CHAPTER FIVE

Mechanisms of Sister Chromatid Pairing

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Contents

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Abstract

The continuance of life through cell division requires high fidelity DNA replication and chromosome segregation. During DNA replication, each parental chromosome is duplicated exactly and one time only. At the same time, the resulting chromosomes (called sister chromatids) become tightly paired along their length. This S-phase pairing, or cohesion, identifies chromatids as sisters over time. During mitosis in most eukaryotes, sister chromatids bi-orient to the mitotic spindle. After each chromosome pair is properly oriented, the cohesion established during S phase is inactivated in a tightly regulated fashion, allowing sister chromatids to segregate away from each other. Recent findings of cohesin structure and enzymology provide new insights into cohesion, while many critical facets of cohesion (how cohesins tether together sister chromatids and how those tethers are established) remain actively debated.

Key Words: Cohesion, Cohesin, Sister chromatid pairing, Chromatin remodeling, Chromosome segregation, Establishment, Budding yeast.

1. Introduction

Cells divide to replenish loss caused by trauma, cell senescence and cell death. Cell division also provides for embryonic growth, tissue regeneration and remodeling. Whether simple unicellular or multi-tissued organisms, the fundamental goal of cell division is to produce two viable and identical daughter cells. Continued viability of cell progeny requires that the DNA complement is replicated and that sister chromatids segregate into the newly forming daughter cells.

In eukaryotes, DNA replication and sister chromatid segregation are temporally separated. For instance, checkpoints delay cell-cycle progression until each chromosome is fully replicated and physically intact. The G_2 phase further separates S phase from mitosis (chromosome segregation), allowing for further growth and maturation prior to cell division. The consequence of temporally separating replication from segregation is that sister chromatids generated during S phase must retain their identity as sisters until anaphase.

The processes that identify sister chromatids, tether them together and maintain this tether from early S phase until anaphase onset fall under the general term sister chromatid cohesion. Cohesion requires coordinated

activities of at least four different classes of proteins: structural cohesins, deposition complexes, establishment factors and cohesion regulators. Here, structural cohesins refers to those factors that directly resist poleward-directed kinetochore/spindle forces that are exerted on each sister chromatid. Deposition factors are required for structural cohesins to associate with chromatin. As we will see, proper deposition and the presence of structural cohesins on chromosomes are not sufficient for sister chromatid pairing. A third and essential activity, Establishment, is required. Many researchers extend the role of deposition factors to include establishment. While such views may simplify matters, they do so at the expense of accuracy. Deposition and establishment are indeed quite separate—a position substantiated by the fact that each requires independent complexes and that these processes can be genetically separated by mutational analyses. The fourth class is cohesion regulators that support cohesion and/or promote structural cohesin dynamics.

Pairings between sister chromatids cannot be static. Soluble cohesin complexes are undoubtedly modified to promote deposition onto chromatin. Chromatin-associated cohesins in turn appear to move away from these deposition sites and set up residence at functional cohesion sites. Cohesins are further modified to affect sister chromatid pairing. The nature of this pairing remains highly controversial. Cohesin dynamics must also accommodate condensation—a progressive contraction that reduces the chromosome length but also produces ever-decreasing contact points between sister chromatids (Guacci *et al.*, 1994; Selig *et al.*, 1992; Sumner, 1991). Little is known regarding how cohesin subsets are inactived to accommodate continued condensation while another set persists to maintain sister chromatid pairing. Add to the list cohesin dissociation pathways. In higher eukaryotes, a predominant fraction of cohesins disassociate from chromatin in early prophase with the remaining cohesins dissociating at anaphase onset. Cohesin release during prophase relies on phosphorylation while release during anaphase onset occurs predominantly through proteolysis (Nasmyth *et al.*, 2000; Wang and Dai, 2005; Watanabe, 2005; Yanagida, 2005). Thus, there are numerous and separate pathways that regulate cohesin dynamics—not all of which are present in every organism.

In this review, I focus on mechanisms used by budding yeast to identify and then pair together sister chromatids. The review starts with a discussion on the physical characteristics of structural cohesins and continues with recent models of cohesin enzymology. From this foundation, the review turns to factors that influence cohesin deposition and, subsequent to deposition—how cohesion is established between sister chromatids. I then highlight recent findings of alternate (non-cohesin) sister pairing complexes and end with a discussion regarding advances in linking cohesion pathways to human disease states.

2. Structural Cohesins

Cohesins are required from the beginning of S phase until anaphase onset, but at no time is their presence more obvious than during mitosis when sister chromatids orient to spindle microtubules and, in a tug of war, oscillate back and forth across the spindle equator (Bajer, 1982; Goshima and Yanagida, 2000; He *et al.*, 2000; Pearson *et al.*, 2004; Rieder *et al.*, 1986; Skibbens *et al.*, 1993; Waters *et al.*, 1996). Kinetochore motility and the checkpoint system that regulates both mitotic exit and anaphase onset have been the topic of numerous reviews (Cheeseman *et al.*, 2002; Hyman, 2005; Maiato *et al.*, 2004; Musacchio and Salmon, 2007; Nicklas, 1997; Pinsky and Biggins, 2005). Relevant here are that structural cohesins not only identify sister chromatids over time (from G_1/S to anaphase onset), but play additional roles required for high fidelity chromosome segregation. First, cohesins physically constrain sister kinetochores positionings to promote proper chromosome bi-orientation to spindle microtubules. Second, cohesins create tension by resisting poleward-pulling forces produced by kinetochore/microtubules. In the absence of this tension, cells activate mitotic checkpoints to delay anaphase onset until each sister chromatid pair becomes properly bi-oriented. Third, cohesins provide a unique and irreversible trigger required for cell cycle progression. In this case, cohesin (Mcd1) proteolysis is ultimately controlled by the anaphase promoting complex (APC) and promotes sister chromatid segregation (Dumitrescu and Saunders, 2002; Murray, 2004; Yanagida, 2005).

At the heart of sister chromatid pairing is the question—what cohesin structure resists kinetochore/spindle pulling forces? Before addressing this question, it is worth considering the motifs that comprise each cohesin subunit. The prevailing evidence indicates that functional cohesin complexes exist minimally as a quaternary structure that contains Mcd1/Scc1, Smc1, Smc3, and Irr1/Scc3. Because cohesins have been the focus of many excellent reviews (Huang *et al.*, 2005; Losada and Hirano, 2005; Nasmyth, 2005), only the main features of these subunits are discussed below. I start with a description of Mcd1/Scc1 (herein termed Mcd1)—arguably the most critical of all the cohesin subunits.

2.1. Mcd1

Wildtype cells arrest in metaphase with sister chromatids tightly paired if the APC is kept inactive through experimental means (conditional mutations or mitotic checkpoint activation via microtubule depolymerization). Under these conditions, researchers predicted that cohesion mutant cells would instead precociously separate their sister chromatids. The co-discovery that

Mcd1 is required to maintain cohesion from S phase into mitosis clearly documented a protein-based mechanism for sister chromatid pairing—a notion posited over a decade before (Guacci *et al.*, 1997; Koshland and Hartwell, 1987; Michaelis *et al.*, 1997). Mcd1 and other cohesins bind chromatin throughout the time that sister chromatid pairing is maintained, further supporting a protein-based mechanism of cohesion. Cohesin complexes are highly concentrated at centromeres and, to lesser levels, bind cohesin associated regions (CAR) positioned at roughly 10–12 kb intervals along the chromosome arms. CAR sites are AT-rich and often occur at intergenic transcription units. Chromatin pelleting and ChIP methods capable of detecting loci-specific protein further showed that Mcd1 binds DNA loci known to function in sister chromatin pairing (Blat and Kleckner, 1999; Glynn *et al.*, 2004; Guacci *et al.*, 1997; Laloraya *et al.*, 2000; Lengronne *et al.*, 2004; Megee and Koshland, 1999; Megee *et al.*, 1999; Michaelis *et al.*, 1997; Tanaka *et al.*, 1999; Toth *et al.*, 1999).

Mcd1 expression is tightly cell cycle regulated: it is repressed during G_1, induced early in S phase (or very late in G_1) and continues into mitosis (Guacci *et al.*, 1997; Michaelis *et al.*, 1997). Upon expression, Mcd1 quickly associates with Smc1,3 and Irr1/Scc3. These other cohesin subunits are present at constant levels through the cell cycle, but bind chromatin only in the presence of Mcd1 (Ciosk *et al.*, 2000; Gruber *et al.*, 2003; Guacci *et al.*, 1997; Haering *et al.*, 2002; Hartman *et al.*, 2000; Michaelis *et al.*, 1997). As such, Mcd1 appears to be the master regulator of cohesin assembly. Both soluble and chromatin-bound quaternary cohesin complexes exist upon Mcd1 expression. It remains unclear if quaternary structures are loaded onto chromatin or to what extent cohesin assembly reactions are coupled to chromatin association (see Section 4).

A third facet of Mcd1 function is that it harbors two cleavage sites (Uhlmann *et al.*, 1999). Mcd1 is cleaved by separase Esp1 and this proteolysis is critical for chromosome segregation. Esp1 is a protease that is kept inactive through Pds1 binding. APC-dependent degradation of Pds1 releases/activates Esp1. For this reason, Pds1 is termed an anaphase inhibitor and its presence used to identify pre-anaphase cells. Mcd1 degradation by Esp1 in turn allows sister chromatids to segregate away from each other (Nasmyth *et al.*, 2000; Pellman and Christman, 2001; Yanagida, 2005). That Mcd1 cleavage temporally corresponds to both cohesin's chromatin-dissociation and sister chromatid separation provides strong evidence that this series of events biochemically defines anaphase onset. However, findings that portions of these pathways can be subverted in a number of cell systems suggest that cohesion dissolution and anaphase onset are still much more complicated (Guacci, 2007; Wang and Dai, 2005; Watanabe, 2005).

In addition to its roles as a master regulator of cohesin assembly and cleavage-dependent regulator of cell cycle progression, Mcd1 associates with the Smc1 and Smc3 cohesin subunits in a particular fashion. In short,

Mcd1 appears to bridge Smc1 and Smc3 heterodimers to produce a closed contiguous ring. As we will see below, whether Mcd1 truly forms a bridge between Smc1,3 subunits has come under recent debate (see Section 2.4 and 6.3). Moreover, whether cohesin structure(s) combine to affect sister chromatid pairing remains one of the most intriguing and debated issues in the field of chromosome segregation (see Section 6).

2.2. Smc1 and Smc3

Smc proteins are comprised of large globular amino and carboxy termini connected by a long α-helical domain. This α-helical domain is interrupted by a centrally positioned hinge. The Smc hinge provides for several key features, many of which have only recently come to light. With regards to structure, the central hinge allows each Smc protein to fold in half. The Smc hinge is really quite flexible. When viewed by EM, enriched bacterial Smc proteins form a number of structures from long linear molecules to proteins folded nearly perfectly in half (Melby *et al.*, 1998). The first consequence of hinge folding is that the α-helical domains that flank the hinge entwine into a single and relatively rigid anti-parallel coiled-coil structure (via heptad turns) approximately 40 nm long (Lowe *et al.*, 2001; Melby *et al.*, 1998). This extended intramolecular coiled coil rod-like domain is occasionally interrupted by small loops. In fact, all Smc proteins contain three loops within the coiled coil rod, the largest comprised of ~50 residues (Beasley *et al.*, 2002). EM micrographs of human cohesin complexes invariably reveal a conspicuous kink within one of the Smc coiled coil rods (Anderson *et al.*, 2002), which may map onto one of these loops. The second consequence of hinge folding is that Smc N- and C-terminal globular domains are brought into registration to form an Smc head domain (Fig. 5.1).

SMC head domains contain highly conserved motifs. The N-terminus of Smc proteins contain a Walker A (P loop) motif that comprises part of an ATP binding/hydrolysis module. The C-terminus contains a Walker B motif, providing the other portion of the ATP binding/hydrolysis module. Hinge folding brings Walker A/B motifs into close opposition. As discussed below, Smc proteins dimerize—forming homodimers in prokaryotes (SMC and the structurally related MukB) and heterodimers in eukaryotes (Smc1,3, Smc2,4 and Smc5,6). There is evidence that Smc1,3 can interact via head-to-head associations and that this binding is promoted by a Signature motif (often referred to as a C motif) that resides within the C-terminal globular domain. For instance, the C motif of Smc1 is thought to bind ATP captured by Smc3's Walker A/B domains. In turn, the C motif of Smc3 is thought to bind ATP captured by Smc1 Walker A/B domains. Because each head binds its own ATP (Walker A/B) and that of its partner's ATP (C motif), two ATPs are sequestered between the Smc1,3 heads. Such ATP binding

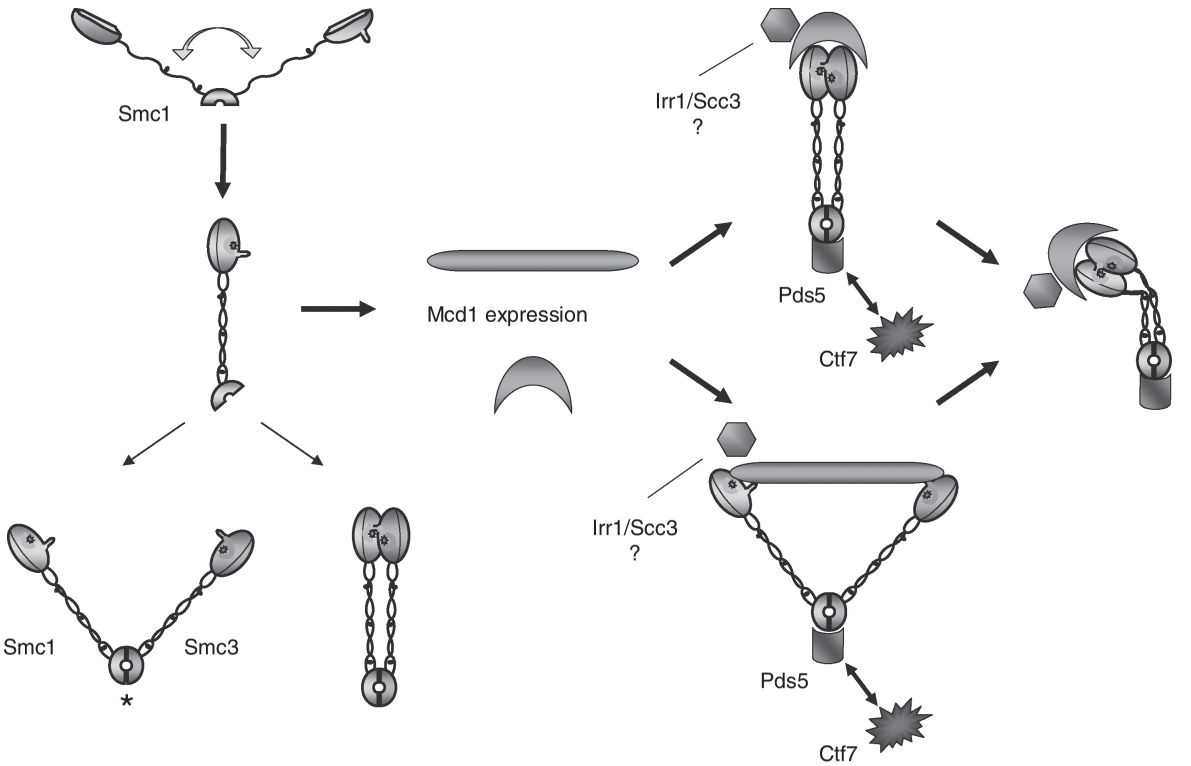

Figure 5.1 (*Continued*)

cassettes (ABC) are typical of many transporters and SMC superfamily members (Hopfner *et al.*, 2000).

