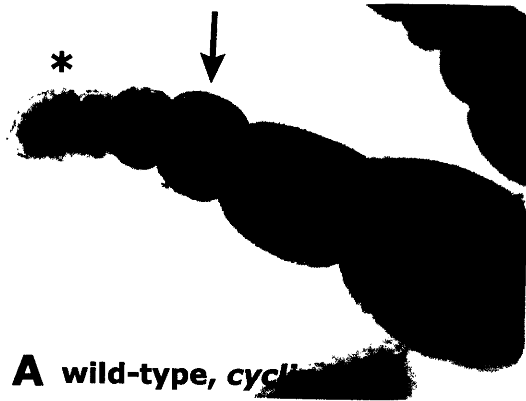
As the *mr* transgenes rescued the larval lethal alleles to adulthood, this allowed us to look at ovaries from these mutants. We speculated that altering levels of *mr* in this manner might reveal phenotypes not present in the female-sterile alleles. We examined nurse cells in mr^3/mr^4 females expressing one of the three *mr* transgenes driven by an *actin*-GAL4 driver. We did not observe any defects in the polyteny-polyploidy transition and in nurse cell chromosome structure with this combination and we conclude, therefore, that overexpression with this driver provides sufficient *mr* for oogenesis in the larval lethal alleles (data not shown).

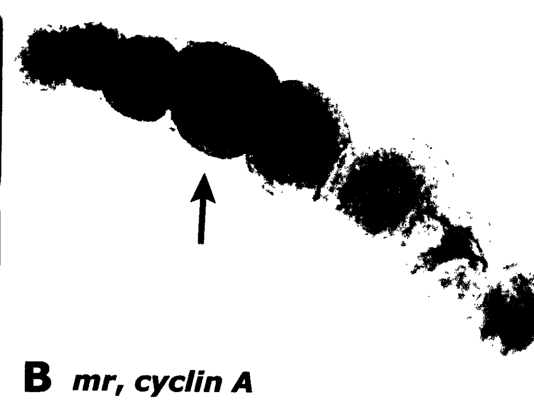
***cyclin A* and *cyclin B* mRNA levels are not altered at the polyteny-polyploidy transition**

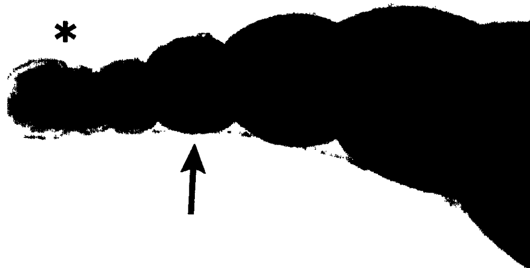
As previously demonstrated, levels of the mitotic Cyclin B protein are abnormally high in nurse cells at the polyteny-polyploidy transition in *mr* mutants (Reed and Orr-Weaver 1997, Chapter 2, Figure 4). The identification of *mr* as a subunit of the APC/C suggested that the inappropriate level of Cyclin B resulted from disruption of the degradation machinery. It is also possible, however, that *mr* affected Cyclin B levels by altering levels of transcription. To determine whether transcription levels of *cyclin B* was increased at the transition, we performed *in situ* hybridization experiments in ovaries using labeled probes for *cyclin B*. We also examined the transcript levels for another mitotic cyclin, Cyclin A. Wild-type egg chambers demonstrate the presence of *cyclin A* (Figure 1A) or *cyclin B* (Figure 1C) transcripts in the nurse cells throughout early egg chamber development. The transcripts have similar expression patterns, appearing first in the late germarium (asterisk in Figure 1A and 1C) and maintaining high levels in nurse cells past the transition (arrowhead in Figure 1A and 1C). *In situ* experiments with control sense probes reveal little non-specific background in these samples (data not shown). The results for *cyclin B* are in agreement with those previously seen, however those for *cyclin A* are not (Dalby and Glover 1992). In the Dalby and Glover study, *cyclin A* transcript was present in the posterior germarium but not in subsequent egg chambers until stages 9-10. These differences may be explained by probe quality or by experimental differences (i.e. hybridization temperature, time for colorimetric development). While the specificity of the probe formally remains a question, we suspect that our experiments reveal levels of *cyclin A* transcript not detectable in earlier experiments. Intriguingly, with this exposure, there is not a dramatic increase in levels of cyclin transcripts at the polyteny-polyploidy transition (arrow in Figure 1A

Figure 1. *cyclin A* and *cyclin B* transcript levels are not altered in the nurse cells at the polyteny-polyploidy transition in *mr*.

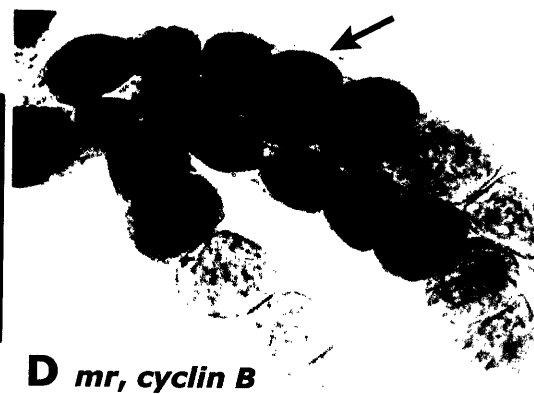
Wild-type or mr^1 ovaries were dissected from females and fixed. Labeled probes were made from cDNAs described in Lehner and O'Farrell 1989 (*cyclin A*) and Lehner and O'Farrell 1990 (*cyclin B*). *In situ* hybridizations were conducted as described in Chapter 2. Until the polyteny-polyploidy transition, the pattern and levels of *cyclin A* and *cyclin B* transcripts are similar in wild-type and *mr* nurse cells. At the transition (black arrow in A-D), no dramatic increase in transcript levels of either cyclin is observed in wild-type or *mr* nurse cells. After the transition, levels of *cyclin A* and *cyclin B* transcript rapidly decrease in *mr* egg chambers as the nurse cells apoptose.







C wild-type, *cyclin B*



and 1C), suggesting that induction of the transient mitosis is not controlled by altering expression of the *cyclin A* and *cyclin B* genes. It is possible, though, that a slight increase in transcript levels occurs at the transition stage that is not detectable with these methods or that a shorter exposure would reveal subtle differences.

mr mutant egg chambers display similar patterns and levels of cyclin expression to wild-type before the transition stage. Again, *cyclin A* and *cyclin B* transcripts appear late in the germarium and are present in the earliest egg chambers. After the polyteny-polyploidy transition (arrow in Figure 1B and 1D), transcript levels of the cyclins decrease in the mutants, likely reflecting the apoptosis seen in late *mr* mutant egg chambers. At the transition stage itself, levels of *cyclin A* and *cyclin B* transcripts are similar to those seen in wild-type transition stage egg chambers (compare at arrows Figure 1A and B, Figure 1C and 1D). Again, this method is unable to reflect subtle variations in transcript levels, but we conclude that *mr* mutants do not affect mRNA levels of *cyclin A* or *cyclin B* at the transition stage. This suggests that the polyteny-polyploidy transition and the phenotypes seen in *mr* are not controlled by changes in gene expression.

PhosphoH1 staining pattern is altered in *mr* mutant nurse cells

In mitosis, CYCLIN B associates with CDK1/CDC2, generating a kinase with many substrates that promote mitotic events such as nuclear envelope breakdown, chromosome condensation and spindle assembly (for review, see Nigg 2001). Degradation of CYCLIN B is necessary to reduce CDK1 activity, a requirement for exit from mitosis to allow cytokinesis and reset replication origins (Wheatley *et al.* 1997, Noton and Diffley 2000). Analysis of transgenic Drosophila embryos expressing a non-degradable form of CYCLIN B show a mitotic arrest,

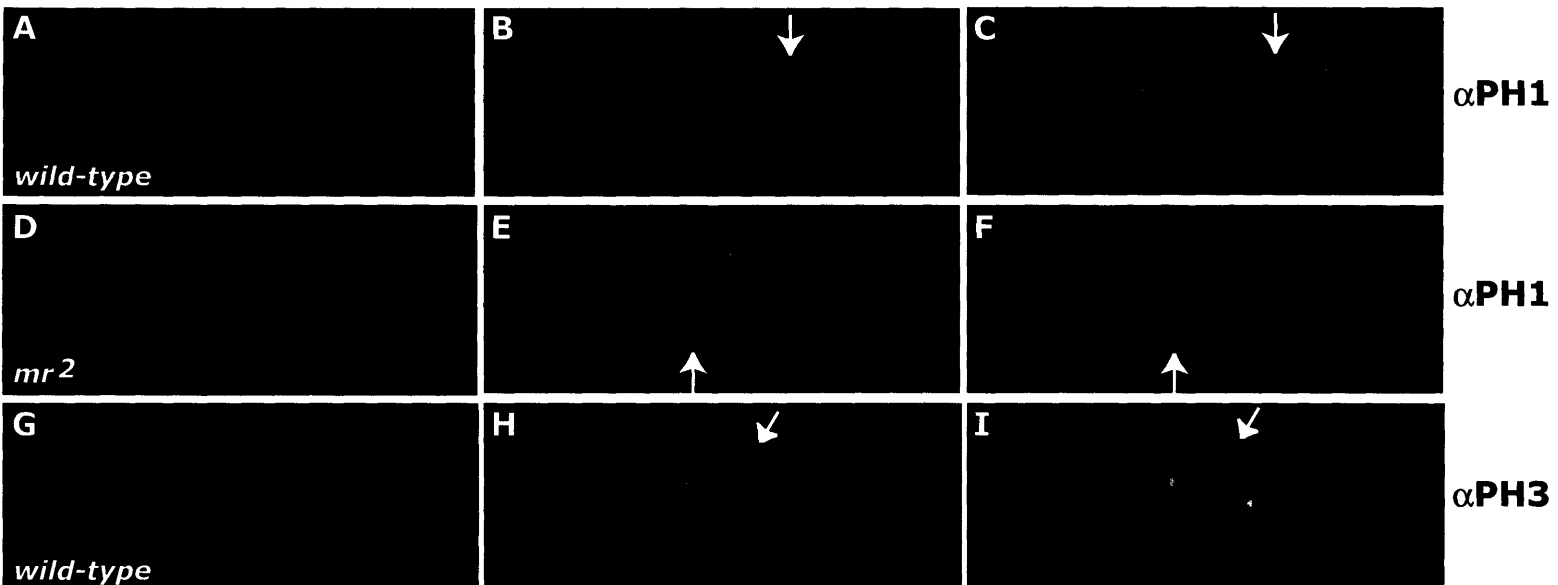
demonstrating that degradation of CYCLIN B is essential for mitotic exit in Drosophila (Sigrist *et al.* 1995). The increased levels of CYCLIN B protein at the polyteny-polyploidy transition stage in *mr* mutants suggested that continuous CYCLIN B/CDK1 activity might be responsible for the persistence of a mitotic-like state in late *mr* egg chambers.

Phosphorylation of histone H1 has been observed to correlate with the cell cycle; levels are low in G1, increase during S-phase and peak before or at metaphase (Bradbury 1992). The subunits of histone H1 kinase have been identified as cyclin and CDK1/CDC2 and thus histone H1 is often used as a substrate to measure CDK1 activity (Bradbury 1992). To look at CDK1 kinase activity in nurse cells, we utilized antibodies against phosphorylated histone H1 (pH1) as an indicator of CDK1 activity and stained fixed ovaries (Figure 2). In wild-type egg chambers, the pH1 antibodies stain follicle cells in a mosaic pattern, which likely reflects the asynchronous mitosis in the follicle cells at this time (Figure 2B and C). At the transition stage (white arrow in Figure 2B and 2C) we noted that some nurse cell nuclei stained for pH1, while others did not. It is possible that this mosaic staining reflects asynchrony in the progression of nurse cell development. We were also surprised to observe that nurse cell nuclei stain for pH1 before the polyteny-polyploidy transition (Figure 2B and 2C) and in later stages following the transition (data not shown). We feel, therefore, that further experiments are required to determine whether the pH1 staining specifically indicates CDK1 activity in nurse cells. As previous have demonstrated that phosphorylation of histone H1 in follicle cells is controlled by CYCLIN E/CDK2 kinase, this result is not particularly surprising (Hartl submitted).

Interestingly, the staining pattern with the pH1 antibody is altered in *mr* mutants. Prior to the polyteny-polyploidy transition the staining pattern is similar; few nurse cell nuclei stain while others do not (Figure 2E). At the transition, though, most *mr* nurse cell nuclei stain with the pH1

Figure 2. Levels of phosphorylation of histone H1 and histone H3 are not altered in the nurse cells at the polyteny-polyploidy transition.

Wild-type or *mr*^{2} ovaries were dissected, fixed and incubated with antibodies as described in Chapter 2. Antibodies for phosphorylated histone H1 (pH1, green in Figures 2B, C, E, F) and phosphorylated histone H3 (pH3, green in Figures 2H, I) were used at 1:100 (Upstate Biotech, Waltham, MA). DNA is visualized by incubation with either propidium iodide or DAPI (red in Figures 2A, C, D, F, G, I). A portion of wild-type nurse cells stain for pH1 in early and late egg chambers (Figure 2B and C) and there is no alteration in levels or pattern at the polyteny-polyploidy transition (white arrow in Figure 2C). In *mr* ovaries, however, the majority of nurse cells stain for pH1 at the transition (white arrow in Figure 2F). pH3 does not stain nurse cells in wild-type ovaries (Figure 2H, I) and there does not appear to be an induction of pH3 staining at the transition (white arrow Figure 2I).



A
wild-type
B
C
αPH1
D
mr2
E
F
αPH1
G
wild-type
H
I
αPH3

antibody, which may indicate high levels of CYCLIN B/CDK1 activity in these nuclei (white arrow in Figure 2F). Additionally, the increased staining continues in nurse cell nuclei past the transition stage. Further experiments, such as observing the pH1 staining pattern in *cdk1* and *cyclin E* mutants, are necessary to determine the precise meaning of this staining pattern and whether it reflects a change in CDK1 activity in *mr* mutant nurse cells. While the nature of the staining remains uncertain, it is intiguing that there is a distinct change in the pH1 pattern in the *mr* mutant and this may prove to be informative.

In order to address further the level of CDK1 activity in polyteny-polyploidy transition, we examined levels of phosphorylated histone H3 (pH3) in nurse cells. Previous work has demonstrated that pH3 signal, detected by antibodies, reflects CDK1 activity in early Drosophila embryos (Su *et al.* 1998). Studies of a $cdk1^{ts}$ (A171T) mutant that reduces CDK1 activity demonstrated loss of pH3 staining on chromosomes, while pH3 signal is maintained in cellularized embryos which express stable versions of CYCLIN A, CYCLIN B or CYCLIN B3 (Su *et al.* 1998). Finally, ectopic induction of CDK1 activity in interphase, via a *cdk1* mutant that cannot be inhibited by the WEE1 kinase, led to induction of pH3 on chromosomes (Su *et al.* 1998). To determine whether pH3 could be utilized as an indicator of CDK1 activity in nurse cells, we utilized antibodies to pH3 and stained wild-type fixed ovaries (Figure 2H and I). This antibody stained certain follicle cells brightly, likely reflecting asynchronous progression through mitosis. We noted, however, that this antibody did not stain nurse cell nuclei at a detectable level in any stage (white arrow, Figure 2I). It is possible that this reflects an absence of CDK1 activity in these nurse cells at the transition stage or that CDK1 activity is below detectable levels with use of these antibodies. We also did not stain *mr* mutant ovaries with the pH3 antibodies in this study and that experiment that may prove informative as well.

