

**ESTABLISHMENT OF SISTER CHROMATID
COHESION DURING DNA REPLICATION IN
*SACCHAROMYCES CEREVISIAE***

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Declaration

I, Vanessa de Sousa Ferreira Borges, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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“Science is an imaginative adventure of the mind seeking truth in a world of mystery.”

- Sir Cyril Herman Hinshelwood, English chemist. Nobel prize 1956.

To my parents

Publications Arising from this Thesis

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Abstract

Establishment of sister chromatid cohesion is a process thought to occur as the replication fork passes chromosomal loci bound by the cohesin complex. After fork passage, cohesin holds together pairs of replication products to allow their recognition by the mitotic machinery for segregation into daughter cells. In budding yeast, cohesin is loaded onto chromosomes during the G1 phase of the cell cycle. During S phase, the replication fork-associated acetyltransferase Eco1 acetylates the cohesin subunit Smc3 to promote the establishment of sister chromatid cohesion. At the time of anaphase, Smc3 loses its acetylation again, but the Smc3 deacetylase and the possible importance of Smc3 deacetylation were unknown. I show that the class I histone deacetylase family member Hos1 is responsible for Smc3 deacetylation. Cohesin is protected from deacetylation while bound to chromosomes but is deacetylated as soon as it dissociates from chromosomes at anaphase onset. Non-acetylated Smc3 is required as a substrate for cohesion establishment in the following cell cycle. Smc3 acetylation during DNA replication renders cohesin resistant against the cohesin destabiliser Wapl. However, because in the absence of Wapl cohesin acetylation is dispensable for cohesion establishment, I have turned my attention to additional 'cohesion establishment factors' replication fork-associated proteins required for efficient cohesion establishment. These include Chl1, Ctf4, Ctf18, Mrc1, Tof1 and Csm3. I have used genetic and molecular assays to investigate the relationship of these cohesion establishment factors with the cohesin acetylation pathway. This revealed a contribution of all of these factors to efficient cohesin acetylation. However, removal of the cohesin destabiliser Wapl corrected the cohesion defect in all of the cohesion establishment mutants, except Ctf4 and Chl1. Furthermore, my genetic analysis revealed pronounced synthetic interactions of these two factors with Eco1. Ctf4 and Chl1 therefore define a subset of Eco1-independent cohesion establishment factors, whose possible mechanism of action I have started to investigate.

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Abbreviations

Ab	Antibody
APC/C	Anaphase-Promoting Complex/Cyclosome
ARS	Autonomously Replicating Sequence
ATP	Adenosine triphosphate
bp	base pairs
BSA	Bovine Serum Albumin
CARs	Cohesin associated regions
CdLS	Cornelia de Lange Syndrome
ChIP	Chromatin immunoprecipitation
Chl1	CHromosome Loss
CIP	Calf intestinal phosphatase
cm	centimetre
Csm3	Chromosome Segregation in Meiosis
CTCF	CCCTC-binding factor
Ctf	Chromosome Transmission Fidelity
DAPI	4', 6-diamidino-2-phenylindole
DNA	DeoxyriboNucleic Acid
DNAse	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DSB	Double Strand Break
dsDNA	Double stranded DNA
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
Eco1	Establishment of COhesion
EDTA	Ethylene diamine tetraacetic acid
FACS	Fluorescence activated cell sorting
g	gram
G1	Gap1
G2	Gap2
GAL	Galactose inducible promoter
GFP	Green fluorescent protein
GIN5	Go, Ichi, Nii, San

h	hours
HA	hemagglutinin
HAT	Histone AcetylTransferases
HDAC	Histone DeAcetylase
HEAT	(Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast kinase TOR1)
HEPES	4-(2-hydroxyethyl)-1-piperazineethansulfonic acid
HR	Homologous Recombination
HRP	Horseradish peroxidase
HU	Hydroxyurea
IF	Immunofluorescence
IP	Immunoprecipitation
kDa	KiloDalton
l	litre
M	Molar
mA	milliAmpere
MAT	Mating type
MCM	MiniChromosome Maintenance
<i>MET</i>	Methionine inducible promoter
min	minute
ml	millilitre
mm	millimetre
mM	milliMolar
Mrc1	Mediator of the Replication Checkpoint
nm	nanometre
nmol	nanomolar
NOC	Nocodazole
OD	Optical Density
ORC	Origin Recognition Complex
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline Tween
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
Pds5	Precocious dissociation of sisters
PEG	Polyethylene glycol

PI	Propidium Iodide
PIPES	1,4-piperazinediethanesulfonic acid
PMSF	Phenylmethanesulfonyl Fluoride
Pre-RC	Pre-Replication Complex
Rad	Radiation
rDNA	ribosomal DNA
RFC	Replication Factor C
RNA	RiboNucleic Acid
RNAse	Ribonuclease
rpm	revolutions per minute
s	seconds
S phase	Synthesis phase
Scc	Sister Chromatid Cohesion
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SMC	Structural Maintenance of Chromosome
ssDNA	Single stranded DNA
TAE	Tris Acetate EDTA
TBS	Tris-Buffered saline
TCA	Trichloroacetic acid
TEV	Tobacco etch virus
Tof1	TOpoisomerase I-interacting Factor
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
ts	Temperature sensitive
U	units
UTR	Untranslated Region
UV	Ultraviolet
v/v	volume per volume
w/v	weight per volume
Wapl	Wings apart-like
wt	Wild Type
YNB	Yeast Nitrogen Base
YP	Yeast Peptone
YPD	Yeast Peptone Dextrose
µg	microgram
µl	microlitre

Chapter 1

Introduction

Chapter 1. Introduction

1.1 Sister chromatid cohesion and chromosome segregation

The development and growth of all organisms requires the faithful reproduction of cells. The information encoded in the genome needs to be equally divided between the two daughter cells. Failure in this process results in missing or supernumerary chromosomes, known as aneuploidy, which is a hallmark of cancer cells (Lengauer et al., 1998). Several processes are essential to accurately segregate the two sister chromatids (the newly replicated chromosomes) between the daughter cells. Among these is sister chromatid cohesion, the physical connection that holds sister chromatids from their synthesis during DNA replication onwards. It is this cohesion that counteracts the pulling between sister kinetochores and enables the bipolar attachment to the mitotic spindle. Once sister chromatids of all chromosomes are bi-oriented in metaphase, sister chromatid cohesion is destroyed at anaphase onset and the two sisters are pulled towards opposite poles of the cell leading to their symmetrical segregation. Absence of cohesion leads to failure in the pairwise alignment of sister chromatids at the metaphase plate and, consequently, to random segregation (Nasmyth, 2005).

The mechanism of sister chromatid cohesion was initially thought to be a passive process. However, since the first discoveries in *Drosophila melanogaster* (Kerrebrock et al., 1992; Miyazaki and Orr-Weaver, 1992) the molecular processes and the specific proteins that mediate cohesion have been extensively studied. Sister chromatid cohesion is important not only for chromosome segregation, but also for homologous recombination-mediated DNA repair and for gene regulation. The correct establishment, maintenance and dissolution of cohesion is achieved both by temporal and spatial regulation. Sister chromatid cohesion is mediated by a protein complex named cohesin and several proteins are involved in this process (Table I). Although great progress has been made, several important questions about the players involved in chromosome segregation and their exact mechanism of action still remain unanswered in the cohesion field.

Table I – Cohesin subunits and regulatory proteins nomenclature.