The Smc1 hinge provides a strong binding surface for the opposing Smc3 hinge (and vice-the-versa) such that hinge–hinge dimerization provides for stable association of Smc1,3 (Fig. 5.1). As opposed to the flexibility of the hinge that allows for intramolecular interactions, Smc1,3 hinge–hinge binding is robust and sterically restricted such that enriched human cohesin complexes exhibit fairly uniform hinge–hinge angles of 88° (Anderson *et al.*, 2002). Parallel studies of *Bs*Smc and *Tm*Smc proteins revealed that four nearly contiguous glycines comprise a binding interface present on each Smc hinge dimerization domain (Chiu *et al.*, 2004; Haering *et al.*, 2002; Hirano and Hirano, 2002). Crystal structure of *Tm*SMC hinge dimers revealed a discontinuity between glycine-rich hinge binding domains to produce a doughnut-like structure (Haering *et al.*, 2002). In prokaryotes that express a single and thus homodimeric Smc, mutations within this glycine-rich domain abrogate interactions on both sides of the hole and thus abolish dimerization. In eukaryotes, Smc proteins form heterodimers and the situation is quite different. Mutation in one set of glycines (disrupting a binding interface either above or below the doughnut's hole) fails to disrupt Smc1,3 hinge binding. Both sides of the hole must be mutated to disrupt Smc1,3 hinge binding. Thus, one glycine-rich hinge domain interface is re-enforced by the glycine-rich interface on the opposite side of the hole with each providing for Smc1,3 dimerization (Chiu *et al.*, 2004).

Figure 5.1 Cohesin complex assembly/structure. Left: Smc1 (blue, top left) is comprised of globular N- and C-termini attached to a flexible hinge via extended helical domains. Hinge folding provides for formation of a head domain capable of binding ATP (small red star). Smc1,3 hinges also provide for dimerization—producing a doughnut like structure (*) at the Smc1,3 base. The physiological relevance of Smc1,3 heterodimers as a subcomplex (Smc3 shown in purple) is unknown (thin arrows). Middle: Mcd1 expression (either green structure) drives formation of a quaternary cohesin complex that contains Smc1,3, Mcd1 and Irr1/Scc3 (Irr1/Scc3 depicted as yellow hexagon). Mcd1-dependent cohesin assembly also recruits Pds5 (dark purple half-barrel) and WAPL (not show—but likely binds Pds5). The locale of Pds5 is speculative, but recent evidence suggest proximity to the Smc1,3 hinge. Genetic and biochemical findings position Ctf7 (large red starburst) near to Pds5, but this association is likely to be highly dynamic and probably does not persist into G2/M phases of the cell cycle in unperturbed cells. Middle and Right: Plausible cohesin structures include an extended rod in which Mcd1 sits atop of Smc1,3 head domains (top middle) or Mcd1 which bridges separated Smc1,3 head domains to form a ring (bottom middle). Irr1/Scc3 appears to bind Mcd1 in an asymmetric fashion. Some studies indicate that the cohesin complexes bend to position SMC heads near to the hinge domains—potentially occluding the cohesin ring lumen (right). Further assembly of cohesins into double-ring or oligomeric structures that tether together two sister chromatids are highly plausible (not shown). See text for details. (See color insert.)

2.3. Irr1/Scc3

Irr1 was identified on the basis of its irregular colony morphology and later termed Scc3 upon its characterization as a cohesin (Kurlandzka *et al.*, 1995, 1999; Toth *et al.*, 1999). In the first case, *irr1/scc3* mutant strains are defective in colony formation on solid substrates and this defect is suppressed by elevated dosage of either Its2 (a putative osmoregulator) or Nog2 (GTPase potentially involved in ribosome maturation). Thus, loss of Irr1/Scc3 indeed appears to affect cellular pathways outside of cohesion (Bialkowska and Kurlandzka, 2002; Kurlandzka *et al.*, 1995). As a bona fide structural cohesin, Irr1/Scc3 (1) co-purifies with Mcd1 and Smc1,3 soluble complexes, (2) binds chromatin and is localized to CAR sites in *Drosophila*, (3) is required to maintain cohesion from G_1/S into mitosis, and (4) dissociates from chromatin at anaphase onset. Co-expression in insect cells of Irr1/Scc3 with either full-length or truncated Mcd1 revealed that Irr1/Scc3 associates with the cohesin complex via binding directly to the Mcd1 C-terminus (Haering *et al.*, 2002). Irr1/Scc3 protein is present throughout the cell cycle, similar to Smc1 and Smc3, but Irr1/Scc3 and Smc1,3 bind chromatin predominantly (but not exclusively) upon Mcd1 expression (Guacci *et al.*, 1997; Michaelis *et al.*, 1997; Toth *et al.*, 1999; Valdeolmillos *et al.*, 2004). As with all other cohesins, Irr1/Scc3 and its role in cohesion are highly conserved through evolution (Stromal Antigens/SAs, STAGs, Rec11, Psc3, DSA1,2) (Chelysheva *et al.*, 2005; Kitajima *et al.*, 2003; Losada *et al.*, 2000; Pezzi *et al.*, 2000; Prieto *et al.*, 2001, 2002; Rollins *et al.*, 2004; Valdeolmillos *et al.*, 2004; Vass *et al.*, 2003).

For a relatively large protein (133 kDa), limited structural information exists for yeast Irr1/Scc3 outside of the conserved Stromal Antigen domains. Irr1/Scc3 orthologs fulfill spatially (centromere vs arm cohesion) and temporally (mitosis vs meiosis) separate cohesion functions (Chelysheva *et al.*, 2005; Kitajima *et al.*, 2003; Lara-Pezzi *et al.*, 2004; Losada *et al.*, 2000; Pezzi *et al.*, 2000; Prieto *et al.*, 2001; Rollins *et al.*, 2004; Valdeolmillos *et al.*, 2004; Vass *et al.*, 2003). In higher eukaryotes, the Irr1/Scc3 homolog SA2 further plays a key role in regulating cohesin release from chromatin during prophase. In these cells, SA2 is phosphorylated by Polo-like kinase, resulting in cleavage-independent cohesin dissociation primarily from the chromosome arms. Cohesin dissociation from centromeres is inhibited by Shugoshin. In budding yeast, there is no prophase cohesin release and Shugoshin (Sgo1) functions primarily in protecting centromeric cohesin during meiosis (Nasmyth, 2002; Watanabe, 2005; Wang and Dai, 2005).

2.4. Cohesins as a complex

Given a basic understanding of cohesin subunits, I return to the question—what cohesin structure identifies chromatids as sisters over time and is capable of resisting robust kinetochore/spindle pulling forces? One set of

studies in particular profoundly influenced the field of cohesion. Independent analyses revealed that the Mcd1 N-terminus binds the Smc3 head domain while the Mcd1 C-terminus binds the Smc1 head (Gruber *et al.*, 2003; Haering *et al.*, 2002). That Mcd1 bridges Smc1,3 heads kept separate by hinge domains that adopt an open ''V'' configuation suggested that cohesins form ''a huge triangular ring'' or ''proteinaceous loop'' of roughly 35 nm in diameter (Gruber *et al.*, 2003; Haering *et al.*, 2002). Ring dissolution by separase-mediated proteolysis of Mcd1 at anaphase onset nicely complements the model that sister chromatid pairing involves a closed and contiguous ring-like cohesin complex (Fig. 5.1).

However seductive, further consideration of the data led researchers to acknowledge that the biochemical and EM data equally supports a variety of cohesin complex structures (Campbell and Cohen-Fix, 2002; Guacci, 2007; Huang *et al.*, 2005; Losada and Hirano, 2005; Nasmyth, 2005). Thus, in addition to a single ring, cohesins have been fashionably compared to hand cuff-like (or catenated) ring pairs, filament-like bracelets, and chromatin snaps. For the outdoorsman, cohesins analogies also include mountaineering carabiners, bicycle locks, and garden gates (Gruber *et al.*, 2006; Haering and Nasmyth, 2003; Hirano and Hirano, 2006; Huang *et al.*, 2005; Skibbens, 2005). There is very persuasive evidence that cohesins associate with higher-order complexes to maintain cohesion. Moreover, double ring configurations and oligomeric cohesin complexes are all fully consistent with the notion that Mcd1 proteolysis produces cohesin dissolution at anaphase onset. On the other hand, efforts directed at isolating cohesin complex oligomers have yet to prove fruitful (Arumugam *et al.*, 2003; Chang *et al.*, 2005; Gruber *et al.*, 2003; Haering *et al.*, 2002; Ivanov and Nasmyth, 2005; Weitzer *et al.*, 2003). Another highly popularized model is that cohesin rings topogically encircle DNA. Despite this popularity, the nature of the structure that tethers two sister chromatids together is unknown and continues to be ardently debated (see Section 6).

3. Cohesin Enzymology

Since the earliest characterization of a *S*tructural *M*aintenance of *C*hromosome protein, or SMC (originally termed *S*tability of *M*ini*C*hromosomes) (Larionov *et al.*, 1985; Strunnikov *et al.*, 1993), analyses of Smc1,3 enzymology produced a confusion of models that remain actively debated in the literature. Most models are predicated on the notions that (1) cohesins assemble into soluble structures—often depicted as rings, (2) subunit contact sites are disrupted for cohesin deposition, and (3) disruption and reformation depend on cycles of ATP binding/hydrolysis. Other scenarios also exist (see Section 4). Despite the relatively simple

nature of the cohesin complex, at least five models of cohesin dynamics reside in the literature. The first four models differ predominantly in which subunit contact site must be disrupted for deposition. In the first two scenarios, a Mcd1 terminus dissociates from either Smc1 or Smc3 (gate opening). A third option is that Mcd1 dissociates fully from Smc1,3—an unlikely model akin to reverse assembly (gate removal). A fourth possibility is that Smc1,3 dissociate from one another by disrupting their hinge/dimerization domains. The fifth model is the simplest of all—cohesin complexes bind DNA without disrupting subunit contacts.

Regardless of the model, most studies concur that Smc1,3 ATPase activities are essential components of cohesin deposition. Sequence alignments of *Bacillus subtilis* SMC, *Sc* Smc1 and *Sc* Smc3 proteins were used to target highly conserved residues for site-directed mutagenesis (Arumugam *et al.*, 2003; Hirano *et al.*, 2001; Weitzer *et al.*, 2003). Point mutations intended to abrogate ATP binding (Walker A and B motifs) and ATP hydrolysis (Signature C motif) in Smc1 and Smc3 produced constructs unable to support cell viability (point mutation of Smc3 C motif provided only for conditional cell growth). All of the mutations produced cohesion defects and most abrogated cohesin deposition, revealing that these motifs are relevant to sister chromatid pairing.

Below, I discuss the analyses of Smc1,3 activities and how those findings influence models of cohesin association with chromatin. In presenting these models, I do not distinguish between cohesins as single rings, double rings or oligomeric complexes. Nor do I discuss these models as requiring cohesins to encircle DNA. In fact, it is equally likely that cohesins make lateral contacts with DNA. The data only make clear that conserved SMC motifs are important for cohesin function and that these activities somehow affect cohesin association with chromatin.

3.1. Gate opening reactions—A swinging gate

Is there direct evidence that Smc1 ATP hydrolysis plays a role in Mcd1 binding? Mcd1 expressed in insect cells is very inefficient in clearing Smc1 from extracts of yeast cells arrested in G_1 (prior to Mcd1 expression). Mcd1 association with Smc1 was greatly increased by the addition of ATP. Since this binding was performed in concentrated extracts, the role of ATP in promoting Smc1-Mcd1 binding could be indirect. However, Mcd1-Smc1 binding in the presence of ATP was significantly reduced when a Walker A Smc1 mutant construct was used instead of wildtype Smc1. This suggests Smc1 binding of ATP is a pre-requisite for cohesin assembly.

Assessed individually, the role for each conserved motif within Smc1,3 is less clear. For instance, C motif mutations in Smc1 abrogated Mcd1 binding in one study while the identical S1130R C motif mutation resulted in robust Mcd1 binding in a parallel study. Complications also arose from

Walker A box mutation analyses: mutations in the Walker A box that abolish Mcd1 binding in one study produced significant (but reduced) binding in the other (Arumugam *et al.*, 2003; Weitzer *et al.*, 2003). One plausible resolution to these conflicting data is that the study in which Smc1 Walker A and C mutants reduced or abrogated Mcd1 binding was performed in extracts that also contained wildtype Smc1 (Arumugam *et al.*, 2003). Wildtype Smc1 simply may out-compete mutant Smc1 for Mcd1 binding, leading the authors to under-estimate the ability of these Smc1 mutant constructs to bind Mcd1. Further complicating these interpretations are findings that Smc1 or Smc3 individually expressed in insect cells both bind Mcd1, suggesting Mcd1 binding can occur in a non-cooperative fashion (Arumugam *et al.*, 2003; Haering *et al.*, 2002; Weitzer *et al.*, 2003).

The finding that Smc1 Walker B mutations abrogate Mcd1 binding predicts that parallel mutations in Smc3 will produce similar effects. Extraordinarily, this prediction was not borne out by experimental analyses: Smc3 protein mutated in any of the Walker A, Walker B, or C motifs bound Mcd1 (Arumugam *et al.*, 2003). These findings support a parallel study showing that Smc3 ATP binding/hydrolysis domains are incidental to Mcd1 binding (Arumugam *et al.*, 2003; Weitzer *et al.*, 2003). Thus, Smc3 binding to Mcd1 is regulated differently (if at all) from that of Smc1. This scenario suggests that Smc3 function has diverged through evolution to provide for more versatile cohesin assembly reactions.

If the C-terminus of Mcd1 binds to Smc3 and the N-terminus of Mcd1 binds Smc1, then both C-terminal and N-terminal fragments produced by Mcd1 cleavage should precipitate with Smc3 (assuming hinge-dependent assembly of Smc1,3 heterodimers) (Gruber *et al.*, 2003; Haering *et al.*, 2002). C-terminal Mcd1 indeed was able to pull down Smc3, but the N-terminal Mcd1 fragment performed poorly in this assay. The authors speculated that Mcd1 binding to Smc1,3 is asymmetric such that C-terminal Mcd1 binding to Smc1 both precedes and promotes N-terminal Mcd1 binding to Smc3 (Arumugam *et al.*, 2003). To test this model, co-immunoprecipitations were performed using headless Smc3 constructs. Headless Smc3 still pulled down Smc1 but Mcd1 association was greatly reduced, in support of a cooperative binding model.

In summary, early models of cohesin regulation suggest that Smc1,3 bipartite complexes coordinate ATP association with asymmetric Mcd1 recruitment. Upon contacting chromatin, ATP hydrolysis promotes Smc1 (or Smc3) to disrupt its contact with Mcd1—opening the gate. After cohesin deposition (often depicted as chromatin entry into the cohesin lumen), ATP re-association promotes Mcd1 re-binding and ring closure. Subsequent studies imply a role for Scc2,4 in regulating Smc1,3 ATP hydrolysis and also in Smc head domain release of Mcd1. Scc2,4 function in cohesin deposition is discussed in detail below (see Section 4).