CDK1 activity may be dispensable for the polyteny-polyploidy transition

Despite the uncertain results from attempts to detect CDK1 activity in nurse cells, we sought to determine whether CDK1 activity was required for the polyteny-polyploidy transition. Flies containing a null allele of *cyclin B*, *cycB^{1}*, reach adulthood but are female sterile (Jacobs *et al.* 1998). Ovaries from these mutant females are reported to have rudimentary ovaries and lay few eggs suggesting a requirement for CDK1 activity in oogenesis (Jacobs *et al.* 1998). The catalytic subunit of the CDK1 kinase is encoded by Drosophila *cdc2* (Stern *et al.* 1993). Multiple alleles of *cdc2* were identified and characterized, including the null allele *Dmcdc2^{B47}* and the temperature-sensitive allele *Dmcdc2^{E1-24}* (Stern *et al.* 1993). Crossing the *Dmcdc2^{B47}* mutation to the *Dmcdc2^{E1-24}* mutation at the permissive temperature (18°C) allows the production of transallelic female progeny (Stern *et al.* 1993). Upon shifting the females to the restrictive temperature (29°C), CDK1 activity is gradually reduced (over a period of five days) and any requirement for CDK1 activity during oogenesis can be evaluated. Previous experiments determined that CDK1 was not required for endocycles in nurse cells, as the nurse cells continued to grow in size over the five day period (Reed and Orr-Weaver 1997). Reduction of CDK1 activity was confirmed by the loss of the mitotically dividing follicle cells after three days (Reed and Orr-Weaver 1997). While CDK1 was determined to not be required for nurse cell endocycles, it was not determined in that study if CDK1 activity was required for progression through the polyteny-polyploidy transition.

To assess whether loss of CDK1 activity had an effect on the polyteny-polyploidy transition, we repeated the experiment described above and dissected ovaries from females kept at the restrictive temperature for three days and five days. Ovaries were stained with a DNA dye

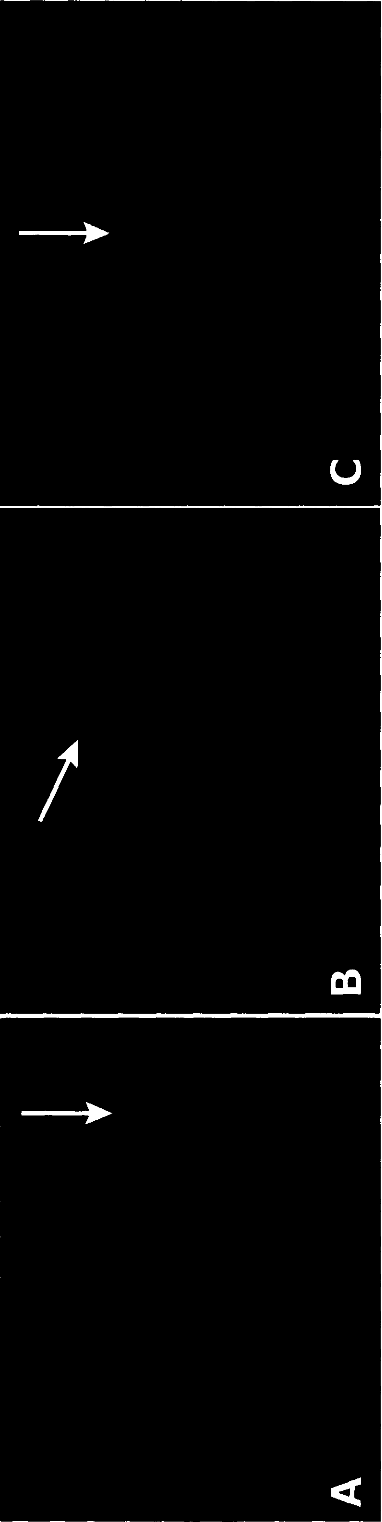
and nurse cell chromosome structure was analyzed. After three days, most follicle cells had disappeared from the developing egg chambers (Figure 3A and 3B). The nurse cell chromosomes, however, were able to pass through the transition properly and demonstrate dispersed, polyploid chromosomes (white arrow, Figure 3A and 3B). After five days, all the follicle cells had been lost, but, again, nurse cell chromosomes were able to disperse (Figure 3C). Unfortunately while these transallelic females show a reduction of CDK1 activity at the restrictive temperature, as reflected by the loss of follicle cells, we cannot rule out that some CDK1 activity persists in the nurse cells allowing the chromosomes to pass through the transition properly. Studies of a stronger temperature-sensitive allele, generated by site-directed mutagenesis, $Dmcdc2^{A171T}$, may be able to answer this question more definitively (Sigrist *et al.* 1995).

***cdc27* and *cortex* mutant ovaries show defects in nurse cell chromosome structure**

To identify additional mitotic regulators involved in the polyteny-polyploidy transition, we analyzed female-sterile alleles of two known cell cycle regulators, *mákos* and *cortex*, and noted abnormal nurse cell chromosome structure that is likely related to the transition. In addition to *mr*, there is one other female-sterile allele of an APC/C subunit called *mákos*. *mákos* was identified by the Berkeley Drosophila Genome Project as the Drosophila homolog of *cdc27* (Spradling *et al.* 1999). mks^1 is a pharate adult lethal allele; the mutants die as pupae with well-developed adult structures (Deak *et al.* 2003). Characterization of mks^1 revealed highly condensed mitotic chromosomes and a high mitotic index in the larval neuroblasts (Deak *et al.* 2003). A weaker allele, mks^2, was identified as a semi-lethal allele with female-sterile escapers, allowing us to look for a phenotype in the ovaries (Deak *et al.* 2003). In mks^2 ovaries, the majority of nurse cell chromosomes progress through the polyteny-polyploidy transition properly

Figure 3. Decreases in CDK1 activity do not block the polyteny-polyploidy transition in nurse cells.

Ovaries with decreased levels of CDK1 were generated by incubating *Dmcdc2*^{B47}/*Dmcdc2*$^{E1-24}$ females at the restrictive temperature (29°C) for three or five days. Following the incubation, ovaries were dissected, fixed and stained with propidium iodide to visualize the DNA (red in Figure 3A-C) as described in Chapter 2. After three days at the restrictive temperature, the follicle cells begin to disappear from the egg chambers, but the nurse cells chromosomes are able to disperse properly (white arrow in Figure 3A and 3B). After five days, all the follicle cells are lost, but the nurse cells again appear to disperse properly (white arrow Figure 3C).

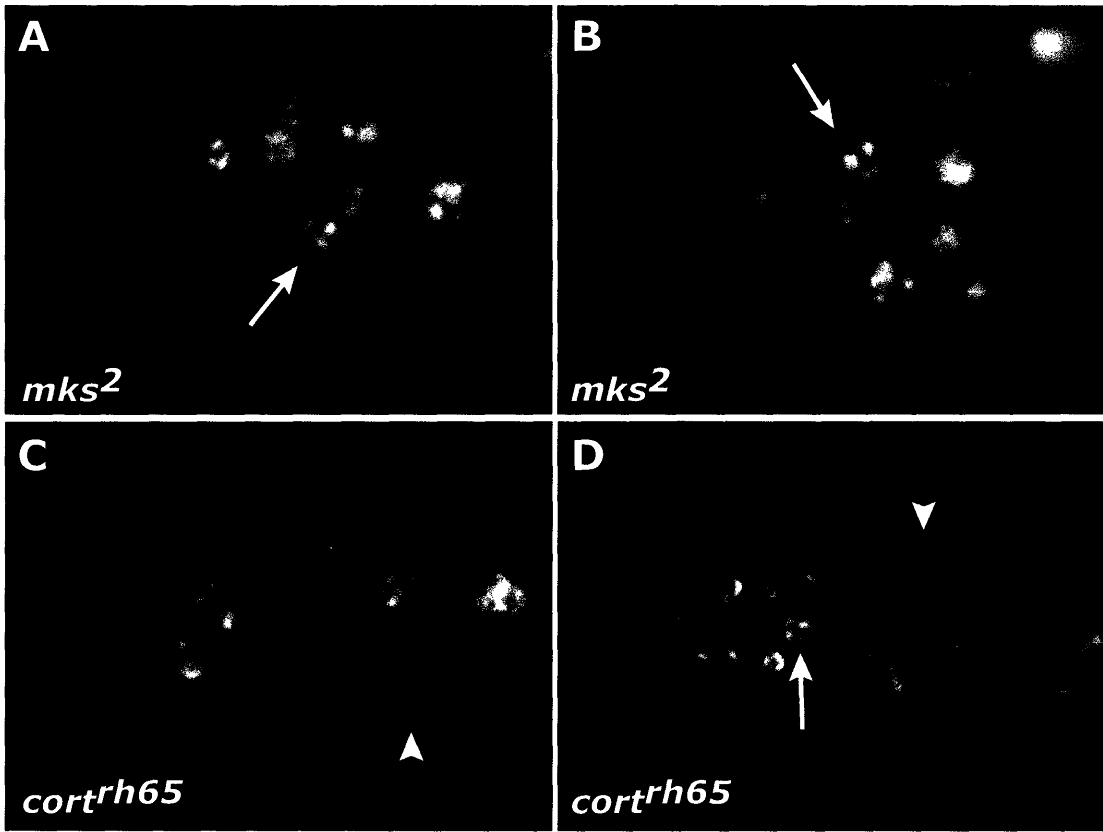

A
B
C

and display dispersed, polyploid chromosomes. In a few rare instances, nurse cell chromosomes are seen that display the condensed chromosomes of the transition state in late egg chambers (white arrow, Figure 4A and 4B). We feel that this phenotype is real due to the previously described role for the APC2 subunit in the transition. However, it remains to be determined whether the rarity of the phenotype reflects differences in allele strengths between the *mr* female sterile alleles and *mks*^{2} or a reduced requirement for MKS in the function of the APC/C at the polyteny-polyploidy transition.

Activation of the APC/C is stimulated by an activator protein; in the mitotic cell cycle members of the CDC20 family activate the APC/C at different times, directing the ubiquitin ligase activity of the APC/C to specific substrates (for review see Peters 2002 and Harper *et al.* 2002). At the metaphase-anaphase transition, APC/C activity is directed through its association with FIZZY/CDC20 (Dawson *et al.* 1993, Dawson *et al.* 1995, Sigrist *et al.* 1995, Visintin *et al.* 1997). Studies of mutant *fzy* ovaries did not reveal a requirement for FZY at the polyteny-polyploidy transition in the nurse cells (Reed and Orr-Weaver 1997). In this experiment, females homozygous for the temperature-sensitive allele, *fzy*^{6}, were raised at the restrictive temperature and the nurse cell morphology from dissected ovaries was examined. While the females failed to produce eggs, suggesting FZY activity had been compromised, the nurse cells did not contain spindles like those seen in the *mr* mutants (Reed and Orr-Weaver 1997). More recent attempts to generate transallelic females with *fzy*^{6} and a null allele, *fzy*^{3}, were unsuccessful, so we examined the ovaries from a female-sterile allele of *cortex*. *cortex* has recently been identified as a member of the CDC20/FZY family and is required for exit from meiosis in Drosophila females (Page and Orr-Weaver 1996, Chu *et al.* 2001). Recent studies in our lab suggest that CORTEX may be a bona fide APC/C activator in meiosis, as levels of PIMPLES and CYCLIN B3 remain

Figure 4. *makos* and *cortex* mutants show defects in nurse cell chromosome structure following the polyteny-polyploidy transition.

Ovaries were dissected from females homozygous for mks^2 and $cort^{rh65}$, fixed and stained with DAPI to visualize the DNA as previously described in Chapter 2. The majority of nurse cell chromosomes progress through the transition properly in mks^2 ovaries. In some instances, however, the nurse cell chromosomes remain condensed and undispersed (white arrow in Figure 4A and 4B). $cort^{rh65}$ and $cort^{QW55}$ mutant ovaries display a striking and unusual phenotype: nurse cell DNA localizes to the periphery of the nucleus giving the appearance of a "crater" in these nuclei (white arrowhead in Figure 4C and 4D, data not shown). Additionally, $cort^{rh65}$ and $cort^{QW55}$ nurse cell chromosomes can remain in the condensed, undispersed state in later egg chambers (white arrow, Figure 4D, data not shown).


A
mks2
B
mks2
C
cortrh65
D
cortrh65

high in *cortex* mutant embryos that are arrested at metaphase II (J. Pesin, personal communication).

Nurse cell chromosomes in *cort*^{rh65} and *cort*^{QW55} ovaries show an unusual structure following the polyteny-polyploidy transition (Figure 4C and 4D). Nuclear structure appears to be disrupted in these nurse cells, as large hollow spaces ("craters") appear in the center of the nucleus and the DNA appears to localize to the periphery of the nucleus (white arrowheads in Figure 4C and 4D). It is possible that this phenotype reflects a disruption in the polyteny-polyploidy transition if dispersion of the sister chromatids is altered in *cortex* mutants. This idea is supported by the infrequent appearance of nurse cell chromosomes that remain in the condensed transition state in later stage egg chambers (white arrow in Figure 4D). It is also possible, however, that this defect is not directly related to the dispersion of the nurse cell chromosomes but rather affects organization within the nucleus, perhaps of the nucleolus in particular. Changes in nucleolar structure have previously been observed to correlate with nurse cell development (Dapples and King 1970, see Discussion). Determining whether the "crater" observed in these nurse cell nuclei correlates to the nucleolus will be an important first step. As this phenotype occurs considerably earlier than the requirement for *cortex* in meiosis II, the disruption of nurse cell chromosome structure in mutant *cortex* ovaries may indicate a new role for CORTEX in oogenesis.