	<i>Saccharomyces cerevisiae</i>	<i>Schizosaccharomyces pombe</i>	<i>Drosophila melanogaster</i>	<i>Homo sapiens</i>
Cohesins subunits	Smc1	Psm1	DmSMC1	hSMC1 α /hSMC1 β
	Smc3	Psm3	DmSMC3	hSMC3
	Scc1	Rad21	DmRAD21	hSCC1
	Scc3	Psc3	DmSA	hSA1, hSA2
Loading	Scc2	Mis4	Nipped-B	NIPBL
	Scc4	Ssl3	CG4302	KIAA0892
Establishment	Eco1 (Ctf7)	Eso1	San, deco	ESCO1, ESCO2
Maintenance	Pds5	Pds5	Pds5	PDS5A, PDS5B
	Wpl1 (Rad61)	Wpl1	Wapl	WAPL
	-	-	-	Sororin (CDCA5)
Dissociation	Pds1	Cut2	Pim	Securin
	Esp1	Cut1	Separase	Separin
	Cdc5	Plo1	Polo	PLK1
	Sgo1	Sgo1, Sgo2	Sgo1	Shugoshin (SGO1)
Replication fork-associated factors	Chl1	SPAC3G6.11	-	DDX11
	Ctf4	Mcl1	-	WDHD1/AND-1
	Ctf18	Chl12	-	CHTF18
	Csm3	Swi3	-	Tipin
	Tof1	Swi1	Timeless	Timeless
	Mrc1	Mrc1	-	Claspin
	Ctf8	Ctf8	-	CHTF8

1.2 The cohesin complex

The first proteins essential for cohesion between sister chromatids were discovered as a result of genetic screens for mutants that had precocious separation of sister chromatids before anaphase in the fruit fly *D. melanogaster* (Davis, 1971; Kerrebrock et al., 1992; Miyazaki and Orr-Weaver, 1992) and in the budding yeast *Saccharomyces cerevisiae* (Guacci et al., 1997; Michaelis et al., 1997). Later studies identified several of these cohesion proteins as subunits of the cohesin protein

complex (Losada et al., 1998; Sumara et al., 2000; Tóth et al., 1999). These initial screens also identified additional proteins that, although are not part of the cohesin complex, have other roles in sister chromatid cohesion (Michaelis et al., 1997; Tóth et al., 1999).

1.2.1 The architecture of the cohesin complex

The cohesin complex is a large ring-shaped protein complex that mediates sister chromatid cohesion. It is composed of four essential core subunits: a heterodimer composed of Smc1 and Smc3, the kleisin subunit Scc1 (also known as Mcd1 or Rad21) and Scc3 (Guacci et al., 1997; Losada et al., 1998; Michaelis et al., 1997; Nasmyth and Haering, 2005). These proteins are conserved from yeast to human (Table I). Further subunits that colocalise with cohesin on chromosomes include Pds5 (Hartman et al., 2000; Panizza et al., 2000; Wang et al., 2002) and Wapl (Gandhi et al., 2006; Kueng et al., 2006).

Smc1 and Smc3 are highly conserved between species and constitute the structural basis for cohesins. They share a common structure with each other and other SMC (Structural Maintenance of Chromosomes) proteins. SMCs are very large proteins that participate in many aspects of chromosome organisation and dynamics. In addition to sister chromatid cohesion, they have roles in chromosome condensation, recombination, gene dosage compensation, DNA repair and transcription regulation (Jessberger, 2002). SMC complexes can be composed of a heterodimer of Smc subunits, as cohesin, or homodimer, as in the bacterial SMC-like complex. Studies in *Escherichia coli* revealed that the bacterial SMC-like complex shared features with cohesin, condensin and Smc5/Smc6 complexes, emphasising the conservation of SMC complexes through evolution (Losada and Hirano, 2005).

Each SMC subunit contains globular domains at the amino- and carboxy-terminal ends separated by a long alpha helical structure, which in turn contains a globular hinge domain in the middle (Figure 1.1). This flexible hinge domain folds back bringing together the two halves of the alpha helix to form long stretches of coiled coil domains. The closely associated N- and C-terminal regions form the head domain that localises on the opposite end of the hinge domain (Haering et al., 2002; Melby et al., 1998). The head domain contains a Walker A motif in the N-terminus and a Walker B and a signature motifs in the C-terminus, which together form a functional ATP binding cassette (ABC) ATPase domain (Hopfner et al., 2000; Saitoh et al., 1994). The

resulting SMC heterodimers form a very large complex with the globular hinge and head domains separated by ~45 nm of coiled coil (Figure 1.1).

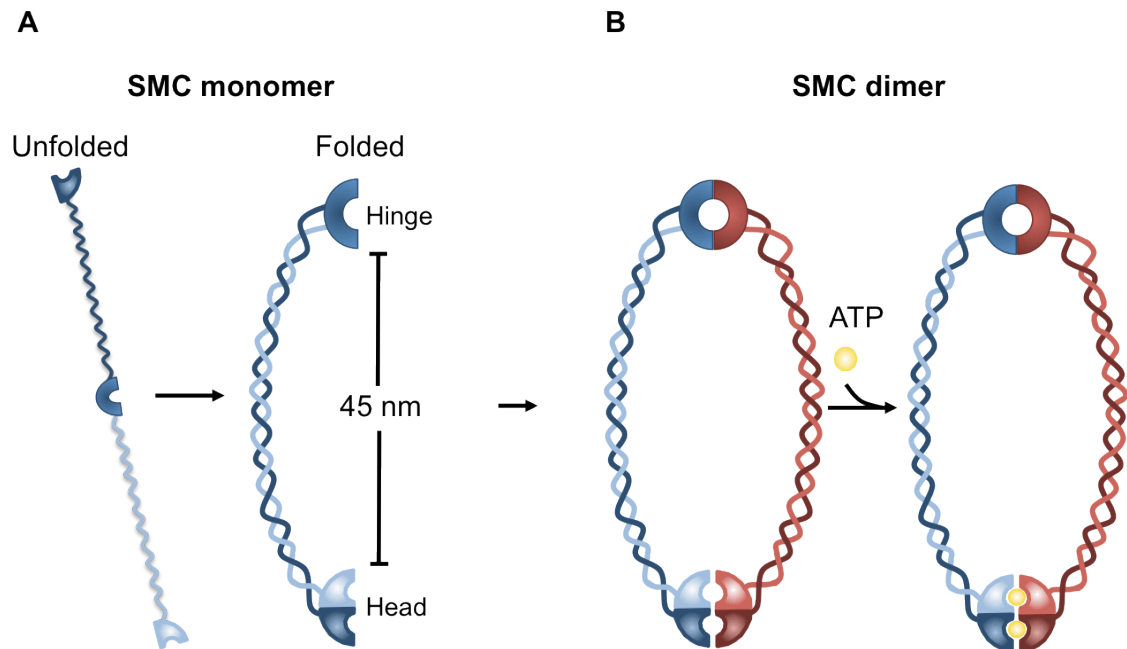


Figure 1.1 – Architecture of Smc proteins and cohesin.

(A) The amino- and carboxy-terminal heads are separated by long stretches of alpha helical coiled coil that contain a globular hinge domain in the middle; the Smc folds back through the hinge domain to form 45 nm long intramolecular antiparallel coiled coils. **(B)** The two SMC proteins dimerise by association of the hinge domains and ATP-dependent interaction of the head domains.

The antiparallel and intramolecular arrangement of the Smc coiled coils has important implications for the structure and function of cohesin. The hinge domains of Smc1 and Smc3 dimerise through hydrophobic interactions (Haering et al., 2002), and are thought to contribute to open and closed conformations due to their intrinsic flexibility. The head domains also interact with each other and this interaction is partly dependent on ATP binding (Haering et al., 2004; Jones and Sgouros, 2001; Löwe et al., 2001). The first images of the cohesin complexes were visualised by electron microscopy studies of human and *Xenopus laevis* cohesin (Anderson et al., 2002) (Figure 1.2A). This showed that cohesins form ring-shaped complexes with the two-armed structure comprising most of the circumference of the cohesin ring.