3.2. Gate opening reactions—A removable gate

That either Smc1,3 head releases Mcd1 to promote chromatin deposition makes some simple predictions. The first of these is that the association of Smc1 to Mcd1 must be labile and reversible for cohesin loading. To test this, the N-terminus of Smc1 was genetically fused to the C-terminus of Mcd1. Once assembled, the resulting cohesin complex would be unable to open via Smc1 release of Mcd1. Surprisingly, the Mcd1-Smc1 fusion construct was fully competent to support cell viability in the absence of both endogenous wildtype Smc1 and Mcd1 proteins. The fusion protein also made cells resistant to the toxic effects of Mcd1 C-terminal overexpression (Mcd1 C-terminal fragments compete with Smc1-Mcd1 binding and is lethal—Gruber *et al.*, 2006; Rao *et al.*, 2001). The viability of cells expressing Mcd1-Smc1 chimeras as the sole source of either Mcd1 or Scm1 function indicates that cohesin deposition occurs independent of Smc1 release of Mcd1.

A swinging gate mechanism of cohesin deposition could instead depend upon labile interactions between Smc3-Mcd1. To test this prediction, the N-terminus of Mcd1 was genetically fused to the C terminus of Smc3. The Smc3-Mcd1 fusion protein sustained cell viability despite being the sole source of both Mcd1 and Smc3 functions (Gruber *et al.*, 2006). Obviously, cohesion deposition is oblivious to Smc3 release of Mcd1 as well as to Smc1 release of Mcd1. Together, these findings confound gate-opening and carabiner models in which either Smc1 or Smc3 ATPase activity is required for Mcd1 release and cohesin loading (Arumugam *et al.*, 2003; Ivanov and Nasmyth, 2005; Weitzer *et al.*, 2003). Alternatively, the cell may be able to open either side of the gate, but how one site versus the other is selected remains a challenge to such a model.

Maybe cohesin deposition requires complete Mcd1 disassociation from Smc1,3. Here, the Mcd1 ''gate'' doesn't just swing open but instead is removed entirely (and then somehow recaptured). To test this model, Mcd1 was fused to both Smc1 and Smc3 (N-terminus of Mcd1 fused to the C-terminus of Smc3 and the C-terminus of Mcd1 fused to N-terminus of Smc1). The resulting Smc3-Mcd1-Smc1 construct was unable to support cell viability (Gruber *et al.*, 2006). At first blush, one might conclude that the Mcd1 gate must be ripped entirely from Smc1,3 to allow for cohesin deposition. However, the authors surmised that cell inviability might instead be due to cohesion relying on the expression of a large and structurally complex fusion protein. Thus, a complementary method was pursued in which Smc1,3 binding to Mcd1 could be re-enforced and in an inducible fashion. An Smc3-Mcd1 fusion protein was generated that also contained an Frb moiety. Frb and FKBP12 dimerize in a rapamycin-dependent fashion (Choi *et al.*, 1996; Ho *et al.*, 1996). Thus, co-expression of Smc3-Mcd1-Frb and Smc1-FKBP12 would generate an Mcd1 gate in which both ends are locked onto appropriate Smc1,3 heads in the presence of rapamycin.

Put another way, expression of Smc3-Mcd1-Frb and Smc1-FKBP12 should produce a ring that that could no longer utilize Smc1,3 ATPase activity. Cells co-expressing Smc3-Mcd1-Frb and Smc1-FKBP12 grew robustly in the absence of rapamycin. In the presence of rapamycin, however, these cells were nearly inviable and capable of producing only severely growth-retarded colonies (Gruber *et al.*, 2006). Analyses of these breakout cells that harbored Frb-FKBP12-stabilized cohesin complexes indeed revealed only moderately elevated levels of cohesion defects. That some population of cells survived at all prompted the authors to suggest that cohesins engineered to bypass Smc ATPase function via rapamycin-induced ring closure "has little discernable effect on cohesion's ability to load onto chromosomes" and "no major deleterious effect on cohesion's ability to establish sister chromatid cohesion" (Gruber *et al.*, 2006). This interpretation raises the formal possibility that Smc ATPase head domains provide only a secondary mechanism for cohesin deposition. It would be interesting to test whether the papillated growth of cells co-expressing Smc3-Mcd1-Frb and Smc1-FKBP12 in the presence of rapamycin might instead occur through second site suppressor mutations or upregulation of alternate pathways.

3.3. SMC Hinges—The gate *de jour*

If cohesin deposition occurs independent of Smc3 release from Mcd1 as well as Smc1 release from Mcd1, what alternatives remain? One option is that cohesin deposition requires Smc1,3 separation through hinge dissociation. To test this model, Smc1 and Smc3 hinge-hinge binding domains were re-inforced using the dimerization motifs described above. Here, FKBP12 was inserted proximal to Smc1's hinge domain (Smc1-FKBP12) and Frb inserted proximal to Smc3's hinge domain (Smc3-Frb). In the absence of rapamycin, expression of either Smc1-FKBP12, Smc3-Frb, or both were competent to support cell viability. The real question could now be addressed: would rapamycin-induced dimerization of the Frb-FKBP12 ligands block cohesin deposition? Addition of rapamycin had no effect on cells expressing either Smc1-FKBP12 or Smc3-Frb alone. However, rapamycin treatment rendered cells expressing both Smc1-FKBP12 and Smc3-Frb completely inviable (Gruber *et al.*, 2006). FKBP12-Frb based stabilization of hinge dimerization also reduced cohesin deposition. These findings suggest a model in which Smc1,3 hinges dissociate to accomodate cohesin loading.

Smc1,3 hinge domains are held together by a doughnut-like structure in which glycine-rich binding surfaces are separated by a small hole (see Section 2.4). The authors posited that only one of the two glycine-rich surfaces disengaged—allowing DNA to enter the hole nestled between the two hinge binding surfaces. Once the DNA was contained within the doughnut lumen, the open binding surface would reseal while the second surface

would open, allowing DNA to pass into the larger cohesin ring lumen. This stepwise hinge dissociation was further posited to depend on conformational changes produced by head domain ATP hydrolysis (Gruber *et al.*, 2006).

The Smc1,3 hinge dissociation model is challenged on many levels. The first issue is one of size. As noted by the authors (Gruber *et al.*, 2006), the doughnut hole is not large enough to accommodate even naked DNA. Thus, both glycine-rich binding surfaces must simultaneously release to allow DNA to pass into the Smc1,3 lumen (Gruber *et al.*, 2006). Since both binding surfaces of the hinge must, at some point, be simultaneously released, this model requires additional and unknown structural features to block cohesin dissociation—reactions otherwise equated to anaphase onset (Arumugam *et al.*, 2003; Gruber *et al.*, 2003, 2006; Haering *et al.*, 2002; Weitzer *et al.*, 2003). The second issue is one of regulating hinge dissociation via Smc1,3 ATPase activity. Smc1,3 coiled coil domains are interrupted several times by loops that are presumably flexible. Thus, it is not clear how conformational changes induced by Smc1,3 head ATP hydrolysis would be transmitted down the stalk to rip apart tightly bound hinge domains. A final challenge to the Smc1,3 hinge dissociation model is illuminated by cells in which cohesin hinges are re-enforced by other dimerization ligands (i.e., Frb and FKB12). The hinge dissociation model predicts that hinges ''glued'' together by Rapamycin-dependent dimerization *prior* to DNA replication would preclude progression of the DNA replication fork. In contrast, the results show that S phase kinetics are completely unperturbed by exogenous hinge sealing activities (Gruber *et al.*, 2006).

3.4. Laying on of the hinges

While Smc1,3 enzymology is unarguably important for cohesin function, I end this section by discussing evidence that supports the notion that cohesin bind laterally to DNA. Even as a ring, cohesins that ''lay'' on top of chromatin would provide for at least two contact sites: the ATPase heads and the hinge dimerization domains. There is evidence that both ends support chromatin binding. Unlike eukaryotic *Sc*Smc1,3, *Bs*SMC ATPase activity is readily detectable. Early studies showed that *Bs*SMC bound DNA independent of ATP. Replacement of the glycine-rich hinge domain with alanines actually improved *Bs*Smc binding to dsDNA—but not to ssDNA (Hirano and Hirano, 2002). While hinge-less Smc mutants no longer bound to DNA, headless mutants (no Smc ATPase domains) appeared quite competent to bind DNA. This observation mirrors early studies that various Smc fragments will bind DNA and that the hinge plays a critical role in this association (Akhmedov *et al.*, 1998, 1999; Hirano *et al.*, 2001). In budding yeast, the Smc1 hinge domain was found to play a critical function in both cohesin deposition and in its proper distribution to CAR sites (Milutinovich *et al.*, 2007).

Other studies support a lateral assocation. Hinge dimerization via glycine-rich surfaces is regulated by proximal sequences. Mutations within a lysine-rich basic patch that flanks the glycine-rich dimerization interface abrogated dsDNA binding and reduced ssDNA binding (Hirano and Hirano, 2006). Notably, even though deposition was abolished, hinge-dependent dimerization itself was unaffected. Mutations within the basic lysine patch also abolished Smc head ATPase activity. These and other findings led the authors to posit that *Bs*SMC homodimers lay down on DNA such that the hinge directly contacts DNA. While the authors further suggested that hinge-DNA binding induces a cycle of head separation and subsequent ATP hydrolysis to open and then close the cohesin ring around DNA (Hirano and Hirano, 2006)—a reasonable alternative is that hinge-DNA binding promotes SMC head attachment to DNA such that the cohesin ring is firmly affixed laterally to chromatin by two separate binding domains.

It is worth speculating that hinge domains that laterally associate with chromatin are tightly regulated. In human cell studies, the Smc3 hinge provides a binding platform for several other proteins. In the first example, the proto-oncogene RET Finger Protein (or RFP) binds directly to the hinge domain of Smc3. Moreover, RFP over-expression and sequestration into the nucleus induced identical effects on Smc3, suggesting that these two proteins functionally associate (Patel and Ghiselli, 2005a). In a second example, Hinderin protein was found to bind Smc3 near its hinge domain. Hinderin appears to compete with Smc1 for Smc3 binding, raising the possibility that Hinderin modulates Smc3's association with Smc1 (Patel and Ghiselli, 2005b). While much less is known about hinge-binding proteins in budding yeast, FRET analyses places the cohesin regulator Pds5 in some proximity (McIntyre *et al.*, 2007).

4. Cohesin Deposition

4.1. Deposition—Scc2 and Scc4

Two slices of bread can be adhered together simply by slathering on sandwich spread (peanut butter, vegemite, etc.). Does such an analogy apply to cohesin deposition and sister chromatid pairing? The differences highlight important aspects of chromosome segregation. For instance, cohesins are not spread along the chromosome length but instead occur only at discrete sites positioned at roughly 10–12 kb intervals (Blat and Kleckner, 1999; Glynn *et al.*, 2004; Laloraya *et al.*, 2000; Lengronne *et al.*, 2004; Megee and Koshland, 1999; Tanaka *et al.*, 1999). Beyond this, however, little agreement exists within the field. For instance, is there a single "coating" of cohesins that holds two sisters together, or do cohesins associate with each

sister (buttering both slices of bread) to generate chromatid pairing? And what of the timing of cohesin deposition—when is deposition essential? Do cohesins stay where they are deposited or are they free to roam the chromosome? There issues are intimately intertwined and their answers will require fundamental changes to current models of cohesin deposition and cohesion establishment (Huang *et al.*, 2005; Losada and Hirano, 2005; Nasmyth, 2005; Skibbens, 2005; Skibbens *et al.*, 2007).

Regardless of the controversies that swirl around the various cohesion models—the observation that cohesins bind chromatin is rock-solid and an excellent starting point from which to proceed. Below, I summarize the role of Scc2 and Scc4 (Scc2,4) in cohesin deposition. From that first section, I discuss the nature of the cohesin complex that is deposited onto chromatin, then turn to studies that map within the cell cycle when cohesins must be deposited. I conclude with analyses of the sites upon which cohesins are deposited and whether cohesin remain adhered to those sites. Other lines of evidence indicate that chromatin remodelers also function in cohesin deposition and this information is reviewed in a following section (see Section 4.2).

A defining phenotype of cohesion mutants is the precocious separation of sister chromatids. In searching for new genes that, when mutated, allow for sister chromatid separation in the absence of APC function, *SCC2* was identified and found to be required, not for cohesion maintenance, but for cohesins to associate with chromatin. In truth, cohesin deposition requires the presence of every cohesin subunit (even Irr1/Scc3)—such that mutations in either *MCD1*, *SMC1,3* or *IRR1/SCC3* abolish chromatin-association of the remaining cohesins. So what differentiates Scc2 function from that of the structural cohesins? Importantly, once sister chromatids become paired, they remain tightly paired despite Scc2 inactivation (using conditional *scc2* mutant strains). Thus, unlike structural cohesins (Mcd1, Smc1,3, or Irr1/Scc3), Scc2 does not function to maintain sister chromatid pairing. Subsequent studies confirmed the role of Scc2, and its binding partner Scc4, in deposition (Ciosk *et al.*, 2000; Toth *et al.*, 1999). Both Scc2,4 proteins and their role in cohesin deposition are conserved through evolution (Bernard *et al.*, 2006; Furuya *et al.*, 1998; Gillespie and Hirano, 2004; Rollins *et al.*, 2004; Seitan *et al.*, 2006; Takahashi *et al.*, 2004; Watrin *et al.*, 2006). Analyses of Scc2 orthologs in *Drosophila* and human cells, Nipped B/NIPBL, profoundly influenced our understanding of several developmental disorders including Cornelia de Lange syndrome, SC phocomelia and Roberts syndrome (Dorsett, 2007 and see Section 7).

What is the nature of the cohesin complex being loaded onto chromatin? For instance, do Scc2,4 assemble cohesin complexes (i.e., drive sequential recruitment of individual subunits)? Co-immunoprecipitations from yeast extracts were used to test the assembly state of cohesin complexes in the absence of Scc2. Loss of Scc2 had little effect on Irr1/Scc3's ability to

pull-down Smc1,3 and Mcd1. Similar results revealed that Smc1, Mcd1, and Irr1/Scc3 also co-immunoprecipitate in the absence of Scc4 (Ciosk *et al.*, 2000). These observations indicate that Scc2,4, although critical for cohesin deposition, are not required for assembly of cohesin complexes in solution. Despite this popularly held view, there is some evidence to the contrary. As described previously, Irr1/Scc3 association with Smc1 requires Mcd1 expression. However, Irr1/Scc3 pulls down robust amounts of Smc1 despite the absence of Mcd1 (G_1 arrested cells) in *scc2* mutant cells. Similar results were obtained for experiments involving *scc4* mutant cells (Ciosk *et al.*, 2000). These findings formally raise the possibility that Scc2,4 may regulate deposition in part by inhibiting inappropriate cohesin assembly reactions in the absence of Mcd1.