HP1 and PIPSQUEAK are localized to nurse cell polytene chromosomes

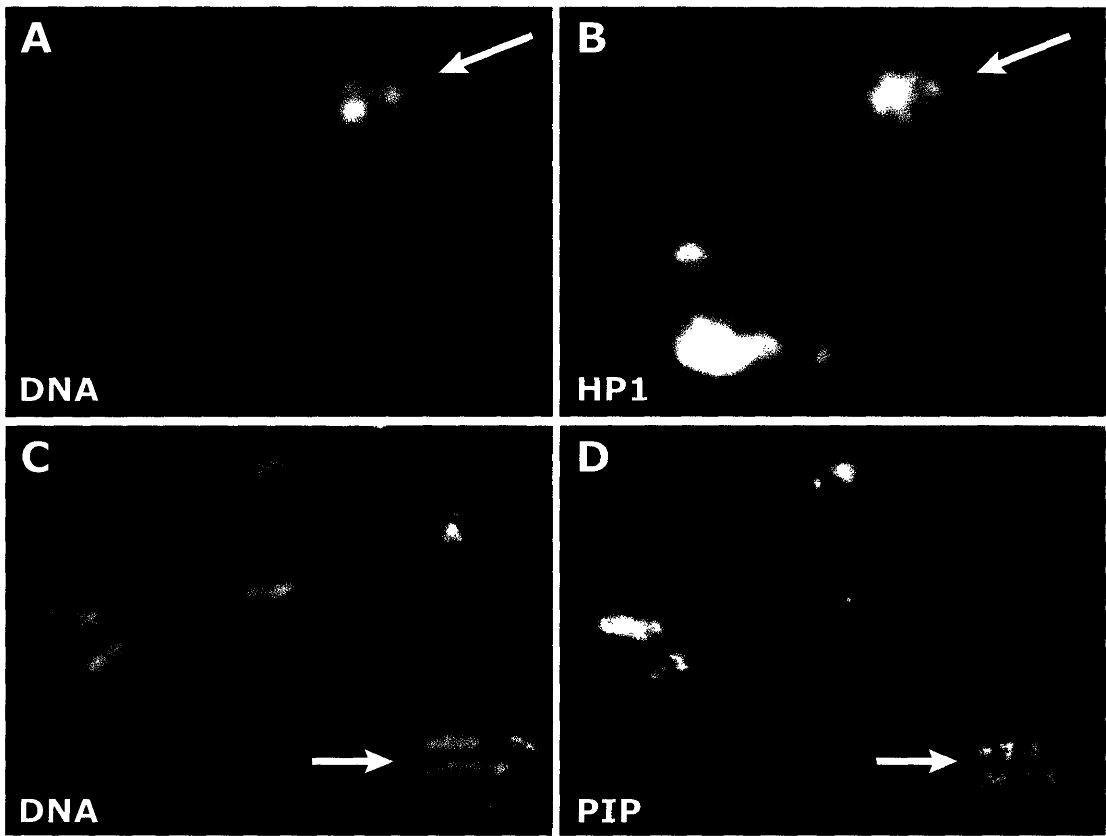
To our knowledge, previous experiments in the field to localize proteins onto wild-type nurse cell chromosomes have not been successful. Our attempts to localize the cohesin complex onto wild-type nurse cells chromosomes required identification of positive controls to validate our immunohistochemical techniques. We looked for proteins that were expressed in the nurse

cells during oogenesis and likely bound to DNA. Incubation of squashed nurse cell chromosomes with anti-phospho histone H1 antibodies and anti-histone antibodies did not reveal the presence of these proteins on the chromosomes, but we feel that this is most likely due to antibody quality. We were able to see localization onto nurse cell chromosomes with two other antibodies though: heterochromatin protein 1 (HP1) and PIPSQUEAK. HP1 is a highly conserved heterochromatin-associated protein whose chromodomain binds a methylated lysine residue on histone H3 (for review see Maison and Almouzni 2004). A Drosophila HP1 antibody, C1A9, has been demonstrated to bind the centric beta-heterochromatin of salivary gland polytene chromosomes, in addition to specific sites along the chromosome arms and all of chromosome 4 (James *et al.* 1989). This antibody also demonstrates the presence of HP1 at the centromeric regions of squashed nurse cell polytene chromosomes and possibly at other sites along the arms, although a detailed cytological analysis was not conducted (white arrow, Figure 5A and 5B).

pipsqueak is a member of the posterior group of genes, a number of maternal effect genes required for both abdomen and germline formation, and it is required for the early stages of oogenesis (Siegel *et al.* 1993). PIPSQUEAK has been identified as a transcription factor by its sequence and binds GAGA DNA sequences *in vitro* (Lehmann *et al.* 1998). The *pipsqueak* locus encodes multiple transcripts; the PSQA isoform is a nuclear protein found in nurse cell and follicle cell nuclei during oogenesis (Horowitz and Berg 1996). Staining nurse cell chromosome squashes with an antibody to PIPSQUEAK reveals that PIPSQUEAK is found along the arms of nurse cell polytene chromosomes (white arrow, Figure 5C and 5D). These two experiments demonstrate that it is possible to localize proteins to squashed nurse cell chromosomes.

Figure 5. HP1 and PIPSQUEAK localize to squashed nurse cell chromosomes.

Newly eclosed females were aged 8 hours on yeast and then dissected. Ovaries were briefly incubated in 45% acetic acid before fixation in 1:2:3 acetic acid, ddH2O and glacial acetic acid. Antibody for HP1 was used 1:5 (C1A9, Developmental Hybridoma Studies Bank, James *et al.* 1989) and antibodies for PIPSQUEAK were used 1:500 (gift from Celeste Berg, Horowitz and Berg 1996). DNA is visualized by DAPI (Figure 5A and 5C). HP1 localizes to centric regions on squashed, polytene nurse cell chromosomes (white arrow Figure 5B). PIPSQUEAK localizes along the length of squashed, polytene nurse cell chromosomes (white arrow Figure 5D).


A
DNA
B
HP1
C
DNA
D
PIP

Discussion

Here we describe findings that suggest the details of the transient mitosis induced following the fifth endocycle in Drosophila nurse cells. Overexpression of an APC/C subunit does not affect the polyteny-polyploidy transition, indicating that APC/C activity in the endocycle likely is not controlled by protein levels of the subunits. Additionally, it appears that the transient mitotic character does not correlate with an increase in mitotic cyclin transcript levels, because by *in situ* hybridization experiments, mRNA levels are not detectably elevated at the transition. As transcript levels of *cyclin A* and *cyclin B* are not increased in *mr* mutants prior to or at the transition, it appears likely that *mr* mutants alter CYCLIN B levels via the effect on the degradation machinery. How then are levels of CYCLIN B induced at the transition? It has been previously demonstrated that translation of *cyclin B* transcripts in the Drosophila oocyte can be kept inactive until a particular developmental time by regulators that bind the 3' UTR (Raff *et al.* 1990, Dalby and Glover 1992, Dalby and Glover 1993). It is also intriguing to speculate that translation of *cyclin B* and other mitotic regulators may be developmentally regulated at the polyteny-polyploidy transition, promoting entry into a transient mitosis. It should be noted, however, that a change in levels of mitotic cyclins at the transition has not been detected by standard immunofluorescence.

The presence of high levels of CYCLIN B in *mr* nurse cells, detected by an antibody in immunofluorescence studies, suggests the persistence of a mitotic-like state in these mutants (Reed and Orr-Weaver 1997). As CYCLIN B activity in mitosis depends on its association with CDK1 and progression through mitosis involves the activity of the CYCLIN B/CDK1 kinase, we feel it is likely that CDK1 activity is present at the polyteny-polyploidy transition in nurse cells. Experiments to detect CDK1 activity specifically at the transition have been difficult to interpret,

as we do not have a convincing marker of CDK1 activity or the absence of CDK1 activity. We have also been unsuccessful in determining whether CDK1 activity is required for the transition as our studies have been inconclusive. Thus, it still remains to be demonstrated that the mitotic-like state in nurse cells utilizes the same regulators as the mitosis of the canonical cell cycle. At this time we cannot rule out that CYCLIN B/CDK1 may not have a role in the mitotic-like state or may have a non-essential, minor role.

Further characterization of *mákos* and *cortex* mutant phenotypes in the nurse cell may prove to be informative. As shown here, *mákos* (*cdc27*) mutant nurse cell chromosomes remain in the condensed polytene state in a few rare instances. It is likely that the rarity of this phenotype is due to the weakness of this female-sterile allele. The generation of germline clones with the stronger allele, mks^1, could answer this question. Recent studies of APC/C subunits in Drosophila have begun to reveal distinctions in the activities of these subunits (Kashevsky *et al.* 2002, Huang and Raff 2002, Bentley *et al.* 2002, Deak *et al.* 2003). Therefore, it is also possible that while MR (APC2) is absolutely required for the polyteny-polyploidy transition, MKS may not be. It will be interesting to determine the identity of the APC/C subunits that act at the polyteny-polyploidy transition and compare this complex to the APC/C that acts in the modified S-M cycles of early embryogenesis and the APC/C that acts in the archetypal cell cycle in the larval neuroblasts. While it seems likely that the core components are the same in these complexes, differences in accessory subunits may reveal differences in the regulation of these complexes.

The phenotype seen in *cortex* mutant ovaries may prove to be informative as well. Following the four incomplete mitoses that generate the 16 cell cystoblast (the oocyte and nurse cell precursors), all the cyst cells enter a premeiotic S phase. Multiple nuclei assemble

synaptonemal complexes, a proteinacous structure indicative of meiotic recombination (Spradling 1993). Within a short time, the structure is restricted to the pro-oocyte, and the nurse cells exit meiosis and begin endocycling (Spradling 1993). It is intriguing to speculate that the germline-derived nurse cells never fully abandon their meiotic character and thus utilize a meiosis-specific activator of the APC/C, CORTEX, at the polyteny-polyploidy transition. This may also have interesting implications for the nature of the cohesin complex on nurse cell polytene chromosomes. In meiosis, the SCC1/MCD1/RAD21 subunit is replaced by REC8 in many organisms (for review see Lee and Orr-Weaver 2001). It is possible, therefore, that the cohesin complex in nurse cells is more similar to meiotic cohesin complexes than the mitotic cohesin complex. It is important to note, however, that a REC8 homolog in Drosophila or any female meiosis-specific cohesin subunit has not yet been identified, so this hypothesis remains extremely speculative.

Previous observations have detailed the development of a large nucleolus in Drosophila nurse cells that is dispersed after the polyteny-polyploidy transition (Dapples and King 1970). It has been speculated that the transition to polyploidy in the nurse cell chromosomes may promote rapid ribosome synthesis by dispersing the regions of the nucleolus (Dej and Spradling 1999). Thus, it may also reflect a defect in the transition for *cortex* mutants if the "crater" seen in *cortex* mutants correlates with the nucleolus. Experiments directly correlating ribosome production rates with polyploid versus polytene chromosomes remain to be conducted. Increases in the rates of RNA synthesis have been noted as oogenesis proceeds, although these appear to correspond to increases in gene copy number produced by the endocycle (Mermod *et al.* 1977). It is also possible that the *cortex* phenotype is not related to the polyteny-polyploidy transition and that

this reflects a previously undescribed role for CORTEX possibly in nuclear organization during oogenesis.

Finally, we demonstrate the ability to localize two proteins, HP1 and PIP, onto squashed nurse cell chromosomes. Attempts using the same protocol to localize DRAD21, DSMC1 and DSMC3 onto these chromosomes were unsuccessful. We do not conclude, however, that this indicates the absence of cohesin complex on polytene nurse cell chromosomes. Differences in antibody quality and the degree of association between the protein and DNA must be taken into consideration and may account for the negative result. Therefore, we still believe that the cohesin complex is integral to polytene chromosome structure in the nurse cells.

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Appendix Two

Studies of a tissue-specific underrepresented ORF, *stellate*

Julie A. Wallace and Terry L. Orr-Weaver

* J.A.W. performed the real-time PCR and FISH experiments.

Introduction

Variations of the endocycle itself are often used in developmental contexts (for review see Edgar and Orr-Weaver 2001). In addition to modifying the extent of mitotic character in the endocycle, cells also are able to vary the character of S phase. In Drosophila, early in nurse cells differentiation an endocycle with a full S phase is utilized, while later nurse cells and the larval-specific tissues employ an endocycle where S phase is truncated and late replicating sequences are not replicated. Additionally, in certain endocycles, total genomic DNA replication is altered such that only specific regions are replicated (amplification). By suppressing "licensing" of origins, endocycling cells are then able to fire specific origins multiple times in a single S phase, producing amplified genomic regions. During oogenesis, the somatic follicle cells end their full genomic replication in endocycles and proceed to program in which certain regions are amplified (for review see Claycomb and Orr-Weaver 2005). Follicle cells of Drosophila therefore have provided an excellent model system in which both genetics and cell biology can be employed to understand how DNA replication can be locally controlled.

The major amplicons in the follicle cells are located on the *X* and *3rd* chromosomes and demonstrate biological relevance for amplification, because they encode the chorion proteins that form the eggshell (Spradling 1981). To identify the other amplicons in the follicle cells, our lab created and utilized a microarray spotted with single ESTs from the Drosophila Unigene collection (Claycomb *et al.* 2004). By comparing levels of hybridization between embryonic DNA and follicle cell DNA, this study identified ORFs in the Drosophila genome that are differentially represented in the follicle cells. Differential representation is most simply explained as resulting from differential replication. Copy numbers were measured by comparing the level of representation of a locus in follicle cell or salivary gland DNA to embryonic DNA.

This experiment identified an EST on the *X* chromosome, *CG32605*, which is underrepresented in the follicle cells (copy number of 0.22 and 0.16 in separate experiments), but fully represented in the salivary glands (copy numbers of 0.81 and 0.90). As little is known about the nature of underreplication or how replication of these regions is regulated, we analyzed this region to determine if the underrepresentation arose from underreplication and to provide insight into the mechanism and biological relevance of underreplication.

Results

The *stellate* locus is differently represented in a tissue-specific manner

The cDNA used in the microarray experiments, GM04658, encodes STELLATE, an ORF that shows amino acid similarity to a casein kinase II regulatory subunit (Livak 1990, Palumbo *et al.* 1994, Bozzetti *et al.* 1995). *stellate* genes are found in tandem repeats in the euchromatin of the *X* chromosome (12D3-4) and in the heterochromatin of the *X* (h26) (Hardy 1984, Shevelyvov 1992, Palumbo *et al.* 1994). In addition, a suppressor of *stellate*, *Su(Ste)*, that shows high similarity to *stellate* itself, is found on the *Y* chromosome (Livak 1984). Using primers to the *stellate* repeat itself, we confirmed the results from the microarray by real-time PCR (Table 1). In follicle cells, the majority of cells in stage 13 egg chambers, *stellate* had a copy number of 0.22 by microarray analysis. Copy numbers in the real-time PCR analysis were determined by dividing the relative fluorescence for the experimental locus product by the relative fluorescence of a non-amplified control product (*polymerase* α) from chromosome *3R* (for further details see Claycomb *et al.* 2004). By real-time PCR analysis, the *stellate* follicle cell copy number was 0.41 ± 0.01, a value similar to that determined by the microarray experiments. DNA from stage 1-8 egg chambers was used as a

Table 1: Relative representation of the *stellate* locus as determined by microarray and real-time PCR analysis

Genomic DNA tissue	Relative representation as determined by microarray analysis [a]	Relative representation as determined by real-time PCR analysis [b,c]
Stage 13 Egg Chambers	0.22	0.41 ± 0.01[d]
Stage 1-8 Egg Chambers	N/A	0.62 ± 0.04
Salivary Glands from Mixed Larvae	0.90	N/A
Salivary Glands from Female Larvae	N/A	2.03 ± 0.15
Salivary Glands From Male Larvae	N/A	2.61 ± 0.24

a: Microarray experiments were described in Claycomb *et al.* 2004.

b: Primers used in these experiments were to sequences found within the *stellate* cDNA GM04658, which was used in the microarray experiment.

c: Relative representation is calculated by dividing relative fluorescence for the experimental locus products by the relative fluorescence of a *3R* non-amplified control product (*polα*) for a given stage.

d: Standard deviations from real-time PCR experiments were determined as described in Claycomb *et al.* 2002.

control, as the follicle cells are mitotically dividing in these stages and have not begun a program of differential replication. By real-time PCR, the copy number of *stellate* in stage 1-8 egg chambers was 0.62 ± 0.04, a value suggesting minimal underreplication of this locus.

In the salivary gland microarray experiments, genomic DNA was extracted from a population of both female and male larval salivary glands. *stellate* had a copy number of 0.90 from this sample. To determine whether the presence of *stellate* repeats on the *Y* had any effect on the *stellate* copy number in the mixed sample, we isolated genomic DNA from separate female and male larval populations. By real-time PCR, *stellate* had a copy number of 2.03 ± 0.15 in female salivary glands and 2.61 ± 0.24 in male salivary glands. Although we can't rule out a minimal contribution by the *Su(Ste)* repeats on the *Y* to the total *stellate* copy number, the *stellate* copy number in female and male salivary glands is similar and correlates with the copy number determined by the microarray experiments. Therefore, the results from the microarray and real-time PCR experiments are similar for both follicle cells and salivary glands, and we conclude that *stellate* is truly underrepresented in follicle cells but fully represented in salivary glands.