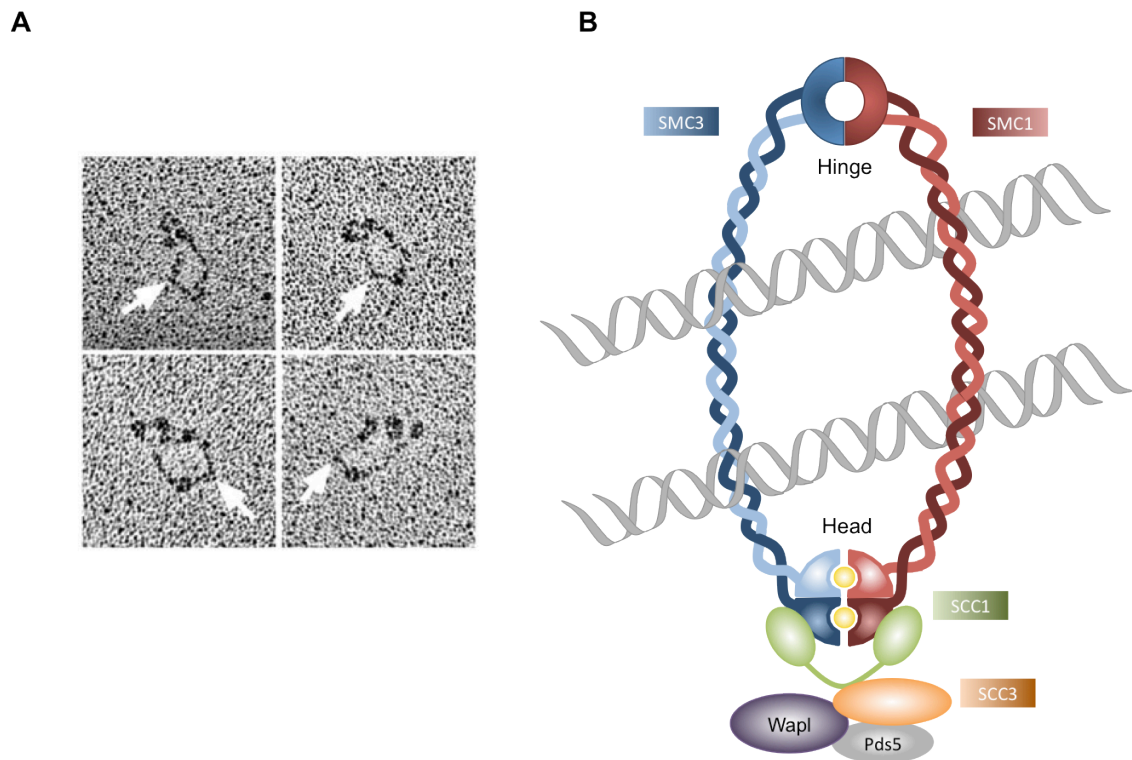


Figure 1.2 – The ring structure of cohesin.

(A) Electron micrographs of the human cohesin complex. Cohesin appears as a ring-shaped structure with the angle of the hinge wide open and the arms clearly separated from each other. Arrows indicate kinks that can often be observed in the coiled-coil of one of the SMCs of cohesin. The regulatory subunits form a globular complex that associates with the head domains of the SMC heterodimer (from (Anderson et al., 2002)). **(B)** Cohesin complex composition. The four core subunits of cohesin associate with each other to form a ring structure. This ring is closed on one side by interactions of the SMC hinge domains. Smc1 and Smc3 fold back on themselves and associate with Scc1 at their head domains. Scc1 links the fourth subunit, Scc3, which in turn associates with Wapl and Pds5.

The SMC head domains are physically connected by the kleisin Scc1 (Sister chromatid cohesion) subunit (Haering et al., 2002), also known as Mcd1 (Mitotic chromosome determinant), creating a tripartite proteinaceous ring. Scc1 acts as a bridge between the SMCs, interacting with the Smc3 head domain via its N terminus and with the Smc1 head domain via its C terminus (Haering et al., 2002). Scc1 was suggested to be a central regulator of cohesin, due to its proximity to the ATPase active site and its ability to connect the heads of the Smc proteins. Indeed, the

cleavage of Scc1 by separase at anaphase onset (Uhlmann et al., 1999) is the basic mechanism underlying chromosome segregation.

The fourth core component of the cohesin complex is the Scc3 subunit, also known as Irr1 (Irrregular), which in vertebrate cells exists as two closely related homologues (SA1 and SA2) (Losada et al., 2000; Sumara et al., 2000). Scc3 interacts with cohesin in an Scc1 dependent manner, binding to its C-terminal region (Haering et al., 2002). Altogether, these four core subunits form the ring-shaped cohesin complex (Figure 1.2).

1.2.2 Cohesin-associated proteins

In addition to the core cohesin subunits, other proteins associate more loosely with the ring complex. One of these proteins is Pds5 (Precocious dissociation of sisters), an accessory protein composed of HEAT repeats (Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast kinase TOR1) (Denison et al., 1993; Panizza et al., 2000; van Heemst et al., 1999), which are known to function in protein-protein interactions. In vertebrates, two Pds5 orthologs can be found, Pds5A and Pds5B (Losada et al., 2005; Sumara et al., 2000). Despite being essential for cohesion in budding yeast, Pds5 is non-essential in fission yeast. In vertebrates, reduction in the levels of Pds5A or Pds5B led to partial defects in sister chromatid cohesion (Losada et al., 2005; Zhang et al., 2007). Thus, different organisms seem to have different requirements for Pds5 function.

Pds5 also associates with Rad61 (Radiation sensitive) (Gandhi et al., 2006; Kueng et al., 2006), which was first described in *D. melanogaster* as the wings apart-like (Wapl) protein. Wapl promotes the dissociation of cohesin from chromosomes and it is generally believed that, together with Pds5 and Scc3, it has an ‘anti-establishment’ activity (Nasmyth, 2011). However, studies in budding yeast suggest an ‘anti-maintenance’ rather than an ‘anti-establishment’ activity for Wapl (Lopez-Serra et al., *submitted*).

A third cohesin interactor, sororin, is essential for proper cohesion during G2 phase (Diaz-Martinez et al., 2007; Rankin et al., 2005; Schmitz et al., 2007). However, in contrast to Pds5, Wapl and the core cohesin subunits, sororin has only been identified in vertebrates so far, and its mechanistic function is not known yet.

1.2.3 Cohesin-related complexes

1.2.3.1 *Bacterial SMC-like complexes*

Bacterial SMC-like proteins contribute to chromosome organisation and compaction and chromosome partitioning. They form homodimers, as revealed by studies of the *Bacillus subtilis* SMC and of the SMC-like MukB protein in *E. coli* (Hirano and Hirano, 2002; Melby et al., 1998). Extensive structural and biochemical characterisation of both *E. coli* MukB and *B. subtilis* SMC have been carried out to date. Despite showing a limited sequence homology with other SMC proteins, MukB shares a similar two-armed structure, as seen by electron microscopy (Melby et al., 1998). MukB interacts with two other proteins, MukE and MukF (Yamanaka et al., 1996; Yamazoe et al., 1999). Similarly, *B. subtilis* SMC interacts with the kleisin ScpA and ScpB (Mascarenhas et al., 2002; Soppa et al., 2002; Volkov et al., 2003). Inactivation of these non-SMC proteins display similar defects in chromosome structure and segregation as the corresponding SMC. Although only one of these two complexes, MukBEF or SMC-ScpAB, is normally found in a given species, a third complex was found to be broadly present in diverse bacteria, MksBEF (MukBEF-like SMC proteins) (Petrushenko et al., 2011).