The molecular mechanism by which Scc2,4 affects cohesin deposition onto chromatin is not clear. Specifically, the studies discussed above are unable to differentiate between models that Scc2,4 modify pre-assembled cohesins for deposition or simply couple cohesin assembly to deposition. The first of these models is predicated on observations that Smc1,3 mutations that allow for cohesin assembly abolish ATP hydrolysis and cohesin deposition (Arumugam *et al.*, 2003; Weitzer *et al.*, 2003). That both Smc1,3 ATP binding/hydrolysis motifs and Scc2,4 are required for cohesin deposition thus led to the model that Scc2,4 directly regulates Smc1,3 ATPase activity. In one scenario, Scc2,4 promotes the hydrolysis of ATP molecules bound to Smc1,3 heterodimers, which drives the Smc1,3 heads apart. Mcd1 recruitment and ATP re-binding generates a tripartite cohesin ring. Repeated cycles could then allow pre-formed soluble cohesins to be deposited onto (or around) chromatin. However, this mechanism is complicated by observations that cohesins can associate with chromatin early in the cell cycle. Thus, if cohesin rings indeed encircle DNA, soluble cohesin rings might be required to open twice: the first time for deposition during G_1 and a second time during S phase to allow for passage of the DNA replication fork (Bylund and Burgers, 2005; Lengronne *et al.*, 2006). It is not clear if both ring opening events would be under Scc2,4 control. Nor is it clear what would preclude cohesin's dissociation from chromatin upon the second ring opening. Confusing the issue further are conflicting models that Scc2,4-dependent cohesin ring opening could affect dissociation of either (1) Smc1 from Mcd1, (2) Mcd1 from Smc3, (3) Mcd1 from both Smc1,3, or (4) Smc1 from Smc3 (Arumugam *et al.*, 2003; Gruber *et al.*, 2006; Weitzer *et al.*, 2003).

Current analyses of Scc2,4 also provide room for less a complicated mechanism of deposition. Here, I consider a scenario of cohesin deposition in which Scc2,4 simply tethers a cohesin subunit to chromatin during complex formation without affecting assembly reactions per se. In this way, Scc2,4 juxtaposes cohesin assembly reactions that normally occur in solution next to chromatin. Thus, while Scc2,4 does not assemble cohesins,

it couples assembly reactions to deposition. In support of this model, there is evidence that Scc2,4 associates with chromatin in G_1 well before Mcd1 expression and that Scc2,4 remains chromatin-associated into mitosis. This allows for continued deposition. In addition, some amount of Smc1 is chromatin-associated in G_1 and first appears at Scc2,4 chromatin-associated loci (Ciosk *et al.*, 2000; Lengronne *et al.*, 2004). Based on these findings, a model emerges in which Scc2,4 tethers cohesin subunits (such as Smc1) to chromatin prior to Mcd1 expression. Mcd1 expression early during S phase then promotes cohesin assembly with Smc1 both in solution and also tethered to chromatin by Scc2,4. If correct, this model obviates a need for Scc2,4 regulation of Smc1,3 ATPase activity. It also avoids the unwieldy requirement for soluble cohesin rings to undergo multiple open/close reactions (Bylund and Burgers, 2005; Lengronne *et al.*, 2006). That Scc2,4 may simply juxtapose assembly reactions to chromatin predicts that cohesin complexes assembled in solution may be dead-end products. On the one hand, this prediction is in accordance with findings that sister chromatid pairing reactions, even when induced outside of S phase, are always accompanied by de novo Mcd1 synthesis (Strom *et al.*, 2004, 2007; Unal *et al.*, 2004, 2007). On the other hand, this model is clearly overly simplistic in that cohesins deposited during G1 are certainly dynamic in their chromatin associations.

4.1.1. What time is it? The Deposition Clock

In higher eukaryotes, Scc2,4 activity is in part regulated through chromatin recruitment. Scc2,4 chromatin binding requires assembly of pre-replication complexes (ORC, Cdc6, and Mcm2–6) and is precluded by geminin—a replication licensing inhibitor (Gillespie and Hirano, 2004; Takahashi *et al.*, 2004). In budding yeast, however, Scc2,4 activity is largely unregulated: cohesin deposition can and does occur during G_1, S, G_2, and M-phase. However, cohesins deposited after S phase can participate in sister pairing only upon re-activation of the establishment pathway. These and studies in other cell systems reveal that not all deposited cohesins participate in sister chromatid pairing—timing matters (Ciosk *et al.*, 2000; Kim *et al.*, 2002; Lengronne *et al.*, 2006; Milutinovich *et al.*, 2007; Strom *et al.*, 2004, 2007; Uhlmann Nasmyth, 1998; Unal *et al.*, 2004, 2007). Thus, the key question for deposition in unperturbed cells becomes—G_1 or S phase? As we will see below, the issue is of considerable consequence to cohesion establishment models: some of which position cohesin deposition immediately behind the DNA replication fork during S phase while others position deposition in front of the DNA replication fork during G_1 (Skibbens *et al.*, 2007).

Mapping Scc2,4 function relative to the DNA replication fork has provided consistent results—but alternate interpretations of when cohesin deposition is essential. It is clear that G_1 synchronized *scc2* mutant cells released from this arrest and that proceed through the cell cycle at a

restrictive temperature become inviable in perfect coordination with S phase progression. It is also true that G_1 synchronized *scc2* mutant cells that proceed from G_1 to the beginning of S phase (i.e., α-factor released into hydroxyurea) at a restrictive temperature and then proceed through S phase at the permissive temperature remain viable (Ciosk *et al.*, 2000; Lengronne *et al.*, 2006; Uhlmann *et al.*, 1998). On the one hand, these finding strongly suggest that cohesin deposition is essential only during S phase: cells progressing into and through S phase without Scc2,4 become inviable while cells progressing through G_1 phase without Scc2,4 remain viable. This interpretation is supported by both yeast mutational and vertebrate cell photobleaching studies that reveal that cohesins associate with chromatin unstably and remain quite dynamic during G_1, compared to the stable cohesin-chromatin binding during S phase and into mitosis (Gerlich *et al.*, 2006; Haering *et al.*, 2004).

Conversely, these same findings can be interpreted as showing that Scc2,4 are required during G_1 phase. In this scenario, *scc2* mutant cells become inviable during S phase because they have progressed beyond G_1 when cohesin deposition is essential (Bernard *et al.*, 2006; Lengronne *et al.*, 2006). In terms of the experiment described above, *scc2* mutant cells might remain viable despite progressing through G_1 at the restrictive temperature because the mutant protein quickly refolds and loads cohesins immediately when placed back to the permissive temperature near the G_1/S border (Bernard *et al.*, 2006). A G_1 phase role for Scc2,4 is further supported by findings that early S phase *scc2* mutant cells shifted to the restrictive temperature remain viable (Lengronne *et al.*, 2006). However, it remains unclear whether the mutant Scc2 protein was inactivated under the reported conditions—especially since conditional proteins assembled into higher-order complexes (i.e., Scc2,4 heterodimer formation and chromatin association) can become refractile to temperature shifts. Future endeavors are required to resolve the critical issue regarding when cohesin deposition is essential.

4.1.2. Scc2,4 association with chromatin and cohesins

Early analyses of cohesins to a great extent revolved around location (Blat and Kleckner, 1999; Glynn *et al.*, 2004; Laloraya *et al.*, 2000; Megee *et al.*, 1999; Tanaka *et al.*, 1999). Do Scc2,4 also reside at these loci and, if not, how do Scc2,4 affect cohesin deposition? In addressing this issue, initial endeavors to find interactions between Scc2,4 and structural cohesins were unproductive (Ciosk *et al.*, 2000). For instance, mass spectroscopy analyses of immunoprecipitated complexes identified a heteroduplex comprised of Scc2 and Scc4, but structural cohesins were not detected. Subsequently, Scc2,4 were reported to associate with chromatin but ChIP methods capable of assessing loci-specific chromatin associations failed to co-localize Scc2 to cohesin-decorated loci.

In attempting to identify other deposition-associated factors, the issue of deposition-cohesin complex interactions was revisited. A complex identified through TAP-tagged Scc2 enrichment produced minor bands that mass spectroscopy identified as Smc1 and Smc3. Mcd1p (but not Irr1/Scc3) may also be present in the sample—although the presence of Mcd1 could not be confirmed via complementary methods (Arumugam *et al.*, 2003). The authors' interpretation that Scc2 (and Scc4) bind at least transiently to cohesin is certainly enticing, despite the fact that the reciprocal purification of TAP-tagged Mcd1 failed to identify either Scc2 or Scc4. Further supporting the notion of transient Scc2,4-cohesin interactions are analyses by ChIP that revealed that cohesins are first recruited (or assembled) onto Scc2,4-decorated sites and that, post-deposition, cohesins became repositioned to typical CAR sites (Lengronne *et al.*, 2004).

The notion that cohesins are first deposited/assembled onto Scc2,4-decorated loci and then migrate to appropriate CAR sites is quite persuasive but still raises some interesting challenges. One concern is that cohesins are enriched at Scc2,4-decorated loci in the absence of Scc2. At face value, this observation contradicts the model of Scc2,4 in deposition (those authors speculated that an early step in Scc2-dependent loading was not abrogated in this *scc2* allele—Lengronne *et al.*, 2004). A second concern is that Scc2,4 levels occur at orders of magnitude under that observed for cohesin binding. Moreover, there appear to be fewer Scc2,4-decorated loci than observed for cohesins. To achieve the elevated levels of cohesins that occur at chromatin loci, Scc2,4-decorated sites should be highly engaged in continuous cohesin-association reactions. Time course studies of deposition reveal that this is not the case (Lengronne *et al.*, 2004). This disconnect is quite evident at centromeres, where Scc2,4 levels and distribution do not adequately coincide with the greatly elevated cohesin levels and distribution.

Are Scc2,4 solely required for cohesin's association with chromatin? Observations that Mcd1 deposition to Scc2,4-decorated loci are unaffected when Scc2 function is abolished and that a significant amount of Smc1p remains chromatin-associated throughout the cell cycle in the absence of Scc2 suggest not (Ciosk *et al.*, 2000; Lengronne *et al.*, 2004). In fact, early biochemical analyses demonstrated that Smc subunits alone are capable of binding DNA (Akhmedov *et al.*, 1998, 1999; Ciosk *et al.*, 2000). These finding raise the possibilities that cohesins can associate with chromatin independent of Scc2,4—that an alternate mechanism of cohesin deposition exists.

4.2. Deposition—Chromatin remodelers

Studies of various cell systems reveal that cohesins perform multiple roles in higher-order heterochromatin. In work from *Drosophila*, cohesin complexes function as boundary elements of transcriptionally repressed domains. Additionally, cohesins may further sequester factors important

for enhancer-promoter activation to provide for additional mechanisms that disrupt proper chromatin structure and gene regulation (Dorsett, 2007). In fission yeast, it is well documented that Swi6 (HP1 in *Drosophila*) sets up centromeric outer core heterochromatin structures that are required for cohesin recruitment (Pidoux and Allshire, 2004, 2005). Moreover, there is strong evidence that these relationships persist in vertebrate cell models. For instance, human SNF2 (ISWI) and all four cohesin subunits (Smc1, Smc3, Irr1/Scc3, and Mcd1/hRad21) were found to comprise a large NuRD-like chromatin remodeling complex. The association between cohesins and SNF2 is physiologically relevant in that point mutations that abolish SNF2 ATPase-dependent chromatin remodeling activity also disrupts human Rad21 (Mcd1) chromatin association. Thus, the NuRD-like chromatin remodeling complex plays a key role either in cohesin deposition or in stabilizing cohesin's chromatin association (Hakimi *et al.*, 2002). The role of chromatin structure in budding yeast cohesion has been viewed conservatively but is now coming into its own (Riedel *et al.*, 2004).

Growing evidence from budding yeast reveals an important link between cohesion and chromatin structure. First, cohesins are highly concentrated at centromeres. Centromeres are transcriptionally repressed and nuclease resistant—indicative of higher-order chromatin structure. Cohesin recruitment/stabilization onto centromeres requires core kinetochore components (Eckert *et al.*, 2007; Kiburz *et al.*, 2005; Weber *et al.*, 2004). Second, cohesion is required to maintain transcriptionally repressed centromeres (Doheny *et al.*, 1993). For instance, analyses of *ctf7* mutant cells reveal that transcription proceeds through centromeres despite wildtype levels of centromeric cohesins (Ivanov and Nasmyth, 2005; Milutinovich *et al.*, 2007). Thus, it is not the absence of cohesins that allows for transcription read-through at centromeres, but presumably the absence of Ctf7's function in creating higher-order structures—possibly through cohesin pairing reactions (see Section 4). Beyond evidence from analyses of chromatin structure, chromatin remodelers are now linked to cohesion pathways.

DNA accessibility and chromatin structure are tightly regulated by numerous complexes. In general, chromatin remodelers use ATP hydrolysis to either move or displace nucleosomes to expose or sequester DNA sequences (Elgin and Workman, 2000). Other chromatin modifiers act via post-translational modification of histones (acetylation, methylation, phosphorylation, ubiquitination, etc.) to alter nucleosome structure. A series of findings now reveal a link between cohesin deposition and chromatin remodeling complexes in budding yeast, adding to the wealth of studies provided from other model organisms.

4.2.1. RSC

In budding yeast, Ctf13 is an inner core kinetochore protein that binds to the centromere (Cheeseman *et al.*, 2002; McAinsh *et al.*, 2003). In searching through ~4700 yeast gene deletions strains, Baetz and colleagues (2004)

found that *RSC1Δ* and *RSC2Δ*, among other gene deletions, exacerbate *ctf13* mutant cell phenotypes. Rsc1 and Rsc2 are components of different RSC (*R*emodel the *S*tructure of *C*hromatin) complexes—both of which are SWI/SNF ATP-dependent chromatin remodelers (Martens and Winston, 2003; Wang, 2003). Cells deleted for Rsc2 exhibit elevated chromosome loss phenotypes and (conditional) synthetic lethality when combined with either kinetochore (*cse4*, *ctf13*, *ctf14/ncd10*, and *okp1*) or cohesion (*ctf7*, *scc2*, *ctf18*, and *ctf8*) gene mutations. In fact, both *rsc1* and *rsc2* mutant strains exhibit at least modest cohesion defects, indicating that RSC-based chromatin remodeling promotes sister chromatid pairing. Rsc2 function maps to early in the cell cycle—making a structural role in cohesion maintenance during G_2/M unlikely. Notably, *rsc2* mutant cells contain chromosomes decorated with cohesins (global deposition assayed via chromosome spreads), suggesting that RSC is not required for global cohesin deposition (Baetz *et al.*, 2004). Thus, how RSC functions in cohesion remained unclear.

In pursuing the role of RSC components in 2μ plasmid partitioning, a connection between RSC and cohesion was again uncovered (Huang *et al.*, 2004a,b). *rsc2* deletion was found to be lethal when combined with *mcd1* conditional alleles, but note that a mutation in *STH1*, an ATPase component of RSC, and an independently derived *rsc2* deletion strain instead suppress *mcd1* mutant cell phenotypes (Huang *et al.*, 2004a,b). In either case, these findings support the view that RSC plays a critical role cohesion. ChIP-based mapping revealed that Sth1 is uniquely positioned to cohesin-binding sites both at centromeres and along chromosome arms. Intriguingly, Sth1 precedes Mcd1 deposition to CAR arm sites by roughly 15 min. However, Sth1 is constitutively present in the centromeres. These findings suggest that Sth1-RSC function in cohesin deposition and that constitutive Sth1 centromere-association enhances cohesin's deposition onto that locus. Is the role of RSC in cohesion loci-dependent? For instance, Mcd1 associates with centromeres normally in *sth1* or *rsc2* mutant cells (Baetz *et al.*, 2004). However, Mcd1 deposition along chromosome arms is either completely abolished or significantly reduced in *sth1* or *rsc2* mutant cells (Huang *et al.*, 2004a). Loci-specific effects thus may in part account for the apparent discrepancy that was previously noted between these studies (Riedel *et al.*, 2004).