The euchromatic *stellate* repeat locus is fully represented by real-time PCR analysis

As the total *stellate* copy number in the aforementioned experiments included both the heterochromatic and euchromatic *stellate* loci, we sought to determine whether the repeats at both loci were underrepresented. To address the replication properties of the euchromatic *stellate* repeat locus, we utilized real-time PCR using primers specific for unique sequence at the euchromatic locus. Primers were determined to be specific if they generated a PCR product from the 12D locus on the *X* chromosome and not from other *stellate* loci, as determined by blasting

the primer sequence against the *Drosophila* genome. By this method, we generated three real-time PCR primer sets unique to the genomic region adjacent to the 12D locus. In the follicle cells, the euchromatic *stellate* locus had copy numbers of 1.72 ± 0.21 and 1.25 ± 0.15 with primer sets 2 and 3, respectively (Table 2). These results suggest that, in follicle cells, the euchromatic *stellate* is fully replicated. In female salivary glands, the euchromatic *stellate* locus had copy numbers of 1.00 ± 0.11 and 1.40 ± 0.14 with primer sets 1 and 2. In male salivary glands the results were similar; the euchromatic *stellate* locus had copy numbers of 0.84 ± 0.07 and 0.75 ± 0.06 with primer sets 1 and 2. As in the follicle cells, these results reveal that the euchromatic *stellate* is fully represented and therefore replicated in female and male salivary glands.

The euchromatic *stellate* repeat locus is fully replicated by cytological analysis

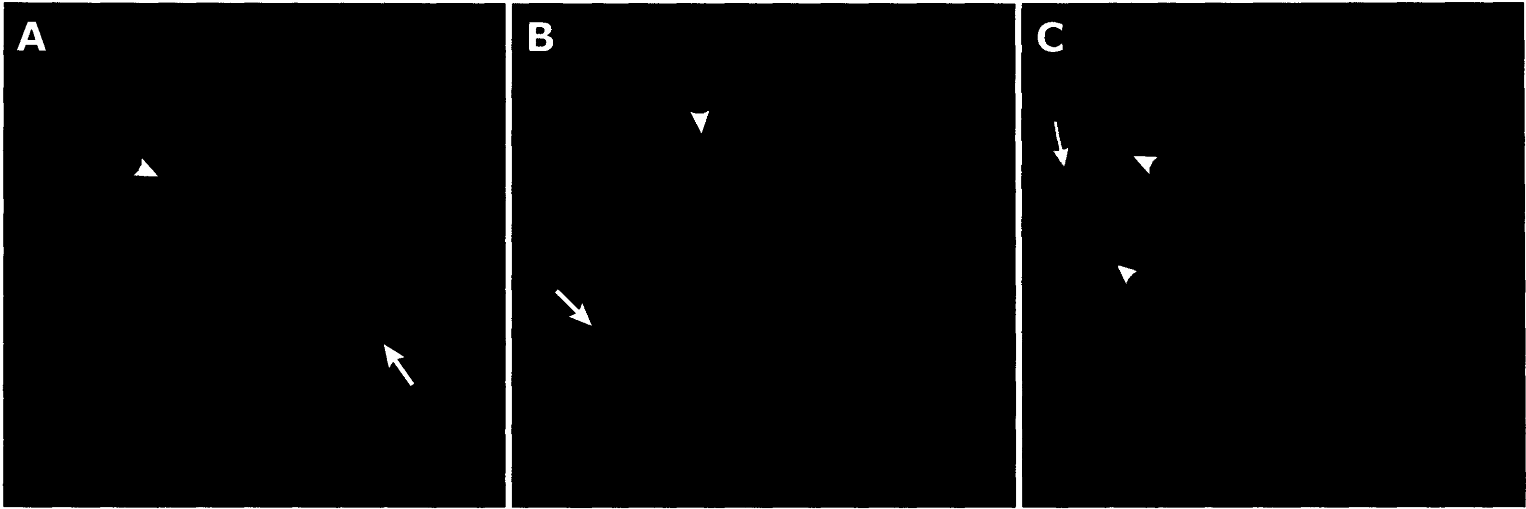
To confirm that the euchromatic *stellate* locus is fully replicated in salivary glands, we cytologically examined the locus in salivary gland chromosome squashes. In these polytene chromosomes, the width of the chromosome at a particular locus reflects the level of polytenization of the locus. To do this, we created fluorescently labeled FISH probes to the *stellate* sequence and to the fully replicated *rosy* locus and hybridized the probes to squashed chromosomes from male larvae (Figure 1). By this method we confirmed that *rosy* and the euchromatic *stellate* locus were replicated to similar degrees (compare *rosy* band, white arrowhead, and *stellate* band, white arrow, in Figure 1A and 1B). Additionally in some squashes we were able to localize the other previously described *stellate* loci in the heterochromatin of the *X* (yellow arrow, Figure 1C) and on the *Y* chromosome (yellow arrowhead, Figure 1C). These

Table 2: The euchromatic *stellate* locus is fully represented in follicle cells and salivary glands

Genomic DNA Tissue	Relative representation determined with Primer Set #1	Relative representation determined with Primer Set #2	Relative representation determined with Primer Set #3
Stage 13 Egg Chambers	N/A	1.72 ± 0.21	1.25 ± 0.15
Salivary Glands from Female Larvae	1.00± 0.11	1.40 ± 0.14	N/A
Salivary Glands from Male Larvae	0.84 ± 0.07	0.75 ± 0.06	N/A

Figure 1: Euchromatic *stellate* locus is fully replicated in salivary gland chromosomes

(A,B) Cytological examination of chromosome width at a fully replicated control locus, *rosy* (white arrowhead), and the *stellate* locus (white arrow), demonstrates that euchromatic *stellate* is replicated in salivary glands. (C) Other *stellate* loci are visible in this particular squash, including the heterochromatin of the *X* chromosome (yellow arrow) and on the *Y* chromosome (yellow arrowhead). Fluorescent probes to *rosy* and *stellate* were generated from cDNAs GH08847 and GM04658 respectively, using Molecular Probes ARES™ Alexa Fluor® DNA Labeling Kits (Eugene, OR). Salivary glands were dissected from male larvae, fixed and squashed as described in Chapter 3. Pretreatment and hybridization of slides was conducted as described in Zhang and Spradling 1994. Slides were stained with DAPI, mounted in Vectashield and imaging was performed using a Zeiss Axiophot microscope and Spot CCD camera and imaging software.


A
B
C

results confirm the real-time PCR data; the euchromatic *stellate* region is fully replicated in salivary gland chromosomes.

Discussion

Tissue-specific differential replication has been described and studied for amplified regions, such as the chorion genes in Drosophila follicle cells, but little is known about regions that are underreplicated. By microarray and real-time PCR analysis, we demonstrate that *stellate* is underrepresented in the follicle cells and fully represented in salivary glands. The differences in representation in these experiments likely reflect differential replication at *stellate* loci in these different tissues. The identification of *stellate* as repeats within multiple loci allowed us to examine the replication properties of these repeats in two contexts, euchromatin and heterochromatin. In the salivary glands, the euchromatic *stellate* locus is fully represented by real-time PCR and by cytological analysis, and previous experiments have demonstrated that the heterochromatic *stellate* is underreplicated in salivary gland polytene chromosomes (Shevelyvov 1992). In follicle cells, the euchromatic *stellate* locus is fully represented, as determined by real-time PCR. Therefore, we hypothesize that the differential DNA replication in follicle cells occurs at the *stellate* repeats found in heterochromatin and that this underreplication may be more severe than that seen in the salivary glands.

Underreplication can occur by several, likely related, means. In some cases, S phase may be truncated such that late replicating sequences, which often correlate with repetitive DNA, are underrepresented, as seen in the late stage nurse cells and the larval specific tissues (Hammond and Laird 1985a, Hammond and Laird 1985b, Lilly and Spradling 1996, Dej and Spradling 1999). In some examples, underreplication appears to be an active process, and this is

particularly apparent in the large polytene chromosomes of Drosophila salivary glands. A mutation in the *SuUR* gene (Suppression of UnderReplication) allows full polytenization of underreplicated regions along the euchromatic chromosome arms, known as intercalary heterochromatin (IH), and localization of the SuUR protein to these sites on wild-type chromosomes suggests that SuUR directly affects their replication (Belyaeva *et al.* 1998, Makunin *et al.* 2002, Zhimulev *et al.* 2003). The *SuUR* mutant adults are normal with respect to morphology, viability and fertility (Belyaeva *et al.* 1998). SuUR can affect replication in oogenesis, however, as overexpression of *SuUR* in follicle cells suppresses amplification of the 66D chorion gene cluster and leads to eggs that lack chorions (Volkova *et al.* 2003). Although a role for *SuUR* in underreplication in follicle cells remains to be determined, it seems possible that SuUR may play a role in the underreplication of heterochromatic *stellate* repeats.

Why does a cell go to so much trouble to block replication of certain regions? For the *stellate* locus, the answer to this question is likely already known for one tissue. Initial studies of *stellate* began with a unique phenotype: males lacking a *Y*-chromosome (*XO* males) showed the presence of proteinaceous crystals in their primary spermatocytes (Hardy 1984). The formation of these crystals was shown to be a direct consequence of overexpression of *stellate*, and *Su(Ste)* on the *Y* chromosome appears to silence the *stellate* loci (Hardy 1984, Livak 1984, Aravin *et al.* 2001). It seems quite likely, therefore, that underreplication of *stellate* loci may be an additional mechanism to ensure the silencing of *stellate*. It is unclear, however, if overexpression of *stellate* affects tissues other than spermatocytes. The biological relevance for underreplicating other sequences may be less obvious. Both the tissues discussed here, the follicle cells and the salivary glands, are highly metabolic and specialized cells. The follicle cells produce proteins and enzymes that form the chorion, yolk and vitelline envelope of the egg; the salivary glands

produce large amounts of secretory enzymes to maximize the larvae's intake of nutrients. As both these tissue types degenerate after serving their purpose, it may not be necessary for the cell to maintain a full complement of the polyploid genome in these tissues. This may save the cell some energy and allow the rapid developmental pace to continue. Additionally, underreplication may assist in downregulating gene expression in regions. The *Bithorax Complex* (*BX-C*), a group of homeotic genes required in embryogenesis, is underreplicated in salivary gland polytene chromosomes and these genes are not expressed in the salivary gland (Moshkin *et al.* 2001). The underreplication of this locus has been demonstrated to be dependent upon *SuUR*, as it is fully polytenized in *SuUR* mutants (Moshkin *et al.* 2001). In the fully polytenized state, however, the *BX-C* region is still late replicating and able to bind the repressive POLYCOMB protein, so any impact of underreplication on silencing remains to be demonstrated (Moshkin *et al.* 2001). While the mechanism and relevance of underreplicating the heterochromatic *stellate* remain elusive, further studies of underreplication during development will likely prove to be quite interesting and provide a greater understanding of how replication can be altered for specific goals.

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Appendix Three

Replication of Heterochromatin: A Model for Epigenetic Inheritance

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Abstract

Heterochromatin is composed of tightly condensed chromatin in which the histones are deacetylated and methylated, and specific non-histone proteins are bound. Additionally, in mammals, the DNA within heterochromatin is methylated. As the heterochromatic state is stably inherited, replication of heterochromatin requires not only duplication of the DNA but also a reinstallment of the appropriate protein and DNA modifications. Thus replication of heterochromatin provides a framework for understanding mechanisms of epigenetic inheritance. Recent studies have identified roles for replication factors in reinstating heterochromatin, particularly functions for ORC, PCNA, and CAF1 in recruiting the heterochromatin binding protein HP1, a histone methyltransferase, a DNA methyltransferase, and a chromatin remodeling complex. Potential mechanistic links between these factors are discussed. In some cells, replication of the heterochromatin is blocked, and in Drosophila this inhibition is mediated by a chromatin binding protein SuUR.

Overview

In recent years the crucial role of epigenetics has become increasingly apparent, as many human diseases have been linked to epigenetic defects (for review see Jiang et al. 2004). Gene expression is controlled not only by DNA sequence elements but also by the configuration of proteins in the chromatin and by methylation of the DNA itself. In mammals some genes are imprinted such that expression of the paternal or maternal alleles are blocked, in mammalian females one X chromosome is inactivated for expression, and in a variety of organisms genes in proximity to heterochromatin are repressed. The epigenetic as well as genetic states are inherited, making it important to decipher mechanisms for the establishment and maintenance of the epigenetic state. In this review we discuss recent advances in our understanding of replication of heterochromatin, an extreme epigenetic state that serves as an excellent model for elucidating how chromatin structure and DNA methylation are regulated.

Heterochromatin was first recognized cytologically as regions of the genome that were highly condensed throughout the cell cycle, as distinguished from euchromatin in which condensation was visible only during mitosis (for reviews see Dillon and Festenstein 2002, Henikoff 2000). Heterochromatin plays critical roles in chromosome structure and transmission, and most eukaryotic centromeres are surrounded by blocks of heterochromatin. In Drosophila, heterochromatin comprises up to 30% of the chromosome, and in fission yeast it is clearly established that centric heterochromatin blocks transcription across the centromere that could cripple its function in chromosome segregation (Ekwall et al. 1997). Similarly, the heterochromatic nature of telomeres is important for their function. Molecularly, heterochromatin consists mainly of highly repetitive satellite DNA and moderately repetitive elements like transposable elements (Dillon and Festenstein 2002, Henikoff 2000). Transposable

elements tend to accumulate in heterochromatin where reduced expression may limit their mobility and restrict accumulation (reviewed in Henikoff 2000, Schramke and Allshire 2004). There is also a sparse distribution of single copy genes in heterochromatin, and gene expression generally is repressed. Although the expression of most genes is repressed by heterochromatin, there are essential genes such as the Drosophila *light* gene that can be expressed only in a heterochromatic environment (Wakimoto and Hearn 1990).

The ability of heterochromatin to repress gene expression is exemplified by situations in which the expression of genes normally located in euchromatic regions is reduced or abolished if they are translocated next to heterochromatin (Dillon and Festenstein 2002, Henikoff 2000). This transcriptional repression is seen in cases in which chromosomal rearrangements, such as inversions, have placed previously expressed euchromatic genes adjacent to heterochromatin. Even on normally configured chromosomes, the expression of euchromatic genes adjacent to the heterochromatin is repressed. This occurs adjacent to centromeres, and in yeast repression also occurs next to the silenced mating type loci (Dillon and Festenstein 2002, Henikoff 2000). This type of positional repression is often unstable, with the genes being expressed in some clonal cells but not others, making it clearly an epigenetic phenomenon that has been termed Position Effect Variegation (PEV). Investigation of heterochromatin provides several advantages for understanding the mechanism by which it is replicated and how the heterochromatic state is epigenetically heritable. PEV serves as a powerful phenotype for genetic studies in yeasts and Drosophila, and key proteins controlling chromatin have been identified by the ability of mutations in the genes that encode them either to suppress or enhance PEV (reviewed in Schotta et al. 2003a). In particular, roles in promoting heterochromatin were confirmed for a conserved heterochromatin binding protein, HP1, and a histone methyl-transferase enzyme, SU(VAR)3-9,

by their identification as suppressors of PEV in Drosophila (Schotta et al. 2003b). In addition to genetics, the size of heterochromatic blocks and stability of heterochromatin permit biochemical studies and cytological visualization both of chromatin-bound proteins and chromatin modifications (Dillon and Festenstein 2002, Maison and Almouzni 2004).