1.2.3.2 *The condensin Smc2/4 complex*

Condensin is a large protein complex essential for chromosome organisation, compaction and segregation during mitosis, and has additional roles in a wide range of chromosome functions, supporting genome stability, cell differentiation and development. There are two different types of condensins, condensin I and II, with non-overlapping functions. Both condensins are almost ubiquitous in eukaryotes, with only a limited number of organisms having condensin I only, whose function in mitotic segregation is conserved in almost all eukaryotes. The condensin complex is composed of two SMC proteins, Smc2 and Smc4, and three unique non-SMC subunits, a kleisin (Brn1 in budding yeast) and two HEAT repeats (Ycg1 and Ycg4 in budding yeast), that associate in a ring-like structure similar to cohesin. Remarkably, condensin-like complexes can also be found in bacteria and archaea. Thus, condensin's role as chromosome organiser appears to be evolutionary conserved (Hirano, 2012).

1.2.3.3 The Smc5/6 complex

The Smc5/Smc6 complex plays multiple roles in maintaining genome stability, although the precise role of this complex in DNA repair is not yet known. Biochemical studies in *S. cerevisiae* and *Schizosaccharomyces pombe* identified two subcomplexes, one formed by Smc5, Smc6, and Nse2 and the other by Nse1, Nse3 and Nse4 (Sergeant et al., 2005). A third subcomplex was found in *S. pombe* composed of Nse5 and Nse6 (Pebernard et al., 2006). In budding yeast, all components of the Smc5/Smc6 complex are essential (Chen et al., 2009) (Hazbun et al., 2003) (Zhao and Blobel, 2005) (Palecek et al., 2006), and all but Nse5 and Nse6 are required in fission yeast (Andrews et al., 2005; Lehmann et al., 1995; Pebernard et al., 2004; Pebernard et al., 2006). In contrast, the Smc5/Smc6 complex is not essential for cell viability in plants, chicken and humans, suggesting that the essential role of Smc5/6 is not evolutionary conserved (Potts et al., 2006; Stephan et al., 2011; Taylor et al., 2008; Watanabe et al., 2009).

1.2.4 Models for cohesin chromosome tethering

Despite the detailed characterisation of the cohesin structure, the mechanism by which cohesin binds chromatin and tethers the two sister chromatids together remains controversial. The ring-like structure of cohesin inspired different models for the mechanism of cohesion (Figure 1.3).

One model proposes that the cohesin complex holds the two sister chromatids together by physical contact between the Smcs and the DNA (Anderson et al., 2002) (Figure 1.3A). However, this model does not explain how sister chromatid cohesion is lost in anaphase upon cohesin cleavage.

The most favored model is the 'ring model', which proposes that cohesin entraps the two sister chromatids together by topologically embracing them inside the ring (Haering et al., 2002) (Figure 1.3B). With a diameter between 30-35 nm (Anderson et al., 2002), the cohesin ring is theoretically large enough to accommodate both chromatids. The ATP hydrolysis by the cohesin heads would allow them to dissociate and the ring to open. The two sister chromatids would then enter the ring to be subsequently entrapped inside by the re-association of the head domains through the binding of ATP and the Scc1 kleisin subunit. This model provides a simple explanation

for the tethering of the sister chromatids as well as the rapid dissociation of cohesin from chromosomes upon proteolytic cleavage of the cohesin ring (Gruber et al., 2003; Uhlmann et al., 2000). It could also explain how sister chromatid cohesion is established concomitantly with DNA replication, as the two replication products would be entrapped inside the cohesin ring after the replisome passage through its centre. Although the simplicity of this model is attractive, it presents several caveats. The static configuration and the size constraints of the cohesin ring do not explain how cohesins perform their functions during DNA replication, transcription, and DNA repair.

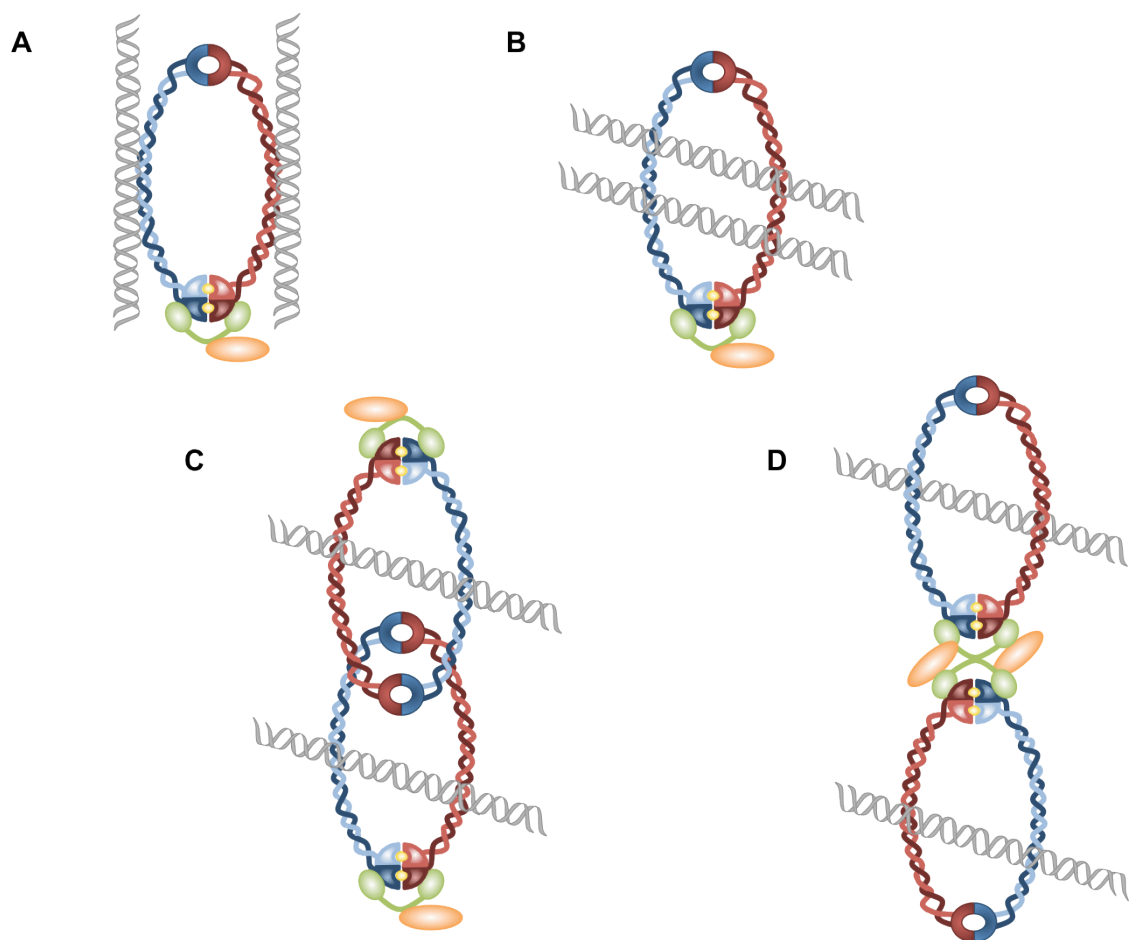


Figure 1.3 – Different models for cohesin chromosome tethering.

(A) This model proposes that the cohesin complex holds the two sister chromatids together by physical contact between the SMC proteins and the DNA. **(B)** The ‘ring model’ proposes that a single cohesin complex topologically entraps sister chromatids inside the ring-shaped structure. **(C-D)** Alternatively, the dimerisation of cohesin complexes may result in the entrapment of each sister chromatid in two different cohesin molecules; this dimerisation can be mediated **(C)** by coiled coil interactions or **(D)** by Scc1, by connecting the SMC heads from the two different cohesin rings.

A variation of the 'ring model' is the entrapment of each sister chromatid in two different cohesin molecules, achieved by the dimerisation of cohesin complexes. In this case, each cohesin ring embraces one, instead of two, sister chromatids. Several configurations of two-ring models have been proposed. The 'handcuff model' proposes that cohesin dimerise through coiled coil interactions (Zhang et al., 2008b) (Figure 1.3C). Alternatively, Scc1 could mediate cohesin dimerisation by connecting the SMC heads from the two different cohesin rings (Figure 1.3D). In contrast to the 'ring model', this configuration would be flexible enough to establish and maintain sister chromatid cohesion.