Both Sth1-RSC and Scc2,4 chromatin associations precede cohesin deposition. So how does Sth1-RSC-dependent cohesin deposition onto chromatin differ from that of Scc2,4? Importantly, Sth1 binding to cohesin subunits Mcd1, Smc1, Smc3, and Irr1/Scc3 are readily detected. Thus, cohesin binding to Sth1-RSC may be more robust than for Scc2,4 in which binding assays produced conflicting results (Arumugam *et al.*, 2003; Ciosk *et al.*, 2000). Also, RSC appears to play more of a context-specific role in cohesin deposition—with Sth1-RSC constitutively located to centromeres. At the molecular level, it is worth speculating that RSC might

promote nucleosome repositioning to facilitate cohesin loading at this locus. In turn, RSC could further promote cohesins' function post-deposition. It will be of great interest to see how future endeavors resolve claims that Scc2,4 are solely responsible for cohesin deposition despite observations that cohesins associate to chromatin in *scc2* mutant cells and that RSC also promotes cohesin deposition or stable chromatin association (Huang *et al.*, 2004a; Lengronne *et al.*, 2006).

4.2.2. INO80

More recent findings document another chromatin remodeling complex as critical for sister chromatid cohesion (Ogiwara *et al.*, 2007). The INO80 complex contains several actin-related proteins (Arp4,5, and 8) in addition to the core ATPase Ino80. Using Arp8 as an INO80 representative, *arp8* mutant cells were found to exhibit context specific sister chromatid cohesion defects. For instance, centromere distal loci exhibited greater levels of cohesion defects than centromere proximal loci in *arp8* mutant cells. While Ino80 does bind chromatin, ChIP-based mapping of Ino80-binding sites revealed that its distribution is not uniquely limited to cohesin-binding sites. Data thus far indicates that loss of Arp8 is inconsequential to cohesin deposition. What then is the role of INO80 chromatin remodeling complex in cohesion? The authors correlate INO80 recruitment to other replication fork factors Ctf18 and PCNA—however, little is known regarding how these latter two factors function in cohesion (see Section 7). Intriguingly, INO80 complex exhibits 3′–5′ helicase activity and can promote nucleosome displacement activities important for subsequent assembly of homologous recombination structures (Shen *et al.*, 2003; Tsukuda *et al.*, 2005). In this light, it is worth speculating that replication forks that stall upon encountering pre-loaded cohesin complexes might employ INO80 complexes to re-initiate replication and promote replication-coupled cohesin pairing (Ogiwara *et al.*, 2007). For now at least, it appears that RSC and INO80 chromatin remodeling complexes may participate in different aspects of sister chromatid cohesion.

Two further findings document that more than just ATPase-dependent chromatin remodeling is at work in sister chromatid pairing reactions. ChIP mapping studies revealed that SFN2 bound every DNA sequence bound by Rad21 (Mcd1). While Rad21 was 100% co-incident with that of SNF2, the converse was not true: SNF2 associated to additional sites devoid of Rad21. This differential binding was abolished by treatment with 5-azacytidine—which decreases DNA methylation. In the presence of hypomethylated DNA, SNF2 not only remained bound to the sites previously identified, but was further recruited to new loci. Importantly, 5-azacytidine treatment produced Rad21 binding in parallel to all sites that contained SNF2—including those that originally were bound only by SNF2 and also to sites previously devoid of both SNF2 and Rad21 (Hakimi *et al.*, 2002).

Thus, overlapping modes of regulating chromatin structure (ATPase remodeling and post-translational modifications of histones) appear to play significant roles in cohesin binding and cohesion.

In summary, evidence from many independent labs document the critical roles for chromatin structure and chromatin remodeling complexes in cohesin deposition and regulation (Baetz *et al.*, 2004; Chang *et al.*, 2005; Doheny *et al.*, 1993; Hakimi *et al.*, 2002; Huang and Laurent, 2004; Huang *et al.*, 2004a; Ogiwara *et al.*, 2007). As a field in its infancy, at least with respect to studies in fission yeast and *Drosophila*, we can look forward to exciting revelations that pursue the link between heterochromatin assembly and sister chromatid pairing reactions in budding yeast.

4.3. Cohesin location—Where they are counts

Relevant to deposition, the Koshland lab analyzed Smc1 constructs in which 5 amino acid residues were inserted randomly throughout Smc1's open reading frame (Milutinovich *et al.*, 2007). Several insertions identified Walker A, C motif and hinge domains as critical for binding Mcd1, cell viability and sister chromatid pairing. Two other insertions were of particular interest in that they did not prevent Smc1 assembly with Mcd1, Smc3, and Irr1,Scc3—but nonetheless rendered cells inviable and unable to promote sister chromatid cohesion. The first of these insertions occurred within one of the loops within Smc1's coiled coil rod domain. The second insertion occurred within the hinge domain. Since both loop and hinge mutant Smc1 proteins bound all other structural cohesin subunits, the search was on to identify the manner in which these two domains abrogated cohesion. The answer was not immediately obvious: cohesin complexes that contain loop or hinge mutated Smc1 protein associated with chromatin in an ATP-dependent manner (loop or hinge mutants combined with C motif insertions failed to bind chromatin), required Mcd1 for chromatin association and exhibited cell cycle chromatin association kinetics that mirrored wildtype kinetics. Cohesin complexes containing mutated Smc1 also bound chromatin as tight as wildtype complexes, based on resistance to KCl disruption (Milutinovich *et al.*, 2007).

If cohesins are completely assembled and loaded onto chromatin in a mechanism indistinguishable from wildtype complexes, why are cells harboring Smc1 loop or hinge mutants inviable and defective in sister chromatid pairing? One possibility is that the cohesin complexes that contain loop or hinge mutated Smc1 are present on chromatin at reduced amounts. Indeed, these cohesin complexes are significantly reduced in chromatin binding. However, the amount of Smc1-mutant cohesin binding to chromatin was similar to wildtype levels that proved sufficient for cohesion (Milutinovich *et al.*, 2007). Thus, while reduced chromatin binding levels may have been a contributing factor—this was not the critical effect.

What then, was the more significant difference? Oddly, cohesins that contained these Smc1 mutants were deficient in recruitment to CAR sites. Thus, cohesin enrichment (and to a lesser extent protein levels) is a critical facet of sister chromatid pairing. Conceptually, this makes sense in that distributed cohesins can no longer re-inforce other cohesins located along the chromosome. Presently, it is not known whether Smc1 loop/hinge cohesins reside at Scc2,4 deposition sites and then fail to migrate to CAR sites, whether these cohesins are deposited independent of Scc2,4 or whether these cohesins are deficient in interacting with chromatin remodelers.

5. PDS5 and WAP1/RAD61—Cohesin Regulators

5.1. Pds5 in cohesion

In budding yeast, *PDS5* was identified in a screen for temperature sensitive mutant strains that exhibited enhanced G_2/M lethality (Hartman *et al.*, 2000). Analyses of *PDS5* was also prompted by Pds5 orthologs *BIMD* in *A. nidulans* and *SPO76* in *Sordaria macrospora*. These factors either bind mitotic chromosomes, associate with SMC-like proteins or prevent mitotic catastrophes and genotoxic sensitivity (Denison and May, 1994; Denison *et al.*, 1993; Holt and May, 1996; Huynh *et al.*, 1986; Panizza *et al.*, 2000; van Heemst *et al.*, 1999). More recently, Pds5 homologs have been identified in fission yeast, *Caenorhabditis elegans* (EVL-14), *Drosophila*, and multiple isoforms (Pds5A and Pds5B) exist in vertebrate cells (Celniker *et al.*, 2002; Dorsett *et al.*, 2005; Losada *et al.*, 2005; Sumara *et al.*, 2000; Tanaka *et al.*, 2001; Wang *et al.*, 2003).

Early studies in budding yeast suggested that Pds5 functioned as a structural cohesin. Similar to cohesin subunits, Pds5 is required for sister chromatin cohesion through G_2/M such that defects in Pds5 result in precocious sister chromatid separation (Hartman *et al.*, 2000; Panizza *et al.*, 2000). Pds5 dynamics also mimics that of cohesins: Pds5 associates with chromatin very early in S phase and requires Mcd1 for this recruitment. ChIP analyses confirmed that Pds5 binds specifically to loci decorated by Mcd1. As for other cohesins, Pds5's recruitment to chromatin depends on Mcd1—despite constant levels of Pds5 throughout the cell cycle (Panizza *et al.*, 2000). Pds5 also dissociates from chromatin at anaphase (coinciding with Mcd1 cleavage)—recapitulating the dynamics of other cohesins (Hartman *et al.*, 2000; Panizza *et al.*, 2000). Pds5 contains tandem HEAT repeats—bi-helical or scissor-like structures that can provide a scaffold that support protein interactions (Neuwald and Hirano, 2000).

The notion that Pds5 is a structural cohesin predicted that cohesin-Pds5 subunit binding will be interdependent. Early studies, however, disagreed on the nature of this relationship and, by extension, on the role of Pds5

in maintaining sister chromatid pairing. Panizza *et al.* (2000) reported that loss of Pds5 severely diminished Mcd1 chromatin binding (Panizza *et al.*, 2000). In contrast, Hartman *et al.* (2000) found that Mcd1 remains stably chromatin-associated in the absence of Pds5 (Hartman *et al.*, 2000). Subsequent studies in budding yeast, fission yeast and vertebrate cells (siRNA Pds5 depletion) supported the latter view that Pds5 has little effect on cohesin complex binding to chromatin (Hartman *et al.*, 2000; Losada *et al.*, 2005; Tanaka *et al.*, 2001; Wang *et al.*, 2002; Zhang *et al.*, 2005).

Other findings indicate that Pds5 is not an integral cohesin subunit. In vertebrate cells, Pds5 dissociates from chromatin at KCl levels much less than that required for cohesin dissociation. Furthermore, Pds5 does not associate with the vertebrate 14S cohesin complex but instead migrates with a 9S sub-complex containing only Smc1,3 (but not Mcd1 nor Irr1/Scc3 isoforms) (Losada *et al.*, 1998, 2005; Sumara *et al.*, 2000). That Pds5 is not a cohesin complex subunit is further supported by immunodepletion analyses. Efficient Pds5 clearance from vertebrate cell lysates leaves Irr1/Scc3 (SA1 and SA2), Mcd1, Smc1, and Smc3 in the supernatant and at levels similar to depletions using control antibody. In combination, these findings reveal that Pds5 is either weakly, transiently or only proximally associated with cohesin complexes—at least in vertebrate cell systems. Given that Pds5 is essential for sister chromatid pairing, but is not a structural cohesin nor functions in cohesin deposition, a likely scenario is that Pds5 regulates cohesin in some fashion.

There is evidence that Pds5's role in cohesin regulation involves chromatin structure. DNA replication produces unwieldy chromatin supercoiling and catanations that in part are resolved by topo-isomerase II (Top2). Not surprisingly, Top2 mutant cells fail to properly segregate their chromosomes upon anaphase onset (Holm *et al.*, 1985; Uemura *et al.*, 1987). Recent evidence showed that elevated levels of Top2 suppress *pds5* mutant cell temperature sensitivity (Aguilar *et al.*, 2005). Elevated levels of Top2 failed to suppress other cohesin mutant cell temperature sensitivity. Thus, Top2 suppression of *pds5* mutant cells is affected specifically through cohesin regulation. Cells harboring *top2 pds5* double mutations exhibit severe growth retardation, suggesting either that reduced DNA decatenation exacerbates mutant Pds5 function in cohesion or that defects in cohesin regulation exacerbate mutant Top2 function in DNA decatenation.

5.2. Pds5 modifications

Pds5 protein levels are fairly constant throughout the cell cycle (Panizza *et al.*, 2000; Stead *et al.*, 2003). In contrast, Pds5's function in regulating cohesins (possibly by altering cohesin's accessibility to dissolution reactions) maps predominantly to the G_2/M portions of the cell cycle in all cell models tested (Hartman *et al.*, 2000; Panizza *et al.*, 2000; Stead *et al.*, 2003; Tanaka

et al., 2001; Wang *et al.*, 2003). These observations raise the question—how is Pds5 activity regulated? Using a high copy suppressor screen, Stead *et al.* (2003) identified *SMT4* as reducing *pds5* mutant cell temperature sensitivity. Smt4 is an isopeptidase that cleaves the small ubiquitin-like modifier SUMO (Smt3 in budding yeast) from target substrates (Li and Hochstrasser, 2000; Meluh and Koshland, 1995; Strunnikov *et al.*, 2001). Subsequent analyses reveal that Pds5 is SUMOylated, Pds5 SUMOylation peaks during S phase and then persists into mitosis. Because over-expressed Smt4 rescues *pds5* mutant cell cohesion defects, the authors posited that hypoSUMOylated Pds5 blocks cohesin dissolution (Stead *et al.*, 2003). This prediction is borne out: elevated levels of the SUMO-conjugating E3 ligase Nfi1/Siz2 exacerbate *pds5* mutant cell temperature sensitivity. Thus, SUMO-dependent regulation of Pds5 critically impacts cohesion maintenance (Hartman *et al.*, 2000; Panizza *et al.*, 2000; Stead *et al.*, 2003; Wang *et al.*, 2003).

Pds5 is regulated through more than one mechanism, although the importance of this alternate form of regulation originally was not apparent. Ivanov *et al.* (2002) reported that Pds5 is acetylated *in vitro* by the cohesion establishment factor Ctf7 (see Section 6). However, only a minute per-centage of Pds5 was acetylated *in vitro* compared to other acetylated targets (Ivanov *et al.*, 2002). Furthermore, acetylation-diminished *ctf7* alleles are competent to support cell viability and high fidelity chromosome segregation (Brands and Skibbens, 2005). Thus, acetylation of Pds5 (at least by Ctf7) is transparent to unperturbed cells.

The importance of Pds5 acetylation became clearer through studies of cells challenged in cohesin regulation. To start with, over-expression of Ctf7 rescues *pds5* mutant cell temperature sensitivity. Ctf7 over-expression had no effect on either *smc1* or *smc3* mutant cell phenotypes, attesting to the specificity of this interaction. Importantly, over-expression of acetylation-deficient Ctf7 alleles failed to suppress *pds5* mutant cell phenotypes, providing the first evidence that Pds5 activity is promoted through Ctf7 acetylation reactions (Noble *et al.*, 2006). Formally, it remains unproven that Pds5 is directly regulated by its acetylation, but observations that Ctf7 (Eso1) and Pds5 associate together in both budding and fission yeast make this a plausible model (Noble *et al.*, 2006; Tanaka *et al.*, 2001). Understanding this relationship will require further experimentation into both Pds5 function in regulating cohesins and Ctf7 function in establishment.

5.3. WAP1/RAD61

WAPL (wings apart-like) was first characterized in *Drosophila* as a heteochromatin regulator (Verni *et al.*, 2000). Subsequent studies revealed that elevated WAPL levels co-incide with cancer progression in human cell studies (Oikawa *et al.*, 2004). WAPL co-sediments in an 8S complex that contains Pds5A and also binds Pds5A (but not other cohesin subunits)

(Kueng *et al.*, 2006). Like most cohesins, WAPL association with chromatin requires Mcd1 expression. Intriguingly, WAPL depletion produces unresolved sister chromatids that contain unusually elevated cohesin levels along the chromosome arms. In contrast, WAPL overexpression produces increased cohesion defects. Thus, WAPL appears to drive cohesins off of chromatin particularly during prophase in higher eukaryotes (Kueng *et al.*, 2006 and see Nasmyth *et al.*, 2000; Wang and Dai, 2005; Watanabe, 2005; Yanagida, 2005 for reviews of cohesin dissociation during prophase). In combination, these findings suggest that WAPL and Pds5A perform antagonisitic activities with regards to cohesin dynamics. In budding yeast, the WAPL ortholog is RAD61.