There are several challenges to faithfully duplicating heterochromatin in each cell cycle. The first concerns the replication of the DNA itself, given the highly condensed state of the chromatin. Most heterochromatin is replicated late in S phase, but the significance of this is unknown (reviewed in Gilbert 2002). It is possible that it takes longer for replication origins to fire within the heterochromatin, but it is also possible that the timing of replication is actively regulated and that limiting heterochromatic replication until late in S phase facilitates reassembly of the epigenetic state of the heterochromatin. In polytene and polyploid cells, the heterochromatin frequently is not replicated, such that these regions are underrepresented (Rudkin 1969, Gall et al. 1971, Leach et al. 2000). This may be a mechanism to optimize the metabolic state of polytene or polyploid cells by dispensing with gene poor regions of the genome. It is important to emphasize that although DNA replication necessitates a mechanism to maintain heterochromatin, it has been shown in yeast that it is possible to establish heterochromatin without DNA replication (Kirchmaier and Rine 2001, Li et al. 2001). The second aspect is how the chromatin is assembled into a heterochromatic state with the appropriate positioning of the nucleosomes, histone modifications, and binding of heterochromatin proteins following replication. The third aspect involves the methylation of DNA sequences.

Because our focus is on the replication of heterochromatin, much of the recent literature on the increasing list of regulators required for the maintenance of heterochromatin is not

discussed here (see Craig 2005 for review). Factors needed to maintain heterochromatin are likely to act both during and following S phase, but in most examples the time of action with respect to replication has not been established. This is true for the exciting finding of the role of noncoding RNAs in heterochromatin. Noncoding RNAs play crucial roles in the establishment of heterochromatin to inactivate the mammalian X chromosome, and the RNAi pathway is important for H3K9 methylation and HP1 localization in the centric heterochromatin. To date, these RNA-mediated mechanisms have not been shown to participate in the replication of heterochromatin, and thus we refer readers to several recent reviews for a full discussion of this topic (Lippman and Martienssen 2004, Schramke and Allshire 2004, Matzke and Birchler 2005). There are several reviews on the use of histone protein variants, another topic not covered in this review (Kamakaka and Biggins 2005, Ahmad and Henikoff 2002).

Here we address these aspects regarding the propagation of heterochromatin. In particular, we discuss: 1) the role of replication proteins in the replication of heterochromatic DNA and the recruitment of heterochromatin binding proteins; 2) a Drosophila protein, SuUR, that specifically controls replication of the heterochromatin; 3) the chromatin assembly factors, specifically CAF1, that act to maintain heterochromatin following DNA replication; 4) the link between DNA replication and DNA methylation; and 5) evidence for roles of chromatin remodeling complexes in the replication of heterochromatin (Table 1).

The replication machinery and replication of heterochromatin

The role of the Origin Recognition Complex (ORC) in heterochromatin

One key concept to emerge from the analysis of the replication machinery and heterochromatin is that replication proteins can act both to replicate the DNA and to recruit the

Table 1: FACTORS IMPLICATED IN TRANSMISSION OF HETEROCHROMATIN

FACTOR	RELEVANT INTERACTIONS	REFERENCES FOR INTERACTIONS
General Replication Proteins		
ORC	HP1, HBO1	Pak et al. 1997, Huang et al. 1998, Iizuka and Stillman 1999, Lidonnici et al. 2004, Prasanth et al. 2004
PCNA	CAF1, DNMT1, MBD1, SETDB1	Chuang et al. 1997, Shibahara and Stillman 1999, Zhang et al. 2000, Sarraf and Stancheva 2004
POLε, δ	PCNA	reviewed in Maga and Hubscher 2003
POLα	PCNA, SWI6	Ahmed et al. 2001, Nakayama et al. 2001
HOAP	ORC, HP1	Shareef et al. 2001, Badugu et al. 2003
Heterochromatin-specific Replication Factors		
SU(UR)	-	-
Chromatin-assembly Proteins		
CAF1	PCNA, HP1, MBD1	Murzina et al. 1999, Shibahara and Stillman 1999, Zhang et al. 2000, Reese et al. 2003
DNA/histone modification enzymes		
DNMT1	HP1, SUV39h1	Fuks et al. 2003a
HBO1	ORC	Iizuka and Stillman 1999
SETDB1	PCNA, CAF1, MBD1	Sarraf and Stancheva 2004
DNA/histone modification binding proteins		
MBD1	PCNA, CAF1, SETDB1	Reese et al. 2003, Sarraf and Stancheva 2004
MeCP2	H3K9 methyltransferase	Fuks et al. 2003b
HP1	ORC, HOAP, CAF1, DNMT1	Pak et al. 1997, Huang et al. 1998, Murzina et al. 1999, Shareef et al. 2001, Badugu et al. 2003, Fuks et al. 2003a, Lidonnici et al. 2004, Prasanth et al. 2004
Chromatin-remodelling complexes		
ACF-ISWI	-	-
WSTF-ISWI	PCNA	Poot et al. 2004

heterochromatin binding proteins that epigenetically confer the heterochromatic state. This is most clear for the origin recognition complex (ORC), an evolutionarily conserved complex consisting of six subunits (for reviews see Bell and Dutta 2002, Leatherwood and Vas 2003). Studies in many organisms have demonstrated that ORC is a link between the processes of DNA replication and heterochromatin maintenance. ORC was identified by the role of the complex in the initiation of DNA replication. In budding yeast, mutations in the subunits of the ORC also disrupt silencing of the mating type loci. Surprisingly, studies have demonstrated that the replication and silencing functions of ORC are genetically separable (Bell et al. 1995, Dillin and Rine 1997). Bell et al. found that the N-terminus of ORC1 in *S. cerevisiae* is specifically required for mating-type repression, but is dispensable for normal growth and, therefore, DNA replication. Dillin and Rine isolated mutants of *orc5* specifically defective in either DNA replication (as determined by 2D gel origin mapping experiments and plasmid loss assays) or mating-type silencing. These mutations were able to complement each other, suggesting that different domains of the protein acted in the two processes, and furthering a model where ORC has two domains that confer separate functions. This separate role for ORC in silencing involves the interaction of ORC with Sir1, the functional homolog of the heterochromatin binding protein HP1 in *S. cerevisiae*, and the recruitment of Sir proteins to loci via ORC's interaction with Sir1 at a small subset of ORC binding sites (Triolo and Sternglanz 1996).

Although the relationship between ORC and heterochromatin in higher eukaryotes is less clear, a role for ORC both in replication and in recruitment of heterochromatin proteins has been described. Analyses of ORC localization during the cell cycle provide evidence that ORC is likely necessary for heterochromatin replication in mammalian cells. Prasanth et al. have recently documented cell-cycle changes in ORC2 localization in MCF7 cells; ORC2 generally

localizes with heterochromatic foci, marked by the presence of HP1α and β, during G1 and early S phase. However, as the cells progress further into S phase, ORC2 localizes to punctate foci that are characteristic of late-replicating pericentric regions (Prasanth et al. 2004). Lidonnici et al. examined localization of tagged, ectopic human ORC1 in mammalian cells and also noted that ORC1 preferentially localizes to the pericentric heterochromatin foci that colocalize with HP1 (Lidonnici et al. 2004). The localization of ORC to heterochromatic foci when they are likely to be replicating in late S phase suggests that ORC is involved in the replication of heterochromatin in higher eukaryotes.

In addition, the phenotype of Drosophila *orc2* mutants indicates an important role for ORC in the proper timing of replication. Generally, euchromatic regions of the genome are replicated prior to heterochromatic regions in S phase. In *orc2* mutants, however, replication of some euchromatic regions is delayed and these regions are inappropriately replicated after heterochromatic regions (Loupart 2000). The authors suggest this intriguing possibility for the phenotype: ORC may have a higher affinity for heterochromatin, and the limited, functional ORC complexes in this mutant are recruited more efficiently to heterochromatin and enable replication of these regions. The euchromatic regions then are less likely to recruit ORC and display delayed replication initiation. Euchromatic and heterochromatic regions may, therefore, require ORC for replication and for coordination of their replication timing.

A role for ORC in the formation of heterochromatin is supported by physical binding between ORC and the HP1 protein. This interaction was first demonstrated in Drosophila, and further studies in human cell culture suggest that this interaction is evolutionarily conserved. Drosophila ORC2 localizes to heterochromatin, particularly centric heterochromatin, in syncytial and cellularized embryos and co-localizes with HP1 on mitotic chromosome spreads (Pak et al.

1997). Immunoprecipitation experiments from Drosophila embryo extracts with ORC, particularly ORC1 and HP1, reveal a physical interaction with the heterochromatin marker and the ORC complex (Pak et al. 1997, Huang et al. 1998). This direct interaction has also been demonstrated in *Xenopus* (Pak et al. 1997) and in mammalian cell lines (Lidonnici et al. 2004, Prasanth et al. 2004). Lidonnici et al. also used fluorescence resonant energy transfer (FRET) to demonstrate an *in vivo* interaction between ORC1 and HP1α.

A second protein, HOAP (HP1/ORC-associated protein), present in heterochromatin not only interacts with ORC but also recruits HP1/ORC to heterochromatin. HOAP was identified based on its ability to co-purify with a protein complex in early Drosophila embryos (Shareef et al. 2001, Badugu et al. 2003). HOAP copurifies with ORC subunits and HP1α and has also been shown to colocalize with ORC and HP1α in cellularized Drosophila embryos and larval brain squashes (Shareef et al. 2001). By incubating Drosophila salivary glands with a competitor peptide, the PETEMNE sequence in HOAP that binds HP1α, Badugu et al. revealed that interaction between HOAP and HP1 is required for the proper localization of HP1, whereas HOAP localization is not disrupted (Badugu et al. 2003). Consistent with a role for the HOAP/ORC complex in recruiting HP1 to heterochromatin and promoting heterochromatin, a mutant in *hoap* suppresses PEV (Shareef et al. 2001). This effect on PEV provides confirmation that HOAP has a functional role in heterochromatin architecture *in vivo*.

Other experiments also suggest that ORC promotes the assembly of heterochromatin in metazoans and that, like in *S. cerevisiae*, ORC's main function in heterochromatin may be to recruit HP1. Like mutants in *hoap*, Drosophila *orc2* mutants also suppress PEV (Pak et al. 1997) and HP1 localization is disrupted in *orc2* mutants (Huang et al. 1998). In mammalian cells, depletion of ORC2 by siRNA resulted in a disruption of HP1α and HP1β foci, leading to a

diffuse nuclear pattern of HP1 localization. Importantly, the heterochromatic HP1 binding site was not disrupted in these cells. In heterochromatin, lysine 9 of histone H3 (H3K9) is often methylated and this site is bound by HP1 (Bannister et al. 2001, Lachner et al. 2001). In cells depleted of ORC2, trimethylated lysine 9 residues on histone H3 were present, so the change in HP1 localization is not a secondary consequence of HP1 lacking nucleosomal sites to bind (Prasanth et al. 2004). These results imply that ORC is necessary to recruit HP1 and that this interaction promotes the formation of heterochromatin.

Given that ORC is likely involved in the replication of heterochromatin, a model can be envisioned in which ORC localizes to heterochromatic sites for DNA replication, recruiting HP1 to those sites to reestablish the heterochromatic state after passage of the replication fork. This simplistic model remains to be demonstrated, and it will be important to determine how ORC recruits HP1 only to heterochromatin. Additionally, it provokes a key question: how do two seemingly opposing forces act on DNA in the same temporal window? Theoretically, replication initiation and heterochromatin packaging are opposing forces, as Leatherwood and Vas proposed in a recent review, because DNA replication requires an open chromatin configuration whereas heterochromatin is by nature in a closed configuration (Leatherwood and Vas 2003). The answer to this question may lie in refining our understanding of the timing of both of these activities in heterochromatin and of other players in these interactions; the HP1-ORC interaction may promote both activities but be regulated to provide different functions at times prior to or following DNA replication. One example of the role other interactions may play in these functions concerns how ORC may promote the opening of heterochromatin for replication. It is likely that disassembly of heterochromatin, to allow DNA replication initiators to bind and replication to proceed, is a slow and energy consuming process and that the cell

would develop mechanisms to promote this process. Interestingly, human ORC1 also physically interacts with HBO1 (histone acetyltransferase binding to ORC), a member of a histone H3 and H4 acetyltransferase complex (Iizuka and Stillman 1999). Acetylation of histones may activate replication by promoting open chromatin states, especially in heterochromatin. Other interactions by HP1-ORC, therefore, may define the activity of this complex during specific periods in S phase.

Another conceivable mechanism to promote DNA replication of heterochromatin would be to facilitate the recruitment of the replication machinery and ORC to these sites. A tantalizing idea has been proposed by Leatherwood and Vas: prior to replication, could the HP1-ORC interaction also function to recruit ORC to heterochromatic sites bound by HP1 (Leatherwood and Vas 2003)? Currently, the answer to this question is complicated, as results are contradictory. First, ORC appears to bind heterochromatic sites even when HP1 has been removed, suggesting that HP1 is not required for ORC localization to heterochromatin. Lidonnici et al. observed that disruption of HP1 localization by treatment with either trichostatin A (TSA), an inhibitor of a subset of known histone-deacetylases, or RNAse A does not disrupt ORC1 localization (Lidonnici et al. 2004). The idea that HP1 could recruit ORC, however, is supported by analysis of HP1 and ORC colocalization through the cell cycle. Lidonnici et al. observed that in synchronized mammalian cells in early G1, HP1β was found at heterochromatic loci, but tagged ORC1 was not. As the cells progressed through the cell cycle, colocalization of ORC1and HP1β at heterochromatic loci increased to 35% in mid G1 and 65% in late G1. These observations might be expected, since HP1 binds to heterochromatin outside of S phase, and they demonstrate the potential for ORC recruitment by HP1. The data of Lidonnici et al. also suggest that the association of ORC1 with heterochromatin requires HP1, as an ORC1 mutant lacking the

HP1 binding domain did not localize to heterochromatin, a result contradictory to a previous experiment (Lidonnici et al. 2004). To explain these two results, the authors propose that HP1 may be required to recruit ORC1 initially to heterochromatin, but isn't required for the stable association of ORC1 with heterochromatin. It seems, therefore, that the HP1-ORC1 interaction could have two functions during the cell cycle: to recruit ORC1 to sites of heterochromatin in G1 and to recruit HP1 to sites of heterochromatic replication in late S phase.