Even though evidence supports a closed ring structure and the potential for trapping DNA, several aspects of how cohesin binds and entraps DNA remains to be determined. Since the cohesin complex is involved in a vast range of cellular processes, whether one model is sufficient to fit all situations remains to be determined.

1.3 Sister chromatid cohesion and the cell cycle

Cohesin association with chromosomes and subsequent dissociation is a dynamic process that takes place every cell cycle. The correct establishment, maintenance and dissolution of cohesion is achieved both by temporal and spatial regulation. Cohesion can be divided into four stages occurring during the cell cycle (Figure 1.4): (1) cohesin loading onto chromosomes in G1, (2) cohesion establishment during DNA replication in S-phase, (3) cohesion maintenance in G2, and (4) cohesion dissolution at anaphase onset. Cohesin association with chromosomes during G1 depends on a separate loading complex constituted by two proteins named Scc2 and Scc4 (Ciosk et al., 2000). During DNA replication in S-phase, additional proteins are required to establish sister chromatid cohesion. The close association between cohesion and DNA replication suggests that cohesion establishment is tightly coupled with the passage of the replication fork (Uhlmann and Nasmyth, 1998). Cohesion between sister chromatids is maintained during G2 and is dissolved during M phase, thereby allowing chromosomal segregation. These four stages of the cohesin cycle are described in more detail in the following paragraphs.

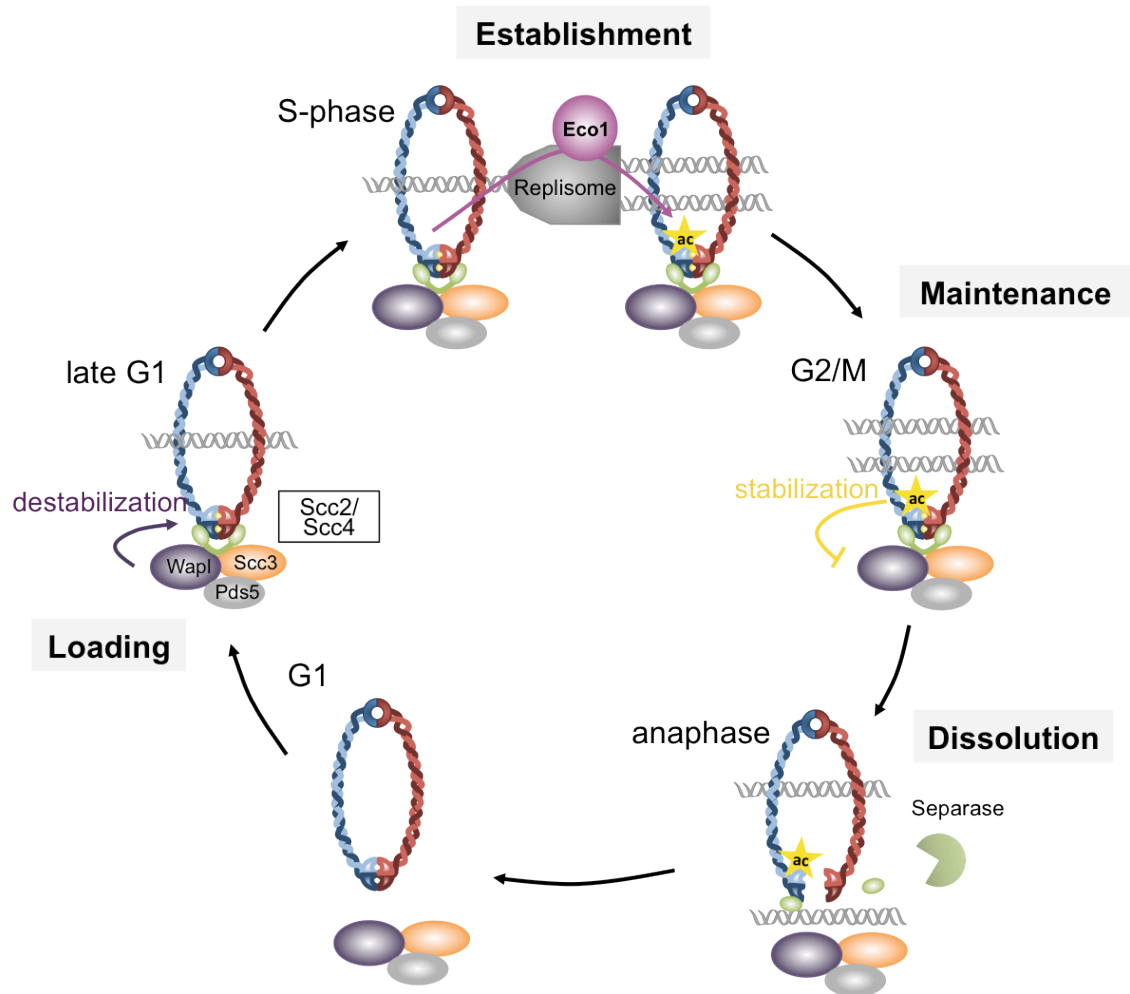


Figure 1.4 – The cohesin cycle in budding yeast.

Cohesin is loaded on chromatin during late G1 in a process that depends on a separate loading complex named Scc2/Scc4. During DNA replication, cohesion is established possibly by topological embrace of the two sister chromatids by cohesin. Sister chromatid cohesion is thought to be coupled to the replication fork passage, and several factors contribute to the establishment of cohesion during S-phase. These include Eco1, which by acetylating Smc3 renders cohesin resistant to the destabilising activity of Wapl. Cohesion is maintained throughout G2 until anaphase onset, when cohesin's Scc1 subunit is cleaved by separase, triggering the segregation of sister chromatids to opposite poles of the cell during anaphase.

1.3.1 Cohesin binding to chromatin

Although the loading of cohesin occurs before S phase, the timing of its binding to chromatin differs among species. In budding yeast, cohesin associates with chromosomes from late G1 phase until anaphase onset, when cohesion is dissolved (Michaelis et al., 1997; Tanaka et al., 1999). In higher eukaryotes, the binding occurs during telophase of the preceding cell division, remaining on chromosomes until the next mitosis (Losada et al., 2000; Sumara et al., 2000).

The localisation of cohesin on chromosomes has been mapped at high resolution in several organisms, revealing non-random distribution and rather specific association sites. In budding yeast, high levels of cohesin binding occur around each centromere, where cohesin is most required to resist the pulling forces of spindle microtubules. In addition, cohesin is also loaded along chromosome arms, on specific Cohesin Associated Regions (CARs) (Blat and Kleckner, 1999; Glynn et al., 2004) (Laloraya et al., 2000; Lengronne et al., 2004). This association with CARs is more discrete, spanning around 0.8-1.0 kb (Laloraya et al., 2000), and occurs on average at 10 kb intervals. Although no specific sequence has been identified in CARs, they are frequently found in AT-rich intergenic regions between convergent transcription sites (Glynn et al., 2004; Lengronne et al., 2004). In higher eukaryotes, cohesin is found associated with the CCCTC-binding factor (CTCF) (Parelho et al., 2008; Rubio et al., 2008; Wendt et al., 2008).