6. Cohesion Establishment and CTF7

Establishment is quite different from cohesin deposition and cohesion maintenance in that the latter two activities are insufficient to form sister chromatid pairing bonds. Here, I address the question ''how is cohesion established between nascent sister chromatids?'' The answer revolves around both cohesin structure and the only essential establishment factor identified to date—Ctf7/Eco1. *CTF7* as a complementation group was first identified from a collection of mutants that exhibited defects in proper *C*hromosome *T*ransmission *F*idelity (Spencer *et al.*, 1990). Portending a future in chromatin structure, *ctf7* mutants allow transcription to proceed through centromeres that are normally transcriptionally quiescent (Doheny *et al.*, 1993). Subsequent to these studies, two groups independently cloned Ctf7/Eco1 (Skibbens *et al.*, 1999; Toth *et al.*, 1999). In combination, those studies showed that cells devoid of Ctf7/Eco1 exhibit sister pairing defects at levels identical to those of structural cohesin mutants, even though chromosomes are fully decorated with cohesins—an observation later documented in full (Ivanov and Nasmyth, 2005; Milutinovich *et al.*, 2007). Cell cycle mapping experiments further revealed that Ctf7 is essential during S phase but not during G_2/M. Thus, Ctf7 is essential for cohesion but unique from both deposition factors and structural cohesins. The name Ctf7 is favored here to acknowledge original endeavors associated with this locus (Doheny *et al.*, 1993; Skibbens and Hieter, 1998; Spencer *et al.*, 1990).

6.1. Hula Hoops and Handcuffs—A historical perspective of cohesion establishment

Genomes are comprised of numerous repetitive DNA elements, motifs, gene families and homologous chromosomes. The cell must thus contend with the problem of pairing specificity: tethering together only the products

of chromosome replication while precluding the pairing of non-sister chromatids. Imagine for a moment the consequences of an explosion in a glue factory—despite the ''efficiency'' in distributing the glue, little functional pairing is likely to occur. Instead, cells in some orderly fashion must apply glue (deposition), identify which sisters to glue together (establishment) and maintain that pairing (structural role) until anaphase onset. Historically, two models were posited to overcome the issue of pairing specificity which were previously termed *Replication-coupled cohesin pairing* and *Replication through a ring* (Skibbens *et al.*, 2007).

6.1.1. Replication-coupled cohesin pairing

The first set of clues into the molecular mechanism of establishment were that Ctf7 interacts in multiple ways with DNA replication factors (Skibbens *et al.*, 1999). For instance, cells harboring alleles of *CTF7* exhibit synthetic lethality when combined with mutations in either *POL30* (Proliferating Cell Nuclear Antigen—PCNA) or *CTF18* (Replication Factor Complex, or RFC, component that loads PCNA onto chromatin). Moreover, *POL30* (PCNA) is a high-copy suppressor of *ctf7* mutant cell lethality. PCNA and most RFCs are essential for cell viability and promote processive DNA polymerase activity (Indiani and O'Donnell, 2006). The findings that Ctf7 functions during S phase and interacts with inter-related DNA replication factors suggests that Ctf7 translocates with the DNA replication fork to pair together cohesins associated on each of the emerging nascent sister chromatids (Skibbens, 2000, 2005). Two tenets of this model are that (1) cohesins are deposited immediately behind the DNA replication fork and (2) cohesins are deposited onto each of the two emerging sister chromatids. Ctf7's job is then to join together the opposing two cohesin complexes (much like Handcuffs) to establish cohesion. Replication-coupled cohesin pairing is attractive because it explains the specificity of sister pairing: only DNA molecules that emerge from the replication fork are acted upon by the fork-tethered Ctf7. This model is consistent with data indicating that cohesins must be loaded onto chromatin during (and before the end of) S phase to participate in sister chromatid pairing (Ciosk *et al.*, 2000; Uhlmann and Nasmyth, 1998).

6.1.2. Replication through a ring

Subsequent studies suggested that V-shaped Smc1,3 heteroduplexes are connected at the open end by a Mcd1 bridge to form a ''huge triangular ring''—much like a giant hula hoop. Cohesins as ring structures thus led researchers to posit an alternate establishment model in which cohesins are loaded ''before the commencement of replication'' such that ''cohesion might be generated as the replication fork passes through the ring—entrapping both sister chromatids inside'' (Gruber *et al.*, 2003; Haering *et al.*, 2002). Replication through a ring suggests that sister chromatid

pairing is passively established: only G_1 cohesin deposition and DNA replication are required. Oddly, the previously-defined roles for Ctf7 and the active participation of DNA replication factors in sister pairing were excluded in devising this establishment model (Gruber *et al.*, 2003; Haering *et al.*, 2002). Regardless, the simplicity of the Replication through a ring model made it incredibly seductive.

6.2. Evidence before the fall(s)

Both models have enjoyed a swell of supporting data. Starting with Replication-coupled cohesin pairing, a long list of DNA replication factors that function in sister chromatid pairing marched into the limelight—led by RFC components. Deletion of *CTF18* not only renders cells inviable when combined with an allele of *CTF7*, but cells devoid of Ctf18 exhibit significant cohesion defects (Hanna *et al.*, 2001; Mayer *et al.*, 2001; Skibbens *et al.*, 1999). Within budding yeast, there are four RFC complexes: each comprised of one large RFC subunit (Rfc1, Ctf18, Rad24, and Elg1) bound to four small RFC subunits (Rfc2-Rfc5) (Majka and Burgers, 2004). Each of these RFC complexes binds to the establishment factor Ctf7 and many of the RFC subunits (Ctf18, Rfc4, and Rfc5) produce cohesion defects when mutated (Hanna *et al.*, 2001; Kenna and Skibbens, 2003; Mayer *et al.*, 2001). RFC complexes play different but also often overlapping roles in DNA metabolism. Rfc1-RFC is essential for DNA replication and loads/unloads PCNA from DNA. Ctf18-RFC also loads/unloads PCNA, but functions in DNA replication/repair checkpoint functions. Rad24-RFC instead loads/unloads the orthologous PCNA-like sliding clamp (composed of Mec1, Rad17, and Ddc1) but still promotes DNA repair similar to Ctf18-RFC. Elg1-RFC maintains genome integrity—but the identify of its associated sliding clamp is less clearly defined (Bellaoui *et al.*, 2003; Bermudez *et al.*, 2003; Green *et al.*, 2000; Hanna *et al.*, 2001; Kanellis *et al.*, 2003; Kenna and Skibbens, 2003; Kondo *et al.*, 1999; Mayer *et al.*, 2001; Naiki *et al.*, 2000, 2001; Paciotti *et al.*, 1998). Of these unique complexes, only Ctf18-RFC is known to promote efficient cohesion.

Beyond RFC complexes, numerous other DNA replication/repair factors promote sister chromatid cohesion. The Polα DNA polymerase-binding factor Ctf4, at least three DNA polymerases (Trf4/Polσ, Trf5, and Pol2), and PCNA all exhibit various levels of cohesion defects when mutated (Edwards *et al.*, 2003; Mayer *et al.*, 2004; Moldovan *et al.*, 2006; Wang *et al.*, 2000, Warren *et al.*, 2004; Xu *et al.*, 2004). Numerous DNA helicases (Chl1, Srs2/Hpr5, Rrm3, and Sgs1) also support efficient sister chromatin pairing (Mayer *et al.*, 2004; Petronczki *et al.*, 2004; Skibbens, 2004; Warren *et al.*, 2004). These helicases function in a diverse array of DNA metabolism including replication, stalled replication fork re-initiation, DNA repair, homologous recombination, Ty1 transposition, meiotic synapsis, etc.

(Bjergbaek *et al.*, 2005; Bryk *et al.*, 2001; Ivessa *et al.*, 2003; Liberi *et al.*, 2005; Lo *et al.*, 2006; Papouli *et al.*, 2005; Pfander *et al.*, 2005; Robert *et al.*, 2006; Torres *et al.*, 2004a,b; Yamana *et al.*, 2005). How and when these various DNA helicases contribute to sister chromatid pairing may depend on either the encountered chromatin state (replicating, nicked, double strand break, recombination) or the additional factors recruited to resolve these structures. For instance, PCNA undergoes multiple SUMO and ubiquitin modifications in response to DNA challenges—some of which appear to alter Ctf7 function and/or promote DNA helicase binding (Hoege *et al.*, 2002; Matunis, 2002; Moldovan *et al.*, 2006; Skibbens *et al.*, 1999; Stelter and Ulrich, 2003). Finally, several S phase checkpoint factors such as Mre11, Xrs2, Mrc1, Tof1, and Csm3 contribute in some way to sister chromatid pairing (Edwards *et al.*, 2003; Mayer *et al.*, 2004; Moldovan *et al.*, 2006; Petronczki *et al.*, 2004; Wang *et al.*, 2000; Warren *et al.*, 2004; Xu *et al.*, 2004). That DNA replication/repair and checkpoint pathways promote efficient sister chromatid establishment support some form of Replication-coupled cohesin pairing.

Replication through a ring models also benefited from further analyses. Several studies strongly support the notion that cohesin rings topologically entrap DNA. For instance, cohesins reportedly resist dissociation from chromatin despite exposure to elevated KCl levels. Moreover, cohesin dissociation is promoted by DNA linearization—suggesting that cohesins might slide off of the free ends (Ciosk *et al.*, 2000; Ivanov and Nasmyth, 2005). Both of these interpretations are now under active debate in that cohesin-binding to chromatin may not be as resistant as initially reported and that chromatin linearization does not uniquely promote cohesin dissociation (Guacci, 2007). Despite this and other disparities, the perception of cohesin structures as single rings that encircle DNA has significantly influenced current cohesion models. Regulation of cohesin ring open/close reactions to affect cohesin deposition (and by extension, establishment) further supports (and in fact is required by) Replication through a ring (Arumugam *et al.*, 2003; Gruber *et al.*, 2006; Ivanov and Nasmyth, 2005; Weitzer *et al.*, 2003). Finally, Separase-mediated proteolysis of Mcd1 nicely complements a ring model—but equally applies to any oligomeric cohesin structure (Campbell and Cohen-Fix, 2002; Huang *et al.*, 2005; Nasmyth, 2005; Skibbens *et al.*, 2007).

6.3. The fall(s)—Replication-coupled cohesin pairing

New findings severely undermine both of the above models. In this section, I highlight the key observations that conflict with the original view of Replication-coupled cohesin pairing. One prediction of the Replication-coupled cohesin pairing model is that Ctf7 binds and translocates with the DNA replication fork to affect sister chromatid pairing (Skibbens, 2000, 2005). A recent study tested this directly by comparing BrDU incorporation

(marking replicating DNA) to chromatin immuno-precipitations of establishment factors Ctf4 (Polα binding factor), Ctf18 (RFC subunit), and Ctf7 (Lengronne *et al.*, 2006). Ctf4 and Ctf18 signals mirrored beautifully that of BrDU incorporation, providing the likely outcome for these DNA replication factors (Hanna *et al.*, 2001; Kenna and Skibbens, 2003; Mayer *et al.*, 2001; Miles and Formosa, 1992). Lengronne *et al.* (2006) further claimed that Ctf7 also co-localizes, and may even translocate, with the replication fork as previously posited (Lengronne *et al.*, 2006; Skibbens, 2000, 2005). Ctf7 indeed appears to coincide with the fork at times. Upon close examination, however, the notion that Ctf7 specifically and significantly co-localizes with the replication fork is not substantiated by the data. The data show that Ctf7 is present at loci devoid of Ctf4, Ctf18, and BrdU signals while loci that provide strong Ctf4, Ctf18, and BrdU signals are devoid of Ctf7. Instead, Ctf7 appears to "chatter" along the chromosome and at greatly reduced levels, compared to Ctf4 or Ctf18. In support of this observation, recent chromatin immunoprecipitations were independently performed for Ctf4, Ctf18, and Ctf7 in both S phase and hydroxyurea-treated cells (early S phase arrest). As before, Ctf4 co-localized with progressing DNA replication forks while Ctf18 co-localized to replication forks predominantly in response to HU perturbation. Importantly, Ctf7 failed to co-localize with either progressing or stalled replication forks (Dr. Katsuhiko Shirahige, personal communication). In combination, these studies suggest that Ctf7 is neither stably recruited to nor translocates with the DNA replication fork. While these observations do not exclude a scenario in which Ctf7 is transiently recruited to both replicating and non-replicating loci and then quickly released, Replication-coupled cohesin pairing requires substantial revision.

While early studies mapped both Ctf7 function and cohesin deposition to S phase (Ciosk *et al.*, 2000; Skibbens *et al.*, 1999; Toth *et al.*, 1999; Uhlmann and Nasmyth, 1998), follow-up studies revealed that cohesion is also established during G_2/M in response to DNA damage (Strom *et al.*, 2004; Unal *et al.*, 2004). The establishment factor involved and signal transduction pathway required to induce this pairing activity remained unknown. Still, Ctf7 was a likely target.

In testing the prediction that Ctf7 activity could be induced during G_2/M, both the Koshland and Sjogren labs assessed post-replicative cohesion establishment in response to DNA double strand breaks. In one of these studies (Unal *et al.*, 2007), temperature sensitive *mcd1* mutant cells held at a temperature permissive for mutant Mcd1 function were arrested in G_2/M. This regimen allows for S phase-based cohesion to be established—but cohesion that could be inactivated in G_2/M upon shifting to a temperature that inactivates the mutant Mcd1 protein. Prior to Mcd1 inactivation during G_2/M, two key events were initiated. First, *HO* endonuclease was induced to generate DNA double strand breaks specifically within chromosome III. Second, wildtype Mcd1 was induced so that, if any new sister pairing

reactions occurred, they would be resistant to a subsequent shift in temperature. Inactivation of mutant Mcd1 without induction of both HO endonuclease (DNA damage) or wildtype Mcd1 resulted in sister chromatid separation. However, co-induction of HO endonuclease and wildtype Mcd1 rescued the sister separation defect on chromosome III. Importantly, functional pairing reactions also occurred on chromosomes I and IV. Since neither chromosome I nor IV were damaged, cohesion establishment occurs separate from the site of DNA damage and also separate from any DNA repair-based replication reactions. The authors further showed that Ctf7 is required for this DNA damage-induced cohesion re-establishment. In fact, over-expression of Ctf7 abolished the need for double strand breaks, suggesting that Ctf7 activity is inhibited upon completion of S phase and re-induced by the DNA damage surveillance system. Further analysis revealed Mec1 (PI kinase DNA damage checkpoint factor) induces Ctf7 activity during G_2/M and that establishment depends on Ctf7's acetyltransferase activity (Unal *et al.*, 2007).