Roles of other replication proteins in heterochromatin

The majority of eukaryotic DNA replication is catalyzed by polymerases α, δ, and ε. Polymerase α associates with primase to synthesize and extend RNA primers in initiation and lagging strand synthesis, whereas synthesis of the leading and lagging strand at the replication fork is achieved by either polymerase δ or polymerase ε. Does the replication of heterochromatin require different replication machinery? Are the mechanics for replicating heterochromatin similar to euchromatin? Two studies in *S. pombe* link DNA polymerase α to the establishment of heterochromatin. First, mutations in *pol α* suppress PEV at the mating-type loci, centromeres and telomeres (Ahmed et al. 2001, Nakayama et al. 2001). These phenotypes are likely to reflect a direct role for *pol a* in heterochromatin because this polymerase has been shown to interact directly with Swi6, a protein known to be important in the silencing of mating-type loci and the pombe HP1 homolog, by both affinity column and co-immunoprecipitation. Additionally, mutations in *pol α* affect localization of Swi6 to mating-type loci and to heterochromatic loci in the nucleus (Ahmed et al. 2001, Nakayama et al. 2001). Is the requirement for a DNA polymerase for heterochromatin linked to replication? Like ORC, the interaction between Pol α and Swi6 is complicated by the ability to separate the replication and

silencing functions. The mutation used for the studies by Nakayama et al. is not located in a conserved region required for polymerase activity, nor does it increase UV sensitivity as expected if the catalytic activity were reduced. However, the *pol α* mutations studied by Ahmed et al. do map to regions conserved in α polymerase and, in some cases, to all DNA polymerases. These observations suggest a model where Pol α, at replication forks, is able to recruit and maintain Swi6 to reestablish heterochromatin following replication.

In contrast, genetic studies in *S. cerevisiae* have raised the possibility that replication proteins participate in the formation of heterochromatin independently of and in addition to their actions in DNA replication. It is important to note that establishment of heterochromatin and maintenance of the epigenetic state at DNA replication appear to be distinct processes in *S. cerevisiae*. It has been demonstrated that establishment of silencing can occur independently of DNA replication, in particular, independently of passage of the replication fork (Kirchmaier and Rine 2001, Li et al. 2001). Nevertheless, mutations in many replication factors, including proliferating cell nuclear antigen (PCNA), replication factor C (RF-C), the replication initiation factor Cdc45, polymerase α (Pol α), and polymerase ε (Pol ε) affect silencing either by disrupting it or by suppressing silencing defects (Huang 2002). This could be explained by many of the factors required for euchromatic replication being required for replication of heterochromatic regions. Given that silencing can be established independently of passage of the replication fork, definitive tests of whether replication factors have a replication-independent role in heterochromatin will require the recovery of mutants that affect silencing but not replication.

In human cells, DNA polymerase ε may assist in the replication of heterochromatin. In addition to its role in chromosomal DNA replication, POL ε is involved in DNA repair and the S-phase DNA damage checkpoint (Hubscher et al. 2002). Analysis of the subcellular

localization of the POL ε subunit p261 (the catalytic subunit) in human fibroblasts revealed that PCNA, BrdU and POL ε colocalize in late S phase specifically to the large foci that are characteristic of heterochromatic DNA replication (Fuss and Linn 2002). Interestingly, in early S phase, PCNA and p261 do not colocalize, but are adjacent. The authors suggest that this may indicate a distinct function for POL ε in replication that is not associated with the growing replication fork, perhaps DNA repair. The specific colocalization in late S phase could mean that POL ε synthesizes DNA only at late-replicating heterochromatic loci or may be specifically suited for replication at these foci. This intriguing possibility of a difference in POL ε's participation in euchromatic and heterochromatic replication could reflect the need for a different replication machinery to process rapidly through the complicated "topology" of heterochromatin (Fuss and Linn 2002).

Studies of PCNA suggest a direct link between DNA replication and epigenetic inheritance. PCNA is a member of the DNA sliding clamp family that increases DNA polymerase processivity (for review see Majka and Burgers 2004). In addition to its role in DNA replication, PCNA interacts with a wide variety of cell factors and may be the major scaffold for recruiting and directing chromatin enzymes (for review see Maga and Hubscher 2003). In *S. cerevisiae*, mutations in the *pcna* gene reduce repression of genes near the telomere and at mating-type loci, linking PCNA to silencing (Zhang et al. 2000). PCNA mutants in Drosophila suppress PEV, indicating that PCNA participates in chromatin assembly in higher eukaryotes (Henderson et al. 1994). Again, similar to the replication factors discussed above, it is difficult to determine whether PCNA's activity in DNA replication is required for the establishment and/or whether it serves to ensure that heterochromatin is preserved after DNA replication. PCNA does localize to mammalian heterochromatic loci where it interacts with

CAF1, a chromatin-assembly factor, and chromatin-remodeling enzymes, both discussed below (Figure 1).

These studies demonstrate a requirement for ORC and the DNA replication machinery in heterochromatin but illustrate the complexities in deciphering the exact role of DNA replication factors, particularly whether they play roles independent of their replication activities in the establishment of epigenetic state. If indeed the replication factors have roles in maintaining heterochromatin that are independent of DNA synthesis there must be a means by which these actions are restricted to the heterochromatin. The ability to separate genetically the activities of ORC in replication and silencing demonstrates that it has independent activities, and such genetic analyses on other replication factors is likely to be informative. The question of whether replication of DNA in heterochromatin requires distinct functions from the replication of DNA in euchromatin also merits further investigation.

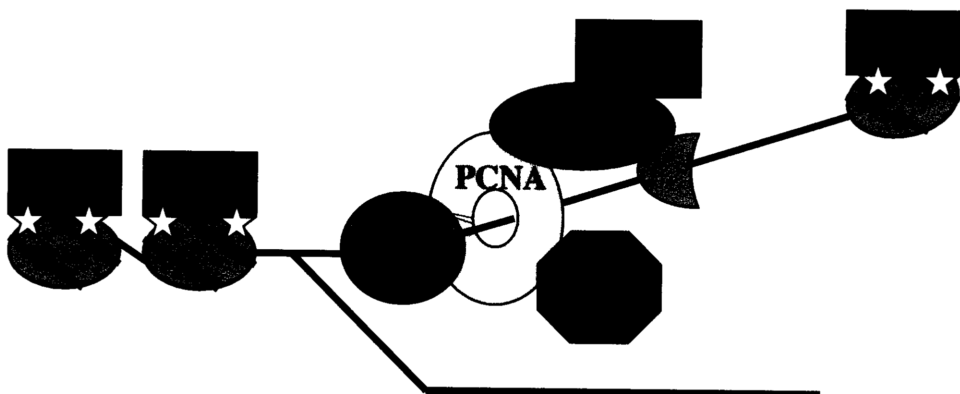
A specialized trans regulator of heterochromatin replication

The Drosophila *SuUR* (suppressor of underreplication) gene encodes an intriguing chromosomal protein that specifically affects the replication of heterochromatin (Belyaeva et al. 1998, Makunin et al. 2002). It is the sole protein identified to date that is uniquely responsible for the replicative properties of heterochromatin. Understanding the role of the SuUR protein requires an appreciation of the parameters of heterochromatin replication during a variant cell cycle, the endo cycle, that gives rise to polyploid or polytene cells (Edgar and Orr-Weaver 2001). In the endo cycle there are repeated rounds of S phase, punctuated by gap phases during which gene expression and cell growth occur, but mitosis does not take place. Endo cycles produce either polyploid or polytene chromosomes which differ in the extent to which the replicated

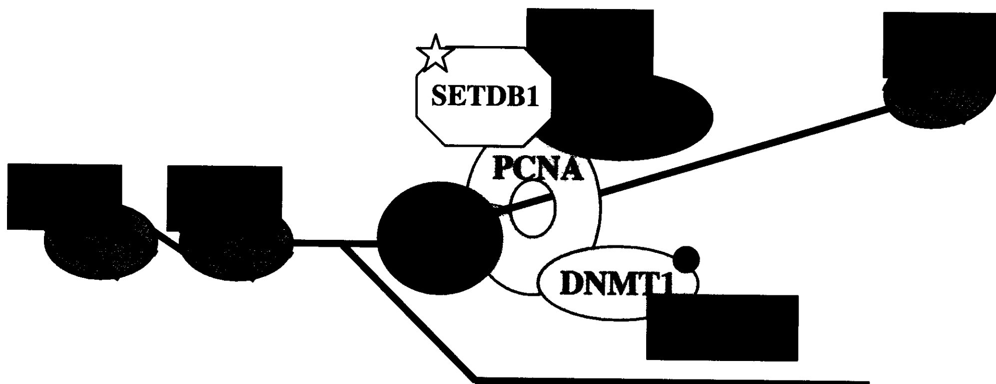
Figure 1: Model of protein-protein interactions at the replication fork that are relevant to the heterochromatic state.

Many characteristics of heterochromatin, like histone modifications, nucleosome positioning and bound proteins, are likely displaced as the replication fork passes through the DNA sequences. The depicted factors are speculated to assist in the reestablishment of the heterochromatic state after the DNA has been replicated. Their interaction with PCNA suggests that they may travel with the progressing replication fork. (A) PCNA acts as a scaffold for nucleosome processes, bringing the chromatin-assembly factor, CAF1, and the chromatin-remodeling factor, ISWI, to nascent DNA. CAF1 deposits histone H3/H4 tetramers on newly replicated DNA, which are joined by two H2A/H2B dimers to form the full nucleosome. ISWI alters the spacing of these nucleosomes on the DNA, forming a regularly spaced array. Additionally, CAF1 binds the heterochromatin protein, HP1, likely keeping the local concentration of HP1 high so that it can quickly bind modified histone H3. (B) DNA methylation and DNA methyl binding proteins must also be reestablished after progression of the replication fork. Again, PCNA is speculated to act as a scaffold, recruiting the DNA methyltransferase, DNMT1, which in turn recruits the MBD2a-3 methyl binding proteins. PCNA and CAF1 also bind MBD1, another methyl binding protein, and SETDB1, a histone H3 methyl transferase. This coordination between DNA and histone modification enzymes is speculated to rapidly promote heterochromatin formation after DNA replication.

A. Nucleosome Modifications



B. DNA Methylation



sister chromatid copies are held in physical register. Polyploid and polytene cells are found throughout the plant and animal kingdoms, most commonly associated with cell types that are highly metabolically active.

Consistent with the implementation of the endo cycle as a means to produce a "factory" cell, in many endo cycling cells S phase is cut short and heterochromatin is not replicated (Edgar and Orr-Weaver 2001). This is evident in Drosophila polytene cells, in particular the larval salivary glands. The approximately 1000 copies of each chromosome pair are aligned to produce a distinctive banding pattern. This banding pattern, however, is present only in the euchromatin; the 20-30% of each chromosome arm adjacent to the centromere that is composed of heterochromatin is not visible in salivary gland chromosomes nor is the heterochromatic *Y* chromosome. Quantitation of DNA doublings in the endo cycle indicates that approximately 20% of the genome is not replicated in each endo cycle S phase (Rudkin 1969, Smith and Orr-Weaver 1991). In addition to the centric heterochromatin and *Y* chromosome, there are regions throughout the euchromatin with constrictions and fragile sites, known as intercalary heterochromatin, that also are underreplicated (Zhimulev and Belyaeva 2003).

Cell cycle regulators controlling the G1-S transition and transcription of genes necessary for S phase have been found to affect underreplication of heterochromatin in the endo cycle. Decreased function of cyclin E or of either subunit of the E2F1 transcription factor results in increased replication of centric heterochromatin in the polyploid nurse cells of the adult ovary (Lilly and Spradling 1996, Royzman et al. 2002). It has been proposed that in the endo cycle S phase is truncated such that late replicating heterochromatin is not copied (Lilly and Spradling 1996). The *cyclin E* and *dE2F1* mutant phenotypes are explained as the consequence of a slowed S phase resulting in the replication of late-replicating heterochromatin. By pulse labeling

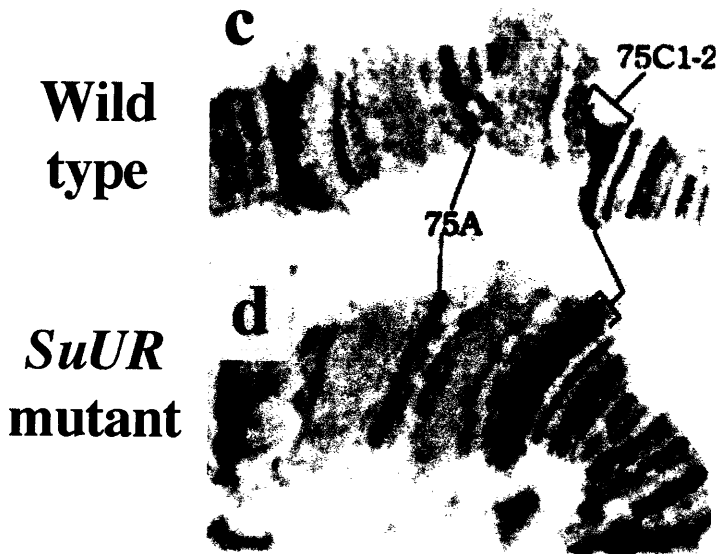
replicating salivary gland DNA and then cytologically examining the pattern of nucleotide incorporation on polytene chromosomes, it was confirmed that the regions adjacent to the centromeres and the constrictions replicate late in the endo cycle S phase (Zhimulev et al. 2003a). Not all late replicating regions are underreplicated, however; only 60 out of 156 late replicating sites correspond to weak constriction points on the polytene chromosomes (Zhimulev et al. 2003a).

The *SuUR* mutant arose spontaneously and was identified because it eliminated the constrictions at intercalary heterochromatin and restored replication to parts of the centric heterochromatin in salivary glands (Figure 2). Quantitation of DNA copy number for several of these intervals demonstrated that, in *SuUR* mutants, the regions are less underreplicated, i.e. they have increased DNA copy number (Belyaeva et al. 1998). Pulse labeling of mutant cells indicates that normally late replicating regions are replicated earlier with the bulk of euchromatic DNA (Zhimulev et al. 2003a). There is suppression of PEV at several loci in the *SuUR* mutant, implying that the wild-type protein is needed for heterochromatin structure (Belyaeva et al. 2003).

The effects of the SuUR protein on heterochromatin structure and replication are dose specific (Figure 2). In the presence of extra copies of the wild-type gene, the number of constrictions and weak points on salivary gland chromosomes increases, and these correspond to late replicating regions (Zhimulev et al. 2003a). Copy number of the DNA decreases at the new sites and is further decreased at the normal constriction points (Zhimulev et al. 2003a, Moshkin et al. 2001). Thus it appears that the SuUR protein leads to underreplication by further delaying the replication of late replicating genes such that they fail to replicate at all during the endo cycle. Increased levels of the protein can dramatically alter polytene chromosome structure,

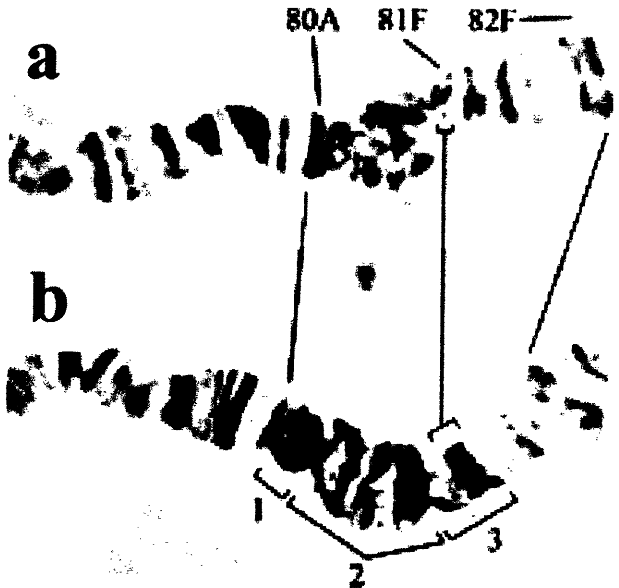
Figure 2: Dosage effects of the *Drosophila SuUR* gene on heterochromatin replication in polytene chromosomes.