In budding yeast, the loading process to chromosome arms and centromeres requires a 'loading complex' composed of the Scc2 and Scc4 proteins (Ciosk et al., 2000) (Figure 1.4). These proteins were found to interact with soluble cohesin, but on chromosomes they do not colocalise with cohesin and do not bind to CARs (Arumugam et al., 2003; Lengronne et al., 2004). Although cohesin has been extensively studied for many years, the interaction between cohesin and Scc2/Scc4 and the nature of the chromosomal sites to which they bind remain poorly understood. It has been proposed that the loader interacts transiently with cohesin to stimulate the ATPase activity of Smc1 and Smc3, leading to SMC dimer hinge opening or Scc1 dissociation (Arumugam et al., 2003). Alternatively, the loader may participate in local chromatin remodeling of target DNA, which is also required for cohesin binding (Baetz et al., 2004; Hakimi et al., 2002; Huang et al., 2004). The *in vitro* loading of human cohesin on DNA by the human loader complex Scc2/Scc4 (Bermudez et al., 2012) has been described. The authors showed that the Scc2/Scc4 loading complex interacts

with the cohesin complex and is required for cohesin loading. Moreover, loading by Scc2/Scc4 depends on dsDNA containing the pre-replication complex (pre-RC) and the replication of cohesin-loaded DNA increased the stability of cohesin associated with DNA.

The Scc2/Scc4 complex is only needed for the loading of cohesin onto DNA, but not for its maintenance, as cohesins rapidly and independently translocate away from the chromosomal loading sites to accumulate at places of convergent transcription (Lengronne et al., 2004). Coupled with the possibility that cohesin entraps DNA by topological embrace, two different models have been proposed to explain how cohesins translocate along the chromosomes (Figure 1.5).

In the 'sliding model', the cohesin rings are thought to be pushed by the transcription machinery, therefore sliding along the chromosomes from their original binding sites to their final places. In this model, cohesin remains topologically bound to the chromosomes. Alternatively, the 'reloading model' predicts that cohesin dissociates from its original binding sites and is re-associated at downstream sites, in a process dependent on the Scc2/Scc4 loader complex (Ocampo-Hafalla and Uhlmann, 2011).

Conserved Scc2 homologs have been identified in all eukaryotic species, including Mis4, Nipped-B, XSc2, and NIPBL, in fission yeast, fruit flies, frogs, and humans, respectively (Furuya et al., 1998; Gillespie and Hirano, 2004; Rollins et al., 2004; Tonkin et al., 2004). Although highly divergent, proteins that are functionally analogous to Scc4 have also been identified in other species (Bernard et al., 2006; Seitan et al., 2006; Watrin et al., 2006).

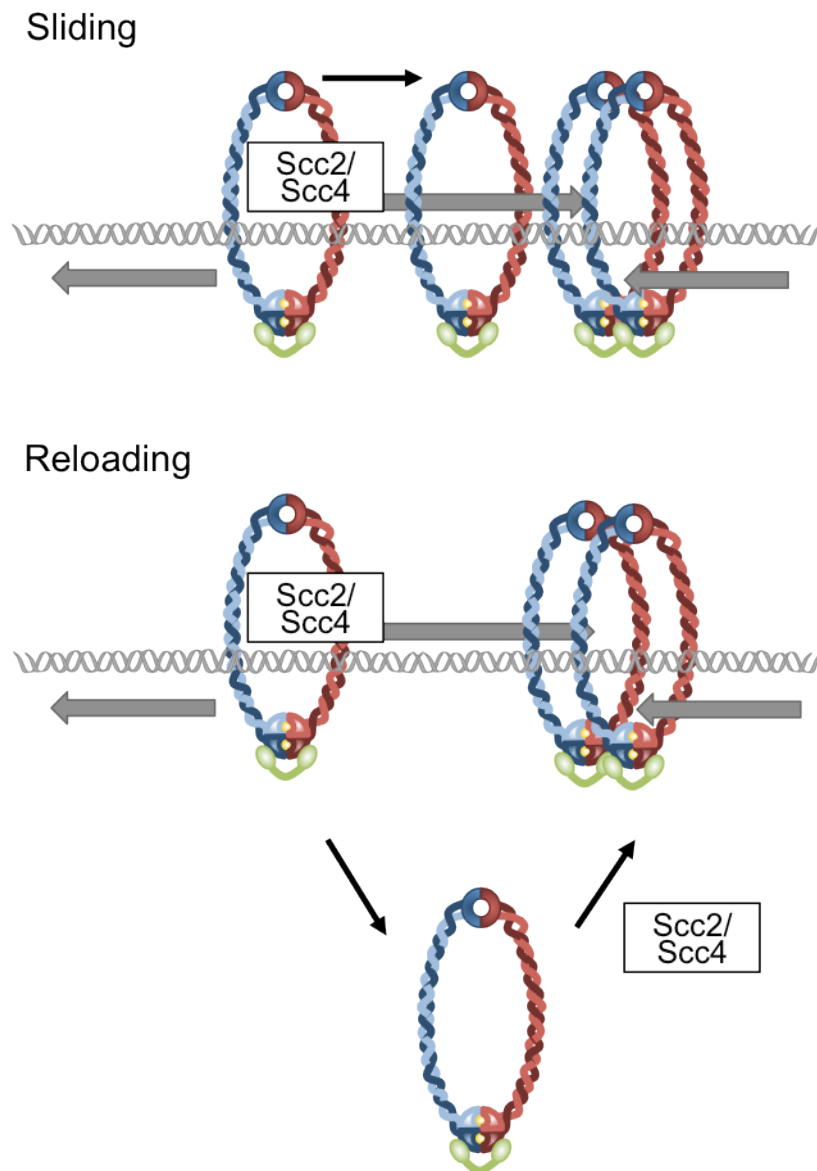


Figure 1.5 – Models of cohesin translocation along chromosomes.

The 'sliding model' predicts that cohesin is pushed by the transcription machinery, sliding along the chromosomes from their original binding sites to downstream sites, while remaining topologically bound to chromosomes. The 'reloading model' predicts that cohesin dissociates from its original binding sites and re-associates at downstream sites, in a process dependent on the Scc2–Scc4 loader complex.

1.3.2 Establishment of cohesion

1.3.2.1 *Building cohesion at the replication fork*

Cohesin loading onto chromosomes is a pre-requisite to establish sister chromatin cohesion, but it is not sufficient on its own. Various observations support the idea that chromatin-bound cohesin exists in two different states: cohesive and non-cohesive. In order to become cohesive, a more stable interaction between cohesin and chromosomes is required. Several lines of evidence suggest that at least a subpool of cohesin becomes stabilised on chromosomes after DNA replication. In budding yeast, expression of Scc1 after S-phase completion leads to binding of cohesin to chromosomes without sister chromatid cohesion being generated (Uhlmann and Nasmyth, 1998). In mammalian cells, approximately one third of all cohesin becomes stably linked to chromosomes during the course of S-phase (Gerlich et al., 2006). This close association between cohesion establishment and the replication fork passage would explain how cohesion is prevented between unrelated sequences, occurring only between newly synthesised sister strands emerging from the replication fork. However, what happens at replication forks to establish sister chromatid cohesion is still poorly understood.

1.3.2.2 *Establishing cohesion in S-phase via Eco1-mediated acetylation*

The discovery and characterisation of the evolutionarily conserved acetyltransferase Eco1 (Establishment of cohesion) was possibly the strongest evidence for the requirement of an additional step for cohesion generation during DNA replication. Although it is not part of the cohesin complex, Eco1 is required for cohesion establishment during S-phase as its inactivation results in a failure to generate cohesion, even though cohesin is still loaded onto chromosomes in G1 (Ivanov et al., 2002; Skibbens et al., 1999; Tóth et al., 1999). Eco1 associates with the DNA replication fork, probably by its physical interaction with the DNA polymerase processivity factor PCNA (Proliferating Cell Nuclear Antigen) (Lengronne et al., 2006; Moldovan et al., 2006). To reach stable sister chromatid cohesion, Eco1 acetylates two evolutionary conserved lysine residues of the cohesin subunit Smc3 (K112 and K113) during DNA replication (Ben-Shahar et al., 2008; Ünal et al., 2008; Zhang et al., 2008a). Smc3 acetylation explains the fundamental mechanism of action of Eco1 in the establishment of cohesion, as substitution of both lysine residues with non-acetylatable

mutations leads to a similar phenotype as *eco1*-deficient cells. Conversely, an acetylation-mimicking mutation results in viable cells even in the absence of Eco1, which is an otherwise essential gene. However, introduction of acetylation-mimicking mutations in Smc3 is not the only condition that makes Eco1 dispensable for sister chromatid cohesion. Inactivation of Wpl1 from budding yeast cells can similarly promote cohesion independently of Eco1 function (Ben-Shahar et al., 2008; Rowland et al., 2009; Sutani et al., 2009), suggesting that Smc3 acetylation blocks the counteracting action of Wpl1 on cohesin. The mechanism of action of Wpl1 in promoting cohesin dissociation from chromosomes is not yet understood. One simple hypothesis is that Wpl1 would control the ATPase activity of Smc3, as this is essential for the association of cohesin with DNA. In turn, Wpl1's effect would be suppressed upon Smc3 acetylation. Alternatively, Eco1-mediated Smc3 acetylation could simply destabilise the interactions between Wpl1 and the cohesin complex.