In the second of these studies (Strom *et al.*, 2007), G_2/M-based loci pairing reactions were performed in the presence of uncleavable Mcd1. *HO* endonuclease was again used to generate a discrete DNA double strand break on chromosome III in G_2/M arrested cells. In this study, HO endonuclease was co-induced with the expression of uncleavable Mcd1. Prior studies showed that encorporation of uncleavable Mcd1-cohesin complexes produces unseparable loci (Strom *et al.*, 2004). HO-endonuclease and uncleavable Mcd1 co-induction provided not only for cohesion establishment on chromosome III but also on unperturbed chromosomes as well (Strom *et al.*, 2007). As before, cohesion establishment required Mec1 activation of Ctf7. In combination, these two studies reveal that (1) cohesion establishment is tightly regulated through the cell cycle, (2) damage-induced establishment requires Ctf7, and (3) Ctf7-dependent establishment in G_2/M is regulated through a Mec1 checkpoint signal (Strom *et al.*, 2007; Unal *et al.*, 2007). Intriguingly, ctf7 acetyltransferase activity is required during G_2/M while the requirement for this activity during S phase establishment in unperturbed cells is unclear (Brands and Skibbens, 2005; Strom *et al.*, 2007; Unal *et al.*, 2007). Relevant to Replication-coupled cohesin pairing is that establishment can occur independent of replication/repair fork components such as RFC, PCNA, or DNA polymerases.

A final prediction of the Replication-coupled cohesin pairing model is that cohesins that participate in sister chromatid pairing should interact in some fashion with cohesins on the opposing sister. Despite a number of efforts aimed at capturing higher-order cohesin structures, these endeavors have yet to prove fruitful. While negative data is typically not compelling, it may be telling that at least simple subunit interactions remain unproven (Arumugam *et al.*, 2003; Gruber *et al.*, 2003, 2006; Haering *et al.*, 2002; Ivanov and Nasmyth, 2005; Weitzer *et al.*, 2003). It may be overly hopeful

to suggest that current studies are unable to detect either 1) catenated rings which do not require specific contact sites between rings or 2) cohesins tethered together through contacts other than direct Smc1,3-Mcd1 binding. At any rate, the observations that (1) Ctf7 does not stably associate with the DNA replication fork, (2) DNA damage-induced establishment occurs independent of DNA replication/repair factors, and (3) cohesin oligomers have yet to be isolated place early versions of Replication-coupled cohesin pairing in jeopardy.

6.4. The fall(s)—Replication through a ring

Numerous lines of evidence reveal that Replication through a ring is untenable as an establishment model. Simply put—size matters. The Replication through a ring model is predicated on the notion that cohesins form a single "huge" ring that is roughly 35 nm in diameter (Gruber *et al.*, 2003, 2006; Haering *et al.*, 2002; Ivanov and Nasmyth, 2005). In contrast, each chromatin fiber in budding yeast is maintained in a histone-based 30 nm state of compaction—more relaxed states appear to be artifacts of chromatin exposed to non-physiological low ionic buffers. This dominant 30 nm chromatin state persists throughout the cell cycle and is required to regulate transcription even through mitosis. Moreover, the tremendous stretching (unfolding) of chromatin that occurs during mitosis in response to spindle forces further discloses the level of DNA compaction (Bystricky *et al.*, 2004; Goshima and Yanagida, 2000; He *et al.*, 2000; Horowitz-Scherer and Woodcock, 2007; Pearson *et al.*, 2001). Throw in the role of condensins that stabilize/promote alternative forms of higher-order chromatin structure along the chromosome length (Belmont, 2006; Swedlow and Hirano, 2003)—and it becomes quite clear that the 35 nm cohesin ring has insufficient girth to accommodate two chromatid pairs. Chromosome compaction is even greater in higher eukaryotes, further compounding the size discrepancy.

How can a cohesin ring thus encircle two chromatids? One possibility is that each chromatid exists as an uncondensed 10 nm fiber or even as 2 nm naked DNA helix (Gruber *et al.*, 2006; Haering *et al.*, 2002; Nasmyth and Schleiffer, 2004). These models thus infer that cohesins reside only at highly localized decondensed loci that may even preclude histones—a stipulation that is again at odds with the data. For instance, there is evidence that cohesin rings are pushed along chromatin fibers by transcription complexes (Lengronne *et al.*, 2004). Here, cohesin ring movement requires that extended lengths of chromatin remain uncondensed (or naked)—not just at specific loci. Given the strict transcriptional regulation of mitotic chromatin, that either naked DNA or 10 nm chromatin states persist *in vivo* from G_1/S to anaphase onset is unlikely.

The cohesin ring may be much smaller than originally posited. In biochemical studies, Smc1 head-only constructs mutated at Walker B were

tested for their ability to interact with Smc3 in the absence of hinge dimerization. Smc1 Walker B mutant constructs bound Smc3 but failed to bind Mcd1. These results imply that Smc1,3 heads associate without an Mcd1 bridge (Arumugam *et al.*, 2003; Weitzer *et al.*, 2003). Recent evidence from FRET analyses further suggests that Smc1,3 head domains are very closely apposed (possibly as close as 3 nm) and that Mcd1 instead sits atop the Smc1,3 heads (McIntyre *et al.*, 2007). Of some issue is that nearly identical FRET signals were obtained for Smc1,3 head domains both in the presence and absence of Mcd1 (assessed in G_1 cells or under conditions that repress Mcd1 expression) and in anaphase cells in which Mcd1 proteolysis is thought to promote cohesin dissolution and Smc1,3 dissociation (McIntyre *et al.*, 2007). A full appreciation for how this FRET analyses relates to *in vivo* cohesin structures must remain for further studies. At any rate, direct head-to-head Smc1,3 binding and loss of Mcd1 as an ''extension bridge'' produce a cohesin ring diameter that is smaller than 35 nm. Moreover, evidence from both budding and fission yeast suggest that the cohesin ring folds to bring the Smc1,3 head domains close to the hinges (McIntyre *et al.*, 2007; Sakai *et al.*, 2003), further limiting (or occluding completely) the cohesin ring lumen (Fig. 5.1). In this light, the notion that cohesins associate laterally with DNA, instead of encircling DNA, is not only consistent with all of the evidence currently in the literature but is at least equally reasonable (Campbell *et al.*, 2002; Huang *et al.*, 2005; Nasmyth, 2005).

Can cohesin's requirement for decondensed chromatin be accommodated by chromatin remodelers that move (or remove) histones from discrete loci? Chromatin remodelers co-localize with cohesins and in some cases even precede cohesin deposition (see Section 4.2). Whether remodeling complexes move with cohesins remains unknown. Nor is there any evidence that remodelers co-localize with Scc2,4 where cohesin deposition is thought to occur (Lengronne *et al.*, 2004). However, chromatin remodelers indeed promote efficient cohesion. Genetic interactions between a heterodimeric transcriptional regulator (comprised of Spt16/Cdc68 and Pob3), the establishment factor Ctf4 and DNA polymerase α suggest a model that cohesion and chromatin structure coordination are critical for S phase progression (Zhou and Wang, 2004). The speculative scenario that chromatin remodelers in turn generate chromatin states for cohesin-binding and migration requires further fleshing out.

Interactions between DNA metabolism complexes and cohesins further challenge the one-ring model. As described above, transcription complexes associated predominantly with a single DNA molecule were reported to push cohesin rings along the chromatin fibers. In contrast, the DNA replication fork, complete with two nascent chromatin fibers and leading and lagging strand replisome machineries, is instead hypothesized to simply pass through the rings (Gruber *et al.*, 2003; Haering *et al.*, 2002; Lengronne *et al.*, 2004). The apparent discrepancies between transcription and

replication machineries responses to cohesin barriers present significant challenges to single ring models.

Are there other considerations, beyond size, that are inconsistent with a single ring model of cohesion? In fact, even early studies challenge the Replication through a ring view of establishment. In the first example, cells devoid of Ctf7 function contain cohesin rings that are deposited onto chromosomes at the appropriate times, at appropriate levels and to appropriate sites. Ctf7 mutant cells also replicate their DNA fully without activation of either DNA damage or unreplicated DNA checkpoints. Despite fulfilling the two criteria for Replication through a ring (cohesin deposition and subsequent DNA replication)—sister chromatids are unpaired (Ivanov and Nasmyth, 2005; Milutinovich *et al.*, 2007; Skibbens *et al.*, 1999; Toth *et al.*, 1999). This phenotype, which we term ''cohesin-without-cohesion'' (Skibbens *et al.*, 2007), is recapitulated in Pds5 mutant cells (Hartman *et al.*, 2000). The observations that cells can load cohesins onto chromatin and then replicate their genomes without tethering sisters together is incompatible with a model that establishment occurs via fork passage through cohesin rings. The cohesin-without-cohesion phenotype also undermines the notion that a single ring tethers two sisters together.

More recent studies indicate that cohesins become tethered to other higher-order chromatin complexes. In testing for the role of silenced heterochromatin in cohesion, Gartenberg and colleagues engineered yeast cells in which silenced *HMR* loci can be excised from the full-length chromosome to produce a mini-circle chromosome (Chang *et al.*, 2005). Post-replication, wildtype cells contained tightly paired circular sister chromosomes. In silencing deficient cells, however, the replicated circle chromosomes were unpaired. Importantly, these separated chromosomes were fully decorated with cohesin rings, in essence recapitulating the cohesin-without-cohesion phenotype observed in Ctf7 and Pds5 mutant cells. From this evidence, the authors posited that a ring on one chromatid binds to heterochromatic silencing complexes assembled on its sister to establish cohesion (Chang *et al.*, 2005). Cohesin-heterochromatin pairing may indeed prevail at silenced loci, but the most parsimonious interpretation of this data is that non-silenced loci are tethered together by ring-to-ring interactions (Skibbens *et al.*, 2007).

6.5. Interim models

Establishment solely by DNA fork passage through a single cohesin ring may soon become a historical footnote. The days of replication-coupled cohesion establishment are similarly numbered. In their place are models that now incorporate an essential establishment factor activity (Fig. 5.2). Unfortunately, these newer models of establishment often remain coupled to the concept of single rings around two sisters—and thus are termed interim models.

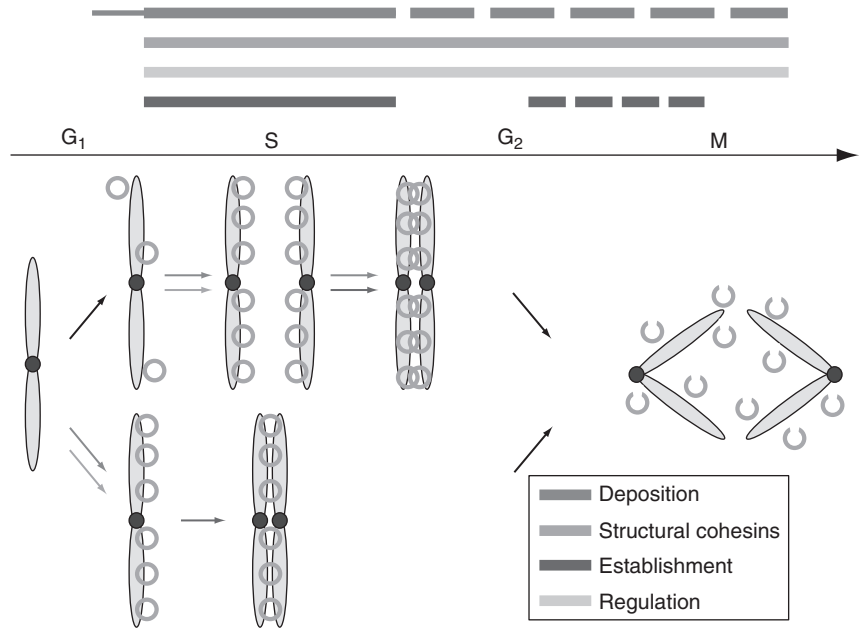

Figure 5.2 Models of cohesion establishment. Top: Portions of the cell cycle when various cohesion pathways perform an essential function. An essential role for Deposition function into G1 remains controversial (thin green line). Deposition occurs beyond S phase, but this activity is not essential (dashed green line). Structural cohesins (purple line) and cohesin regulation via Pds5 (gray line) are required at least from early S phase until anaphase onset during mitosis. Establishment activity via Ctf7 is required during S phase (red line) but can be re-induced (dashed red line) during G2/M in response to DNA damage. Middle and Bottom: Various models of cohesion establishment. Middle—Cohesin deposition and establishment occur concomitantly with DNA replication, although these activities can be separated via mutational analyses. Bottom—cohesions are loaded prior to S phase. Replication and establishment coordination only are required to pair sisters together (Deposition is no longer essential). In most presentations of this model, only single rings are needed to pair sister chromatids together. (See color insert.)

6.5.1. Altered fork geometries

Taking note of the role that Ctf7 and DNA replication factors play in cohesion, one new model was forwarded that replication forks, upon encountering cohesins, partly disassemble (Bylund and Burgers, 2005; Edwards *et al.*, 2003; Lengronne *et al.* 2006). Here, Ctf7 and the RFC/polymerase/clamp factors that encounter a cohesin barrier promote fork disassembly into separate leading and lagging strand replisomes. In this relaxed state, each replisome traverses in turn through the cohesin ring. This altered fork geometry model has numerous deficiencies. For instance, how separated replisomes become re-tethered to re-coordinate leading/lagging strand synthesis remains unclear. This model also fails to explain cohesin-without-cohesion phenotypes reported in many establishment mutant cells (Chang *et al.*, 2005;

Hartman *et al.*, 2000; Skibbens *et al.*, 1999; Toth *et al.*, 1999). A further challenge is that loss of either Ctf7 or establishment RFCs (such as Ctf18) are predicted to retain tethered DNA replisomes complexes. In the absence of altered fork geometry, forks encountering cohesin complexes should stall—producing incomplete replication or inducing bypass replication mechanisms. These predictions thus far are not borne out by the data (Hanna *et al.*, 2001; Mayer *et al.*, 2001; Skibbens *et al.*, 1999).

6.5.2. Ctf7 as a regulator of cohesin ring dynamics

Another recent establishment model encorporates a role for Ctf7 in altering cohesin ring structure in response to the DNA replication fork. Here, fork-coupled Ctf7 either opens G_1-loaded cohesin rings or, once open, tethers cohesin rings to DNA. In either case, the replication fork passes around cohesin rings—not through. After fork passage, the open cohesin rings would close to now surround two chromatin strands (Edwards *et al.*, 2003; Lengronne *et al.*, 2006; Majka and Burgers, 2004; Skibbens *et al.*, 2007). This model makes some clear predictions that at present are not borne out by the evidence. The first of course is that Ctf7 does not appear to stably bind or translocate with the replication fork. Moreover, this model predicts that cells devoid of Ctf7 should either fail to open cohesin rings and thus inhibit fork progression or fail to tether opened rings to chromatin during fork passage, resulting in cohesin dissociation from chromatin. Neither outcome comes to pass—severely undermining the efficacy of replication-around-cohesin models.

In general, another challenge for both of these interim models relates to cohesins deposited after DNA replication. Several studies document that cohesins are deposited onto chromatin during G_2/M when sister chromatids are tightly paired. According to the popular single ring model, the sisters are confined within 35 nm diameter (or less) rings. Thus, it is difficult to conceive why newly deposited cohesins would fail to encompass the tightly confined sister pairs. Yet, numerous lines of evidence demonstrate that cohesins deposited after S phase in unperturbed cells do not participate in cohesion (Strom *et al.*, 2004, 2007; Uhlmann and Nasmyth, 1998; Unal *et al.*, 2004, 2007). Clearly, there is more going on than allowed by these interim models.