(A) In larval salivary gland chromosomes the centric heterochromatin that comprises the proximal 20-30% of each chromosome arm is so severely underreplicated that these segments of the chromosomes are not visible following orcein staining. The region 80 on chromosome 3L and 81 on chromosome 3R are indicated. (B) Mutation of the *SuUR* gene results in replication of the centric heterochromatin such that banded regions become visible, shown here for cytological intervals 80 and 81. (C) In addition to the blocks of heterochromatin flanking the centromeres, underreplication of intercalary heterochromatin can be visualized by missing or thin bands, chromosome constrictions and breaks. These sites also frequently attach ectopically to other chromosome regions. Two sites of intercalary heterochromatin at 75A and 75C1-2 on chromosome 3L are shown. (D) In the *SuUR* mutant, sites of intercalary heterochromatin become more fully replicated. (E) Overexpression of the SuUR protein from extra copies of the gene results in many new sites of intercalary heterochromatin. Two of the sites with pronounced breaks are highlighted by arrows, but there are many regions visible in which the bands are partially missing. Panels A and B are from Belyaeva et al. 1998, panels C and D are from Semeshin et al. 2001, and panel E is from Zhimulev et al. 2003a.



Wild type

SuUR mutant



8 copies *SuUR*+

leading to swellings that resemble DNA puffs (Zhimulev et al. 2003b). Extra copies of the wild-type SuUR gene enhance PEV, also arguing that the protein promotes heterochromatin formation (Belyaeva et al. 2003).

The SuUR gene encodes a protein of 962 amino acids whose N terminus has some similarity to the conserved motifs in the SNF2/SWI2 chromatin remodeling proteins (Makunin et al. 2002). The N terminal half of SuUR is 42% identical to the bromodomain of the Brahma trxG transcriptional activator (Tchurikov et al. 2004). The spontaneous mutation, discussed above, is due to an insertion that leads to loss of the single transcript from the gene, which is normally particularly abundant in females and embryos. As expected from the homology motifs, the SuUR protein binds to chromosomes and is observed at heterochromatin regions of polytene chromosomes (Makunin et al. 2002). It localizes to 113 bands, and 108 are sites of late replication. When overexpressed it localizes to 280 sites. The binding of the protein to affected regions argues that SuUR directly promotes the heterochromatin state and restricts DNA replication. Its mechanism of action remains to be deciphered at a molecular level, particularly whether the primary effect is via heterochromatin structure or via perturbation of the replication machinery. SuUR colocalizes with HP1 at a cytological level, but the relationship between these proteins has not been investigated (Zhimulev and Belyaeva 2003).

Given the dramatic effects of *SuUR* mutants on PEV and underreplication, it is puzzling that the mutant is fully viable and fertile (Belyaeva et al. 1998). Increased levels of protein, however, are deleterious. Continuous overexpression of the protein in the salivary gland results in a small gland, and ubiquitous overexpression results in lethality (Volkova et al. 2003). Overexpression in the follicle cells is capable of repressing replication at specific sites during the amplification of the chorion eggshell genes (Volkova et al. 2003). Thus the organism can

survive without this protein and the resulting increased copy number of heterochromatin regions, but increasing the number of underreplicated domains lead to lethality.

Reestablishment of heterochromatin after DNA replication

As DNA replication requires the ability of the polymerase to contact directly the nucleotide sequence and move processively along the DNA, any proteins bound to the DNA and higher order chromatin would need to be disassembled and then reassembled following the replication fork. Indeed, *in vitro* studies have demonstrated that nucleosomes, the basic unit of chromatin, are disrupted at the replication fork (Gruss et al. 1993). Both euchromatin and heterochromatin, therefore, require factors to recruit and deliver nucleosomes to newly replicated DNA. Although the nucleosome deposition function of these chromatin-assembly enzymes is likely similar in both euchromatin and heterochromatin, it is possible that these enzymes have additional roles in reestablishing heterochromatin after the replication fork. Here we address evidence that chromatin-assembly factor 1 (CAF1) has an additional role in transmission of heterochromatin by recruiting heterochromatin proteins.

Chromatin-assembly factor 1 (CAF1) is a multi-subunit complex that assists in loading newly synthesized H3-H4 tetramers onto chromatin, preferentially following DNA replication and DNA repair (for review, see Ridgway and Almouzni 2000, Mello and Almouzni 2001). As the replication fork progresses, the parental nucleosomes are transiently disrupted into H2A/H2B and H3/H4 tetramers and distributed equally between the two newly formed daughter duplexes. Newly synthesized histones are incorporated with parental histones to form the full nucleosome complex, with two new H2A/H2B dimers binding parental H3/H4 tetramers and vice versa. CAF1 previously has been observed to localize to mammalian euchromatic DNA replication foci

first and later to associate with heterochromatic replication foci once the euchromatic replication is completed (Krude 1995). Additionally, CAF1 physically associates with PCNA, implying that incorporation of new histones directly follows the DNA polymerase (Figure 1a) (Shibahara and Stillman 1999). Heterochromatic histone H4 is characteristically under-acetylated, but newly synthesized histone H4 is acetylated at lysine 5 and lysine 12 regardless of the previous chromatin state. In mammalian cells, acetylated H4K5 and H4K12 are specifically enriched at late replicating foci, but not at early replicating foci, and colocalize with HP1α, HP1β and CAF1 (Taddei et al. 1999). This study also found that the association of acetylated H4K5, H4K12 and CAF1 with late-replicating foci is related to DNA synthesis, as BrdU labeling in a pulse-chase experiment colocalizes with CAF1 at these foci (Taddei et al. 1999). Thus, the default chromatic state post-replication may be more euchromatic or "open" and the reestablishment of heterochromatin is likely to be an active process.

Why might only late-replicating regions be associated with acetylated H4K5 and H4K12? The authors suggest that euchromatic histones may be more rapidly modified, thus making it difficult to visualize the marks in euchromatin (Taddei et al. 1999). Studies of the largest CAF1 subunit in *S. cerevisiae*, Cac1/Rfl2, have suggested a model in which CAF1 plays an integral role in incorporating "heterochromatin competent" H3-H4 tetramers, by virtue of their acetylation pattern (Enomoto and Berman 1998). Interestingly, at mammalian late replicating foci, the hyperacetylated H4 and CAF1 remain associated with the heterochromatic foci for 20 minutes post-replication, revealing a window in which heterochromatin begins its reestablishment. CAF1 continues its association with heterochromatin at least until late G2 (Murzina et al. 1999). It is tantalizing to speculate that the lingering presence of acetylated H4K5, H4K12 and CAF1 at

newly replicated heterochromatic foci may act as a particular mark for heterochromatin and recruit heterochromatin factors to stimulate heterochromatin formation.

Like ORC and POL α, CAF1 also physically interacts with HP1 (Figure 1a). Murzina et al. demonstrated that the largest subunit of CAF1, p150, and several isoforms of HP1 associate through the MOD1 interacting region (MIR) of p150 in mammalian cells (Murzina et al. 1999). However, the purpose of this interaction remains unclear. Interestingly, Murzina et al. found that mutations in MIR that disrupt the CAF1-HP1 interaction did not affect recruitment of CAF1 to either euchromatic or heterochromatic replication foci (Murzina et al. 1999). Additionally, HP1 localization to heterochromatin doesn't appear to require heterochromatic replication, implying that HP1 can localize to heterochromatin by means independent of CAF1's link with fork progression (Murzina et al. 1999). In *S. cerevisiae*, silencing at the *HML* locus can be restored in *cac1 sir3* mutants by expression of *SIR3*, indicating that CAF1 is not required for the establishment of silencing. However, the presence of silencing defects in *cac1* mutants suggests a role for CAF1 in the maintenance and transmission of heterochromatin (Enomoto and Berman 1998).

Visualization of replicating heterochromatin enables a better understanding of the spatial and temporal relationship between DNA replication and heterochromatin assembly. In a recent report by Quivy et al., pulse-chase-pulse experiments, in conjunction with high-resolution microscopy and 3D modeling, reveal the nuclear positioning and architecture of replicating mammalian pericentric heterochromatin domains (Quivy et al. 2004). Imaging replicating pericentric foci demonstrates that DNA synthesis, based on colocalization of a 10-minute pulse of BrdU and PCNA, occurs at the periphery of the pericentric domain. The newly replicated DNA then moves into the interior of the pericentric domain, suggesting that the heterochromatic

region subject to disruption by replication is restricted to the exterior of the pericentric domain. Disruption of higher-order heterochromatin factors, like HP1, by replication seems likely given the disruption of nucleosomes, although HP1 is not visibly delocalized from heterochromatic regions as they replicate (Taddei et al. 1999). This also suggests that disruption of HP1 and alterations of heterochromatin for replication may be a local and transient event, which may promote rapid reestablishment of heterochromatin.

Whereas the architecture of replicating pericentric domains may be specific to pericentric heterochromatin and/or mammalian cells, the studies of Quivy et al. reveal details of heterochromatin reassembly that may be more universal (Quivy et al. 2004). These experiments demonstrate the presence of two pools of nuclear HP1 in these cells: a replication-associated pool and an independent pool. The replication-associated HP1 colocalizes with PCNA, CAF1 and acetylated H4K5 at the periphery, but not methylated H3K9, which is found in the core of the pericentric heterochromatin domain. In contrast to the independent pool of HP1, the replicative pool of HP1 is resistant to RNAse treatment and is detected in knockout cells of *suv39h*, a histone methyltransferase. The replication-associated pool of HP1 does appear to be dependent upon CAF1 for its localization, as knock-down of CAF1 by siRNA to the p150 subunit leads to a loss of HP1 staining at the periphery. These data add to a model in which PCNA recruits CAF1 to loci and CAF1 assists in reestablishing heterochromatin, following passage of the replication fork, by recruiting HP1 to newly replicated foci (Figure 1a).

Reestablishment of DNA Methylation Patterns

Hypermethylation of cytosine bases is another characteristic of silenced chromatin, most prominent in vertebrates. DNA replication and methylation appear to occur concurrently; by

isolating newly synthesized DNA, containing origins of replication, from mammalian cells it was demonstrated that levels of cytosine methylation were equal in the parental and daughter DNAs (Araujo et al. 1998). The methyltransferase DNMT1 has been linked to maintenance of this epigenetic state due to its association with hemimethylated DNA and its interaction with the replication machinery at late-replicating foci (Figure 1b). DNMT1 has been demonstrated to co-purify with *in vitro* DNA replication activity and co-elute with POL α activity, supporting a model in which methylation occurs concomitant with DNA replication (Vertino et al. 2002). Consistent with these observations, DNMT1 localizes to the characteristic sites of mammalian pericentric heterochromatin replication; DNMT1, BrdU and PCNA colocalize at these sites (Leonhardt et al. 1992). Chuang et al. also demonstrated colocalization of DNMT1, PCNA and BrdU at these sites and furthered this by revealing a physical interaction between DNMT1 and PCNA by GST pulldown (Chuang et al. 1997). This interaction supports a model in which PCNA, traveling with the replication fork, acts as a scaffold to recruit a number of chromatin-modifying enzymes (Figure 1b). Recent evidence suggests that, in regard to PCNA's interaction with DNMT1, PCNA may do more than act as a passive loading dock. Data from Iida et al. indicate that DNMT1 is recruited to DNA more efficiently if the DNA is bound by PCNA (Iida et al. 2002). Additionally, DNA methylation assays reveal that PCNA-bound DNA is methylated more efficiently by DNMT1 than a PCNA-free control (Iida et al. 2002). It is intriguing to envision that PCNA is able to ensure that DNA or histone modifications are rapid and specific by enhancing the activities of these enzymes.

In addition to the reestablishment of the DNA methylation pattern, specific methyl-binding proteins that contribute to the silenced state of chromatin must also rebind following replication. A family of proteins consisting of MeCP2 and MBD1, 2, 3 and 4 binds methylated

CpG sequences in vertebrates. Importantly, these methyl-binding proteins are commonly found in complexes with histone deacetylases and chromatin remodeling enzymes, suggesting that these proteins assist in the recruitment of factors that reestablish the heterochromatic state (for reviews see Newell-Price et al. 2000 and Wade 2001). Methyl-binding proteins, particularly the MBD2a-MBD3 complex, also colocalize with DNMT1 in late S phase at mammalian pericentric heterochromatin, but not before (Tatematsu et al. 2000). This suggests that both methylation of the DNA and binding of this mark by methyl-binding proteins occur quickly following replication, although it remains to be demonstrated that these methyl-binding proteins are present on nascent DNA. A link between silencing and replication is also suggested by the fact that MBD1 physically interacts with CAF1 by immunoprecipitation and yeast two-hybrid (Reese et al. 2003). MBD1 and CAF1 colocalize to mammalian pericentric heterochromatin domains, implying that this interaction may have functional consequences (Figure 1b) (Reese et al. 2003). It has not been tested whether PCNA is involved in the MBD1-CAF1 interaction or whether CAF1 may act as a second scaffold behind the fork. The notion that CAF1 can act as a scaffold is furthered by the fact that the MBD1/CAF1 complex associates with HP1, but that HP1 has not been demonstrated to interact physically with PCNA (Figure 1b) (Reese et al. 2003).

Reese et al. also examined the effects of disrupting CAF1 p150 on CAF1-MBD1 localization and on several heterochromatin markers. Overexpression of the C-terminus of CAF1 p150, the domain required for the MBD1 interaction, disrupted localization of CAF1 to pericentric heterochromatin foci (Reese et al. 2003). In addition, this overexpression prevented localization of MBD1 to the heterochromatin foci, but did not seem to disrupt other markers of heterochromatin such as MeCP2 or HP1α. This experiment implies several things. First, CAF1 localization to heterochromatin requires domains outside of its C-terminus. This is not

particularly surprising as the N-terminus of CAF1 p150 is necessary for strong binding to PCNA (Moggs et al. 2000). Second, overexpression of the CAF1 C-terminus acts as a dominant negative, sequestering MBD1 away from full-length CAF1 and disrupting its localization to heterochromatin. This suggests that CAF1 mediates MBD1's localization to pericentric heterochromatin. Finally, the ability of HP1α to localize to heterochromatin in the absence of proper CAF1 localization suggests that other factors assist in recruiting HP1α to heterochromatin. It may be that the methylation of histone H3 can recruit HP1 on its own post-replication, and that this is facilitated by HP1's interaction with CAF1. It is also possible that ORC and replication proteins could recruit HP1 or that unidentified factors assist in recruiting HP1 (Figure 1a). Whether or not these factors normally assist in recruiting HP1α or only in this aberrant state remains to be elucidated.