In addition to Wpl1, point mutations in the cohesin subunit Scc3 and Pds5 were also described to suppress lethality caused by the absence of Eco1 (Rowland et al., 2009; Sutani et al., 2009). One possible explanation is that these mutations abrogate the interactions between Wpl1 and the cohesin complex, indirectly resulting in the absence of the destabilising activity of Wpl1. In vertebrates, an additional mediator of sister chromatid cohesion is sororin (Rankin et al., 2005; Schmitz et al., 2007), which upon Smc3 acetylation binds to Pds5, thereby counteracting Wpl1's destabilising activity (Nishiyama et al., 2010). So far, no homologs of sororin were found in yeast, whereby the mechanism by which Smc3 acetylation counteracts Wpl1 destabilising activity in yeast remains to be determined.

Smc3 acetylation occurs in S-phase during DNA replication and is restricted to chromosome-bound cohesin (Ben-Shahar et al., 2008; Ünal et al., 2008). Acetylation remains stable during G2 phase until anaphase onset, when Smc3 loses its acetylation, remaining undetectable during G1. The enzyme counteracting Eco1's activity and promoting deacetylation during anaphase remained unknown.

1.3.2.3 Additional establishment factors at the fork

In addition to Eco1, several non-essential cohesion establishment factors contribute to efficient sister-chromatid cohesion in as yet unknown ways, although many of these factors are functionally related to DNA replication fork machinery (Hanna et al., 2001; Mayer et al., 2001; Mayer et al., 2004; Petronczki et al., 2004;

Skibbens, 2004; Warren et al., 2004). In contrast to essential cohesion components, these non-essential genes can be deleted without loss of cell viability, although it leads to mild precocious sister chromatid separation. These non-essential factors are required for the establishment of cohesion during S phase, but not for the maintenance of cohesion once it is established. Recent studies divided these factors into two genetic epistasis groups (Xu et al., 2007), where the simultaneous inactivation of members of the different pathways resulted mostly in synthetic-lethal interactions, while deletion of the components of either group did not result in loss of cell viability. The two cohesion pathways identified are mediated through two complexes: the Tof1/Csm3 complex, with Ctf4 and Chl1 as additional members of the same pathway, and the Ctf18-RFC complex, which also contains Mrc1 in the same group.

The alternative Ctf18-Dcc1-Ctf8-Replication Factor C (RFC)-like complex includes some of the first nonessential genes reported to have roles in sister-chromatid cohesion. During DNA replication, this complex is required to load PCNA onto DNA (Bermudez et al., 2003; Mayer et al., 2001), which in turn associates with and promotes DNA polymerase processivity at the replication fork. Deletion of Ctf18 (Chromosome transmission fidelity) results in a reduction of PCNA levels in the vicinity of replication forks in early S phase (Lengronne et al., 2006). Inactivation of the RFC core subunits and PCNA itself also lead to cohesion defects. In addition, PCNA genetically interacts with Eco1 (Moldovan et al., 2006; Skibbens et al., 1999), although it is not known whether PCNA recruits Eco1 to replication forks. In addition to its function in sister chromatid cohesion, Ctf18-RFC is also involved in replication checkpoint activation and may have a role in the DNA damage replication checkpoint (Bellaoui et al., 2003; Crabbe et al., 2010; Kubota et al., 2011; Naiki et al., 2001). Mrc1 (Mediator of the replication checkpoint), the other component in the first epistasis group, is an S-phase checkpoint mediator required for DNA replication, which contributes to the establishment of cohesion in an unknown manner (Katou et al., 2003; Xu et al., 2004).

The second pathway includes Ctf4, a component of elongating replication forks that interacts with several replication proteins, including the GINS (Go, Ichi, Nii, and San) complex and DNA polymerase alpha/primase. The interaction of GINS and Ctf4 has an important role in coupling the MCM (Minichromosome Maintenance) DNA helicase and the DNA polymerase alpha at the replication fork (Gambus et al., 2009; Hanna et al., 2001; Tanaka et al., 2009; Zhu et al., 2007). Ctf4 is also important for the elongation of replication forks (Lengronne et al., 2006), which together with his function

of coupling the DNA helicase and the primase suggests a role for Ctf4 in lagging strand reactions. It has been reported that cells lacking Ctf4 show synthetic lethality with the *dna2-2* mutation (Formosa and Nittis, 1999), which is a nuclease-helicase involved in Okazaki fragment processing. Thus, similar to the prokaryotic replication systems (Tougu and Marians, 1996), Ctf4 could regulate the Okazaki fragment processing during lagging strand replication. In addition, Ctf4 has also been linked to diverse pathways, such as DNA damage checkpoint response, spindle assembly checkpoint, microtubule function and chromosome structure (Tong et al., 2004).

A link between cohesion establishment and lagging strand reactions has also been suggested by the Chl1 (Chromosome loss) helicase (Farina et al., 2008; Gerring et al., 1990; Petronczki et al., 2004; Skibbens, 2004). Chl1 is important to stimulate the flap endonuclease activity of Fen1, a key enzyme for proper and efficient Okazaki flap processing (Farina et al., 2008). Furthermore, Chl1 was shown to physically interact with both Fen1 and Eco1 (Rudra and Skibbens, 2012; Skibbens, 2004). Nonetheless, little is known about the role of Chl1 at replication forks to establish sister chromatid cohesion. In addition to its role in cohesion, Chl1 is also important for transcriptional silencing, rDNA recombination, and aging (Das and Sinha, 2005).

Lastly, this group also contains Tof1 (Topoisomerase I-interacting factor) and Csm3 (Chromosome segregation in meiosis), which together with Mrc1 (from the first epistasis group) form a replication-pausing checkpoint complex (Katou et al., 2003). These factors interact directly with the MCM helicase during both replication fork progression and when the replication fork is stalled (Nedelcheva et al., 2005). They are critical for replication fork pausing at replication fork barriers, but their role during undisturbed S-phase progression is unclear.

Despite all the links between these non-essential factors with the DNA replication fork, it is not yet understood how they contribute to cohesion establishment. Apart from the Timeless-Tipin protein complex (human orthologs of budding yeast Tof1 and Csm3) and the TIM-1 (a paralog of Timeless in *C. elegans*) that were shown to interact with cohesin (Chan et al., 2003; Leman et al., 2010), no other stable interactions between non-essential factors and cohesin are known. A potential role of these proteins could thus be to coordinate the passage of the replication fork through cohesin rings with Okazaki fragment processing, so that loops would be released upon encountering cohesin. This hypothesis proposes that the replisome would successfully pass through the cohesin ring, without disrupting it. Although some evidence based on knowledge of prokaryotic DNA replication suggests that this may be possible

(Lengronne et al., 2006), our current understanding of the organisation of eukaryotic fork components and the way they coordinate their activities at the replication fork is still incomplete. Based on the 'trombone model' for Okazaki fragment synthesis (Hamdan et al., 2009), we could hypothesise that the lagging strand loop is a potential obstacle to the passage of replication forks through cohesin rings (Figure 1.6).