6.5.3. The disappearing Ctf7

A final and very interim model of establishment posits that cohesin deposition and DNA replication are solely sufficient for establishment and that cells would soldier on just fine without Ctf7. In supporting this notion, Uhlmann and colleagues recently stated that ''characterization of proteins involved in sister chromatid cohesion has so far not identified any truly essential cohesion establishment factor'' (Lengronne *et al.*, 2006). Maybe the issue is how one defines ''truly'' or ''essential''. Apparently, cell death in the absence of Ctf7 doesn't count. To support this contention, they make the remarkable argument that establishment activities may not really be

conserved because a Ctf7 homolog has yet to be identified in the parasitic microsporidian *E. cuniculi* (Lengronne *et al.*, 2006). While it is no sleight-of-hand to make establishment activity disappear from the genome of this primordial eucaryote, Lengronne *et al.* (2006) fail to make clear that *E. cuniculi's* early divergence produced significant genetic shrinkage through evolution such that this parasite encodes less than 2000 genes. Further, less than half of these genes are characterized—leaving the field wide open to identify establishment activities in these early cells (Fedorov and Hartman, 2004; Katinka *et al.*, 2001). Note that others similarly noted the lack of Ctf7 and also Pds5 and Scc2 from the *E. cuniculi* genome—but conservatively interpreted this to indicate only that these are not structural cohesins (Nasmyth, and Schleiffer, 2004). Possibly a more prudent response is to consider results from numerous labs that show that Ctf7 is highly conserved through evolution: protein sequence, acetyltransferase activity and its essential role in establishment are now well documented in budding yeast, fission yeast, *Drosophila* and higher eukaryotes including human cell systems (Bellows *et al.*, 2003; Brands and Skibbens, 2005; Hou and Zou, 2005; Ivanov *et al.*, 2002; Tanaka *et al.*, 2001; Vega *et al.*, 2005; Williams *et al.*, 2003). Not surprisingly, Ctf7 homologs are of significant clinical relevance. Mutations in EFO2/ESCO2 (Ctf7 homolog in humans) directly produce developmental abnormalities including Roberts syndrome and SC phocomelia (Vega *et al.*, 2005). More recently, EFO2/ESCO2 was identified as one of only ~12 cell cycle control genes that were significantly upregulated in aggressive melanoma cells (Ryu *et al.*, 2007). Thus, while the issue of Ctf7 function in *E. cuniculi* remains temporally ambiguous, a role for Ctf7 orthologs in most eukaryotic cell systems is unequivocal.

6.6. Ctf7—Putting establishment together

Any new model of establishment in eukaryotic cells (with the possible exception of *E. cuniculi*) must include some role for Ctf7 and conform to all of the available data. Below, I summarize what is known of Ctf7 post-translation modifications and chromatin association. I then highlight recent advances that link Ctf7 to cohesins.

Ctf7 is essential during S phase. After S phase, Ctf7 activity is inhibited in some fashion but can be re-activated in G_2/M. Ctf7 is an acetyltransferase and is itself autoacetylated (whether in *cis* or *trans* remains unknown). The role for Ctf7 acetyltransferase activity during S phase is unclear. However, Ctf7 acetyltransferase activity is critical for establishment induced during G_2/M in response to DNA damage. Post DNA damage, the Mec1 checkpoint kinase activates Ctf7, which in turn establishes cohesion globally along chromosomes and independent of DNA replication/repair factors. Ctf7 is also a phosphoprotein and contains a Cdc28/Cyclin-Dependent Kinase (CDK) concensus target site (Ubersax *et al.*, 2003). *ctf7* mutant

cells exhibit synthetic lethality in combination with either *cak1* (CDK Activating Kinase) or *cdc28* alleles, suggesting that CDKs regulate/participate in some form of cohesion (A. Brands and R. V. Skibbens, in press).

Ctf7's relationship to chromatin is equally intriguing. Ctf7 binds chromatin during S phase. Ctf7 also associates with several DNA replication factors (RFC complexes, PCNA, Chl1 DNA helicase) and contains a zinc finger domain (Kenna and Skibbens, 2003; Lengronne *et al.*, 2006; Moldovan *et al.*, 2006; Skibbens, 2004; Skibbens *et al.*, 2007). Despite these findings, specific loci or chromatin states (i.e., damaged, replicating, or DNA under repair) to which Ctf7 associates remain unknown. Nor is there convincing evidence that any of the DNA replication factors recruit Ctf7 to DNA. In one study, Ctf7 recruitment to DNA was reported to require PCNA (Moldovan *et al.*, 2006). However, subsequent analyses suggest that this interpretation might be predicated on expression level artifacts and that Ctf7, mutated to abolish Ctf7-PCNA interactions, binds chromatin with an efficiency identical to that of wildtype Ctf7 (Skibbens *et al.*, personal communication). It is undoubtedly telling that essential DNA replication factors reported to promote efficient cohesion in truth exhibit only weak cohesion defects when mutated (Edwards *et al.*, 2003; Moldovan *et al.*, 2006). Moreover, most DNA replication factors that promote cohesion are non-essential (Hanna *et al.*, 2001; Mayer *et al.*, 2001, 2004; Wang *et al.*, 2000; Warren *et al.*, 2004). In combination, these observations suggest that replication factors perform redundant or indirect establishment activities (Skibbens *et al.*, 2007).

How does Ctf7 establish cohesion? Several lines of evidence reveal a direct link between Ctf7 and the cohesin regulator Pds5. Ctf7 (and the fission yeast homolog Eso1) binds to Pds5 *in vitro* (Noble *et al.*, 2006; Tanaka *et al.*, 2001). Genetic studies support the notion that this interaction is physiologically relevant. In fission yeast, Ctf7 (Eso1) and Pds5 mutations cancel out the other's effect, suggesting that these factors exhibit antagonistic activities (Tanaka *et al.*, 2001). From this, the authors posited that Eso1 (Ctf7) suppresses Pds5's role in blocking cohesion formation (Tanaka *et al.*, 2001). However, the situation is quite different for budding yeast. In budding yeast, *ctf7*, and *pds5* are synthetically lethal and elevated levels of *CTF7* or *PDS5* suppress the others' mutant cell phenotypes (Noble *et al.*, 2006). Pds5 may affect cohesin accessibility to dissolution. Here, Ctf7 suppression of *pds5* mutant cell phenotypes again suggests an S phase specific Ctf7 role to antagonize Pds5 (Noble *et al.*, 2006; Stead *et al.*, 2003). Preliminary FRET evidence suggests that Pds5 (and possibly Ctf7 via Pds5-association) is recruited to the cohesin hinge dimerization domain (McIntyre *et al.*, 2007). However, this study produced only a moderate signal level and failed to detect any differences between Smc1,3 head associations throughout the cell cycle. Given these caveats, Pds5 (and Ctf7) may lay proximal to and regulate hinge-dependent cohesin open/close reactions—if such dynamics exist *in vivo*.

Ctf7-dependent acetylation has opposing effects on different cohesin mutants. For instance, elevated levels of Ctf7 rescues *pds5* mutant cell phenotypes, but not those of either *smc1* nor *smc3* mutant cells. At first blush, these observations suggest a positive role for Ctf7-dependent acetylation in cohesion regulation. However, elevated Ctf7 exacerbates *mcd1* mutant cell phenotypes. Thus, Ctf7 promotes Pds5 function but antagonizes Mcd1 function. These opposing effects are acetylation-dependent: elevated levels of acetylation-deficient Ctf7 fail to suppress *pds5* mutant cell phenotypes but greatly exacerbate *mcd1* temperature sensitivity (Noble *et al.*, 2006).

New models of cohesion establishment must remain speculative (Fig. 5.2). At present, the evidence favors a model in which Ctf7 directly binds Pds5 to affect cohesion between chromatin-bound cohesins. Ctf7 binding to Pds5 is probably quite transient, given the inability to document Ctf7-binding to chromatin by ChIP. Note however that other methods demonstrate readily detectable Ctf7 recruitment to chromatin, suggesting that Ctf7 interacts transiently but also globally with chromatin. At appropriate intersections, Ctf7 modifies cohesins deposited onto each sister chromatid (or indirectly affects cohesion through Pds5) to tether sister chromatids together. The structural nature of this tether is unknown and it is disappointing to the extent that numerous reviews forward only one or two of the possibilities. Formally, the tethers that connect sisters formally could be either a single ring or any one of many oligomeric structures. While speculative, I favor the notion that two cohesins are required for sister pairing—one on each sister. Cohesion establishment then occurs either through cohesin catenations, lateral snapping together or transition to double-ring configurations. These linkages are not predicated on rings that topologically encircle each sister. In fact, the notion that each cohesin complex—like a taco shell—partially envelopes and thus grabs onto chromatin is consistent with a large body of evidence. Such a lateral chromatin association is likely to be stabilized by both Smc1,3 head and hinge domains. Little is known of the role for Irr1/Scc3 in establishing and maintaining cohesion. Future endeavors that reveal how this factor promotes sister tethering are likely to greatly advance the understanding of cohesion.

7. Alternate Cohesion Mechanisms

7.1. Non-cohesin forms of sister chromatid pairing

7.1.1. ORCs

Even early studies foretold a role for non-cohesins in sister chromatid pairing. Loss of structural cohesins results in only 50–60% cohesion defects at both centromere proximal and distal loci (Guacci *et al.*, 1997;

Michaelis *et al*., 1997; Toth *et al*., 1999). Why not 100%? In fact, analyses of cohesion defects at telomeres produced roughly 100% pairing defects in these same mutant alleles (Antoniacci and Skibbens, 2006). Thus, at least for telomeres, cohesins are the only game in town. That study supported prior evidence that pairing mechanisms at other loci (rDNA and centromeres) utilized not only cohesins but also alternate cohesin structures and cohesin-independent complexes (Chang *et al*., 2005; D'Amours *et al*., 2004; Sullivan *et al*., 2004; Zhang *et al*., 2006). The focus shifted to ORC components and Cdc6–factors previously characterized for their essential role in DNA replication initiation (Cook *et al*., 2002; Duncker *et al*., 1999; Nguyen *et al*., 2001; Sanchez *et al*., 1999; Weinreich *et al*., 2001). In part, this change in focus was driven by observations that ORC performs genetically separable roles in transcriptional silencing and replication initiation and that certain *orc* mutant cells exhibit a mitotic arrest, as opposed to G_1/S (Dillin and Rine, 1997, 1998). Subsequent analyses revealed genetic interactions between ORC components and numerous replication/establishment factors (Ctf4, Ctf18, Dcc1, etc.) and that *orc* alleles greatly exacerbate cohesion defects when present in *mcd1* mutant cells (Suter *et al*., 2004). Intriguingly, significant cohesion defects were not evident when either *orc5–1* or *orc2–1* mutant cells were tested.

Still, the findings that ORC, a complex that remains chromatin-associated throughout the bulk of the cell cycle, contributes to cohesion prompted further investigation. In a succession of revelations predicated on Orc2 depletion (*orc2–1* under GAL-regulated expression and glucose-dependent suppression), cells devoid of ORC were found to activate the mitotic checkpoint pathway and delay in G_2/M (Shimada and Gasser, 2007). It is well established from a number of cell systems that cohesion defects indeed activate this checkpoint mechanism (Hoque and Ishikawa, 2002; Mayer *et al*., 2001; Skibbens *et al*., 1999; Toyoda *et al*., 2002). Orc2 depletion produced significant precocious sister chromatid separation levels that approached that for structural cohesin mutants (Shimada and Gasser, 2007). Importantly, additive cohesion defects occurred by coupling Orc2 depletion to either *smc1* or *ctf7* mutant alleles (although this combined effect was still well below 100%). These findings support the notion that ORCs provide for sister chromatid pairing independent of structural cohesins. Do ORC and cohesins act together at all loci? Results from Antoniacci and Skibbens, (2006) predict that while most loci employ both forms of cohesion, some loci will utilize only cohesins while others may utilize only ORCs. Indeed, Shimada and Gasser, (2007) readily detected ORC-dependent cohesion defects at several loci on different chromosomes (*ARS609*, *ARS1413*, and *TRP1*). However, they also identified the *URA3* locus as not requiring ORC for cohesion maintenance. Thus, ORCs likely provide for sister chromatid tethering similar to but independent of cohesins (Shimada and Gasser, 2007).

7.1.2. Silencing complexes

As discussed above (see Section 6.4), studies from the Gartenberg lab revealed that *sir3* mutant cells precociously separate mini-chromosomes that are fully decorated with cohesins (Chang *et al.*, 2005). Based on this evidence, the authors posited that a cohesin ring on one chromatid binds to heterochromatic silencing complexes assembled on its sister. However, the evidence raises the possibility that silencing complexes assembled onto each sister become tethered together to directly promote sister pairing independent of cohesins. In the absence of Sir3, silencing complex structures are incomplete and can no longer participate in cohesion maintenance. In this respect, note that both ORCs and silencing complexes function in cohesion and assemble onto each of the sister chromatids. Cohesins follow suit in that they also assemble onto single chromosomes. At present, there is general agreement that both ORCs and silencing complexes promote cohesion by the pairing together of separate complexes. It seems inevitable that cohesins may again follow suit.

8. Human Disease States and Future Considerations

Each of the four cohesion-related processes (deposition, maintenance, dissolution, and establishment) are essential and required for proper chromosome segregation. Thus, it is not surprising that each is linked to clinical manifestations including developmental abnormalities (Dorsett, 2007). For instance, Cornelia de Lange Syndrome is produced by mutation of either human Scc2/NIPBL or Smc1 (Krantz *et al.*, 2004; Musio *et al.*, 2006; Tonkin *et al.*, 2004). Cornelia de Lange Syndrome (CdLS) individuals often exhibit heart defects, hearing impairments, missing digits and mental retardation. Roberts Syndrome is another developmentally related malady in which afflicted individuals exhibit growth retardation, craniofacial abnormalities, mental deficiencies and flipper-like appendages. Roberts syndrome directly results from mutations in EFO2/ESCO2 (Vega *et al.*, 2005). EFO1/ESCO1 and EFO2/ESCO2 are both orthologs of yeast Ctf7 (Bellows *et al.*, 2003; Hou and Zou, 2005; Vega *et al.*, 2005).

While the link between cohesion defects and developmental abnormalities is firmly entrenched in the literature, a growing body of evidence places cohesion defects at the heart of cancer progression. Human cancer cell lines are highly upregulated for human securin—the Pds1-like regulator of cohesin dissolution. hSecurin was also identified as human proto-oncogene pituitary tumor-transforming gene (PTTG), which is itself tumorgenic (Cohen-Fix *et al.*, 1996; Zou *et al.*, 1999). More recently, EFO2/ESCO2 (one of four human Ctf7 orthologs) was identified from global

genome-wide microarray-based analyses as one of twelve highly upregulated cell cycle control genes in aggressive melanoma cells (Ryu *et al.*, 2007). This link extends to breast cancer. BACH1 is a DNA helicase that is required for BRCA1-dependent repair of double strand DNA breaks. BACH1 mutant cells exhibit gaps between sister chromatids, as do mutations in other BRCA1 pathways (Cantor *et al.*, 2001, 2004). BACH1 is highly conserved and the yeast ortholog Chl1 not only exhibits cohesion defects but also binds Ctf7 (Mayer *et al.*, 2004; Petronczki *et al.*, 2004, Skibbens, 2004). It is worth speculating that mutations in BRCA1-related pathways directly diminish sister chromatid pairing reactions.

Resolving the current controversies regarding 1) when in the cell cycle cohesin deposition is essential, 2) what structure holds sister chromatids together and 3) how is the final bond between sisters established persist as fundamental issues in chromosome segregation. Clear insight into any one of these activities will undoubtedly profoundly shape future cohesion models. The field of sister chromatid pairing is both exciting and constantly evolving. I hope that readers of this article will come away with an appreciation for the current understanding of the field of cohesion and also that we know much less than is often portrayed.

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