As mentioned previously, in heterochromatin, lysine 9 of histone H3 (H3K9) is often methylated and this site is bound by HP1 (Lachner et al. 2001, Bannister et al. 2001). Reestablishment of histone methylation following replication is linked to establishment of methylated DNA. DNMT1 and DNMT3a, a *de novo* DNA methyltransferase, bind to SUV39H1, a known H3K9 methyltransferase, and HP1β and SUV39H1 associate with DNA methyltransferase activity (Fuks et al. 2003a). Additionally, DNMT3b, another *de novo* DNA methyltransferase, fails to localize in *Suv39h* null cells, and these cells display an altered DNA methylation status at particular sequences, highlighting the importance of the DNA methylation-histone methylation relationship (Lehnertz et al. 2003). Methyl-binding proteins, specifically MeCP2, have previously been shown to recruit H3K9 histone methyltransferase activity in mammalian cells (Fuks et al. 2003b). Recently, Sarraf and Stancheva have demonstrated a similar interaction between MBD1 and SETDB1, an H3K9 histone methyltransferase, by yeast

two-hybrid and immunoprecipitation experiments (Sarraf and Stancheva 2004). In addition, MBD1/SETDB1 associates with CAF1 and PCNA specifically in S phase, and the formation of this complex requires ongoing DNA replication (Figure 1b). By using RNAi to MBD1, Sarraf and Stancheva also revealed that SETDB1's interaction with MBD1 is required to recruit SETDB1 to CAF1 during DNA replication (Sarraf and Stancheva 2004). The interaction between DNA methylation and histone methylation is intriguing, and the rapid transition from newly synthesized chromatin to heterochromatin may be facilitated by this coordination.

Although heterochromatic factors must be synthesized to meet the demands of the daughter genomes, it seems unlikely that the old factors are discarded and fresh factors are incorporated at each round of replication. How then does the passing replication fork keep track of "old" factors and ensure that the proper epigenetic state is reestablishment? Although many details of these questions remain, experiments by Sarraf and Stancheva imply that the fork may transiently displace MBD1 from methylated DNA, but keeps MBD1 close by to incorporate the factor postreplication. MBD1 binding to methylated DNA and CAF1 are mutually exclusive, as shown by *in vitro* binding experiments, suggesting that replication forks may generate a transient CAF1/MBD1/SETDB1 complex by displacing MBD1 from methylated DNA (Sarraf and Stancheva 2004). The CAF1/MBD1/SETDB1 complex also associates with histones H3 and H4 in S phase, hinting that methylation of H3K9 occurs during chromatin assembly (Sarraf and Stancheva 2004). Finally, Sarraf and Stancheva identified the promoter of p53 binding protein 2 as an MBD1 binding site. Using this site as a tool, treatment of the cells with MBD1 siRNA and aphidicolin revealed a requirement for DNA replication to reestablish H3K9 methylation (Sarraf and Stancheva 2004). This result provides evidence that the passage of the replication fork is necessary to reestablish a heterochromatic state at this site in mammals, contradicting results in

yeast experiments. Sarraf and Stancheva propose an intriguing model based on these results: DNA methylation directs H3K9 methylation by SETDB1 at MBD1-bound loci. If the DNA methylation is removed, the recruitment of MBD1/SETDB1 complex to CAF1 is disrupted and results in a gradual loss of methylation following rounds of replication. It will be interesting to determine the details of the DNA methylation and histone methylation relationship: the number and importance of histone methylases that act to restore the heterochromatic state, the importance of replication in recruiting these factors, and whether or not every histone methylase is dependent upon DNA methyl-binding proteins. The details of the coordination between DNA methylation and histone modifications suggest a complex interplay between all these factors. Research to date reveals that there are many players and many variations on interactions, and much remains to be determined about the importance of each factor and the significance of each interaction.

Higher order chromatin structure and the role of chromatin-remodeling complexes

Multiple chromatin-remodeling enzymes, which alter the positioning and spacing of nucleosomes without removal from DNA, have been identified in eukaryotes and shown to play a role in the formation of heterochromatin. Heterochromatin is characterized by regular spacing of nucleosomes and tight compaction, restricting accessibility of the DNA (Wallrath and Elgin 1995, Sun et al. 2001). Chromatin-remodeling enzymes utilize ATP to shift nucleosomes into equally spaced positions and to remove them from promoter regions. Complexes containing Imitation Switch (ISWI) have been implicated in replication and maintenance of heterochromatin (for review, see Corona and Tamkun 2004, de la Serna and Imbalzano 2002. Two ISWI

complexes in particular have been studied in higher eukaryotes and seem to have roles at heterochromatin in S phase. Defining the time of action of these complexes will be complicated, however, as chromatin-remodeling enzymes may be involved in moving nucleosomes to open DNA for replication and/or to reestablish the nucleosome pattern of heterochromatin. Current research on the role of ISWI complexes reveals roles in regulating replication and heterochromatin, although at present it is not clear whether they act primarily to open heterochromatin to promote replication or restrict replication through heterochromatin by maintaining a closed configuration, as detailed below.

Studies in human cells demonstrate a requirement for the ACF1-ISWI complex (ATP-utilizing chromatin assembly and remodeling factor 1) in replication of heterochromatin. Prior to late S phase, ACF1 and ISWI exhibit general nuclear staining. At late S phase, these factors colocalize with BrdU and HP1β at the characteristic pericentric heterochromatin foci (Collins et al. 2002). By disrupting the ISWI interaction domain (BAZ domain) on ACF, this group revealed that ACF1 could localize to pericentric heterochromatin without its interaction with ISWI, but experiments in cell culture suggest that the function of ACF requires ISWI (Collins et al. 2002). RNAi to ACF1 results in a decrease in the number of cells incorporating BrdU at pericentric heterochromatin, but does not alter HP1β localization. In addition, these ACF1 depleted cells show a delay in late S phase, which the authors interpret as a delay in the replication of heterochromatin. To address whether the delay was due to impairment in opening chromatin for replication, the ACF1 siRNA cells were treated with 5-aza-2-deoxycytidine (5A2D), a DNA methylation inhibitor that leads to the decondensation of heterochromatin. The ACF1 siRNA, 5A2D-treated cells no longer accumulated in S phase and demonstrated an incorporation of BrdU in pericentric heterochromatin, suggesting a reversal of the ACF1 siRNA

phenotype (Collins et al. 2002). Depletion of ISWI by siRNA also decreased the rate of BrdU incorporation, but at all stages of S phase. This phenotype was also reversed by treatment with 5A2D, suggesting that ISWI, likely in combination with another regulator, has a role in early and mid-S phase (Collins et al. 2002). Based on the ACF1 siRNA effect on late S phase replication and its reversal by decondensing heterochromatin, the authors conclude that the ACF1-ISWI complex is required in mammalian cells for replication of heterochromatin.

Studies of ACF1 in Drosophila, however, suggest a different role for the ACF1-ISWI complex in heterochromatin. As might be expected for a chromatin-remodeling enzyme, extracts made from *acf1* null mutants assemble nucleosomes arrays less efficiently than wild-type extracts and show a decrease in the periodicity of these arrays on isolated chromatin (Fyodorov et al. 2004). Mutations in *acf1* act as strong suppressors of PEV, suggesting that ACF1 contributes to the formation of heterochromatin (Fyodorov et al. 2004). Observations on DNA replication in *acf1* mutant embryos and larval neuroblasts also indicate that the functions of ACF1 in Drosophila differ from those observed in human cells. Drosophila *acf1* mutant embryos spend less time in S phase during the late embryonic S/M cycles as determined by using time-lapse microscopy to measure the time between the beginning of nuclear cycle 13 S phase and the initiation of chromosome condensation, signaling the beginning of mitosis. Fyodorov et al. observe that DNA replication appears normal in these mutant embryos, citing the absence of morphological defects in chromosome structure and the ability of the chromosomes to pass through mitosis without segregation defects. *acf1* mutant larval neuroblasts, which undergo the canonical mitotic cycle, also spend less time in late S phase and thus appear to progress more rapidly through late S phase (Fyodorov et al. 2004). An accelerated S phase is also observed in mutants with decreased levels of histones, which further suggests that the repressive nature of

heterochromatic DNA replication is relieved by poor chromatin assembly in *acf1* mutants (Fyodorov et al. 2004).

Is it possible to reconcile the observations in human cells and Drosophila? As Fyodorov et al. note, the behavior of cultured mammalian cells and *acf1* mutant embryos and larvae may not be identical (Fyodorov et al. 2004). The ACF1-ISWI may perform slightly different roles in different organisms or at different developmental stages. Fyodorov et al. also propose that the decline in BrdU incorporation at heterochromatic foci in the ACF1 siRNA-treated cells could be due to an acceleration in progression through S phase instead of a delay in late S phase. However, the persistence of PCNA at the heterochromatic loci in the absence of BrdU incorporation agrees more with a model where S phase is delayed. Another possibility may be that ACF1-ISWI is involved in both roles, opening heterochromatin for replication and arranging nucleosome arrays for the heterochromatic state. Perhaps each system or experimental technique is particularly suited to observe predominantly one role over the other. Nevertheless, it is apparent that the ACF1-ISWI complex is important in the propagation of heterochromatin after DNA replication.

Another ISWI-containing complex, WSTF-ISWI chromatin remodeling complex (WICH), has been linked to maintenance of chromatin state in mammalian cells. WSTF and ISWI form a complex in vertebrates that, *in vitro*, can reconfigure disorganized nucleosomal arrays into more regularly spaced and organized configurations in an ATP-dependent manner (Bozhenok et al. 2002). A role for the WSTF-ISWI complex in heterochromatin maintenance is suggested by its localization to mammalian pericentric heterochromatin in late S phase where it colocalizes in large, distinct foci with HP1β (Bozhenok et al. 2002). Based on the localization of WSTF in late S phase, the authors suggest that the WSTF-ISWI complex either facilitates DNA

replication through heterochromatin or has a role in the assembly of heterochromatin reestablishment post-replication. A recent paper from the same lab probed the role of WSTF-ISWI further and revealed that WSTF-ISWI may have a role earlier in S phase (Poot et al. 2004). Poot et al. revisited the early S phase localization pattern for WSTF and asked whether treatment with a high salt wash would reveal distinct foci instead of the previously observed, general nuclear staining. Indeed, with the high salt wash, WSTF localizes to distinct foci in early S phase that partially colocalize with sites of BrdU incorporation (Poot et al. 2004). Importantly, in mid, late and very-late S phase, WSTF nearly always colocalized with sites of active DNA replication. Additionally, WSTF and ISWI physically interact with PCNA and are retained at replication foci via their interaction with PCNA, as demonstrated by the ability of a PCNA peptide to compete WSTF from these sites (Figure 1a) (Poot et al. 2004). This interaction with PCNA is consistent with the observation that WSTF-ISWI is retained at foci post-replication, because PCNA can persist at replication foci after DNA synthesis is complete.

Experiments in which WSTF has been depleted from cells provide evidence for a different role for WSTF-ISWI: WSTF acts to maintain open chromatin structures (Poot et al. 2004). WSTF-depleted cells have small nuclei that are more resistant to DNase I and micrococcal nuclease digestion, indicative of the chromatin in these cells being closed and more packaged. In addition, these cells demonstrated an increase in heterochromatic markers; levels of HP1α and β and histone H3 lysine 9 trimethylation and lysine 27 dimethylation were increased in both total cell extracts and the chromatin-bound fraction (Poot et al. 2004). mRNA levels of HP1α and HP1β were not altered in the WSTF-depleted cells suggesting that the observed increase in protein levels was not due to a release of transcriptional repression by WSTF (Poot et al. 2004). Interestingly, the increase in HP1β levels can be prevented if the cells

are blocked in G1 by treatment with mimosine, indicating that passage through S phase is required for the observed increase in HP1a and HP1B protein levels (Poot et al. 2004). The authors present two possible interpretations for the role of WSTF-ISWI. Nucleosomes may be less mobile in the absence of WSTF-ISWI, thereby promoting formation of heterochromatin. It is also possible that WSTF-ISWI may directly prevent HP1 binding to newly replicated DNA and actively maintain euchromatic structure. These interpretations suggest that heterochromatin is the default state for newly replicated DNA or that newly replicated DNA is highly susceptible to heterochromatin assembly in the absence of an active inhibition factor. It is hard to imagine heterochromatin as a default state; keeping an organism's genome open would require a high level of heterochromatin-inhibition factors and a great deal of energy, but a precedence exists in the requirement of Dot1 in S. cerevisiae to actively block the spread of heterochromatin [REF]. Is there an alternate interpretation for the increase in HP1 and methylation states in the absence of WSTF? Additionally, a role for WSTF-ISWI at euchromatic loci may differ from its role at pericentric heterochromatin. Why would a factor that inhibits the formation of heterochromatin localize to pericentric heterochromatin while it replicates? Could an as-of-yet unknown additional factor regulate whether WSTF-ISWI promotes heterochromatin formation or blocks it? Clearly many exciting questions remain and more are generated as the complicated role of chromatin-remodeling factors is revealed.

Conclusions and Perspectives

We have presented the evidence for roles of replication proteins, histone modification enzymes, DNA methyltransferase, and chromatin remodeling complexes in the reinstatement of heterochromatin at the replication fork in S phase. Many of these functions are likely to be

required outside of S phase for the maintenance of heterochromatin and to be critical for the establishment of heterochromatin at new genomic locations in response to developmental cues such as position effect variegation or X chromosome inactivation. Even within S phase, precisely evaluating the mechanism by which these proteins contribute to heterochromatin replication is impeded by the complexities of distinguishing their roles in DNA replication versus reestablishment of the chromatin. The use of mutations that dissociate DNA replication and chromatin requirements will be a powerful means to decipher these roles.

Conversely, new factors required for maintenance of heterochromatin now need to be analyzed for roles in the replication of heterochromatin within S phase. Among the most exciting new activities needed for heterochromatin are the RNAi machinery and the retinoblastoma tumor suppressor protein. In fission yeast, Drosophila, and mammalian cells the RNAi machinery is required for heterochromatin protein binding, heterochromatic silencing, and centromere function (Pal-Bhadra et al. 2004, Verdel et al. 2004, Huertas et al. 2004, Kanellopoulou et al. 2005, Motamedi et al. 2004, Fukagawa et al. 2004). The Retinoblastoma protein family also has been shown to be required for DNA methylation, hypoacetylation of histone H3, and trimethylation of histone H4, most likely via a direct interaction with the Histone H4 lysine 20 trimethyl transferases Suv4-20 (Gonzalo et al. 2005). Given that Rb is known to be present and act within S phase (Bosco et al. 2001, Krek et al 1995), it is likely that Rb has a function in reinstating heterochromatin during DNA replication. In addition to the predominant histone proteins, there are histone variants that contribute both to the formation of heterochromatin and to protect against the spreading of heterochromatin into euchromatic regions (for review see Kamakaka and Biggins 2005). Although some of these histone variants such as H3.3 do not require DNA replication for their assembly into nucleosomes, the assembly

requirements of other variants and potential roles in replication are in the early stages of investigation.

A crucial issue is how the histone modifications and associated chromatin proteins are templated onto the daughter duplex after replication. Given the interdependency of histone modifications (Czermin and Imhof 2003, Fischle et al. 2003), the semiconservative reassembly of the nucleosome could provide a means to reestablish histone modifications that then could promote proper protein association. The relationship between DNA methylation and histone modification provides an additional template mechanism. Although the problem of templating chromatin architecture is common to both euchromatin and heterochromatin, in the case of heterochromatin it has the increased complexity of requiring the reassociation of heterochromatin binding proteins. Further investigation of the regulation of heterochromatin replication will produce insights into how these modifications and protein associations are templated.

The timing of heterochromatin replication within S phase and the mechanism by which it is delayed until late in S phase is another issue that remains to be unraveled. This is of biological significance in that this delayed timing facilitates the underreplication of heterochromatin in endo cycles. Limiting replication until late in S phase may also facilitate assembly of heterochromatin binding proteins. Defining the means by which the intriguing SuUR protein both affects the timing and extent of heterochromatin replication in endo cycles is likely to provide crucial insights into how heterochromatin replication can be restricted until late in S phase

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