Evidence so far indicates that cohesion is actively established at the replication fork with the assistance of components of the DNA replication machinery. However, the precise mechanism, the link between the different processes and their requirement for sister chromatid cohesion establishment remain poorly understood.

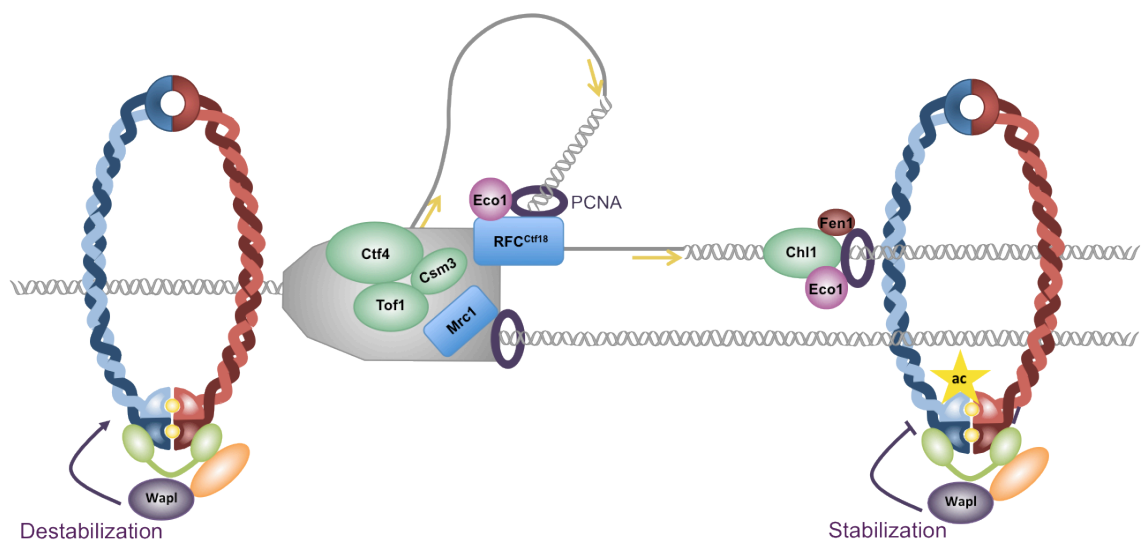


Figure 1.6 – Establishment of sister chromatid cohesion during DNA replication. The acetyltransferase Eco1 associates with the DNA replication fork, probably by its physical interaction with the DNA polymerase processivity factor PCNA. Eco1-mediated Smc3 acetylation blocks the counteracting action of Wapl on cohesin. Several additional cohesion establishment factors have been linked to the replication fork and contribute to efficient sister-chromatid cohesion in as yet unknown ways.

1.3.3 Sister chromatid cohesion in G2

1.3.3.1 *Maintenance of cohesion*

Sister chromatid cohesion must be maintained throughout G2, until anaphase onset. Perhaps the most striking evidence for the high stability of the links generated during cohesion establishment are the mammalian oocytes, which can remain quiescent for many decades and still sustain cohesion before allowing cell division and proper segregation of chromosomes (Petronczki et al., 2003). Maintenance of sister chromatid cohesion does not require cohesion establishment factors like Eco1 (Skibbens et al., 1999; Tóth et al., 1999), but instead specific maintenance factors are required to regulate cohesin-chromatin interactions.

In budding yeast, cohesion maintenance was shown to depend on Pds5 (Stead et al., 2003), a highly conserved protein from yeast to humans (Hartman et al., 2000; Sumara et al., 2000; Tanaka et al., 2001; van Heemst et al., 1999). However, the importance of its function varies between different model organisms. Although Pds5 is essential for cell viability in budding yeast, fission yeast Pds5 can be deleted (Tanaka et al., 2001). In the absence of Pds5 cohesion is still established, and cohesins remain bound to chromatin, but sister chromatid cohesion is no longer maintained (Dorsett et al., 2005; Hartman et al., 2000; Losada et al., 2005; Stead et al., 2003; Tanaka et al., 2001). Pds5 has been implicated in both stabilisation and destabilisation of sister chromatid cohesion. In human cells, Pds5 colocalises with cohesin on chromosomes, and associates with Wapl, Eco1 and sororin (Gandhi et al., 2006; Kueng et al., 2006; Noble et al., 2006; Rankin et al., 2005; Sumara et al., 2000), so perhaps the different interactions of these factors with Pds5 coordinate their functions to promote the cohesive state (Eco1 and sororin) and the non-cohesive state (Wapl).

Several studies suggested that cohesion is also mediated by cohesin-independent mechanisms, including the condensin complex, the origin recognition complex (ORC) and centromere complexes (Lam et al., 2006; Monje-Casas et al., 2007; Shimada and Gasser, 2007; Suter et al., 2004).

1.3.3.2 *DNA damage induced cohesion*

After replication is completed and sister chromatid cohesion has been established in S-phase, cohesin will still continue to associate and dissociate from chromosomes, but will no longer be able to establish cohesion (Uhlmann and Nasmyth,

1998). However, a notable exception happens in G2 in response to DNA double-strand breaks, where cohesion can be established *de novo* (Ström et al., 2004; Strom and Sjogren, 2005). As DSB repair relies on DNA synthesis via homologous recombination (HR), the mechanism of cohesion establishment upon DNA damage in G2 and during DNA replication in S-phase could be the same. However, studies revealed cohesion could still be established for DNA repair upon DNA synthesis blocking, indicating that the damage-induced cohesion is independent of DNA replication (Ström et al., 2007; Ünal et al., 2007). Interestingly, cohesin is not only recruited to the break site but also along entire chromosomes, establishing sister chromatid cohesion in an Eco1-dependent manner (Ström et al., 2004; Ünal et al., 2007). RFC^{Ctf18} is also recruited to the damaged sites and could mediate cohesion establishment by engaging PCNA along with Eco1 (Ogiwara et al., 2007). Furthermore, overexpression of Eco1 in budding yeast is sufficient to trigger cohesion establishment in G2, bypassing the requirement for DSBs (Ünal et al., 2007). Although the mechanism of DNA damage induced cohesion is not fully understood, evidence suggests that the checkpoint kinase Chk1 phosphorylates the cohesin subunit Scc1, facilitating Scc1 acetylation by Eco1 (Heidinger-Pauli et al., 2008; Heidinger-Pauli et al., 2009). Similar to the replication-fork Smc3 acetylation, Scc1 acetylation appears to counteract the function of Wapl to establish DNA damage-induced cohesion. Thus, Eco1 seems to have distinct substrates to establish sister chromatid cohesion during DNA replication and G2.

Reinforcement of cohesion in response to DSBs also occurs in higher organisms (Kim et al., 2010), although the mechanism of cohesion establishment might not be entirely conserved from yeast to humans. The genome-wide reinforcement of cohesion to regulate checkpoint and DNA repair ensures accurate segregation of mitotic chromosomes. This additional sister chromatid cohesion in response to DSB formation could facilitate homologous recombination reactions by keeping sister chromatids in close vicinity. Thus, sister chromatid cohesion is important not only during DNA replication, but also for repair of DNA DSBs to ensure faithful chromosome segregation.

1.3.4 Dissolution of cohesion

Cohesin removal and subsequent chromosomal segregation are irreversible processes, so the cells have developed several mechanisms to ensure faithful

chromosome inheritance. The accurate segregation of chromosomes requires bi-orientation of chromosomes in metaphase. This is generated when every sister chromatid pair is attached to the spindle microtubules emanating from two opposite spindle poles. If kinetochores are wrongly attached, the kinetochore-spindle pole connections must be re-oriented to generate proper bipolar attachment. In budding yeast, a prerequisite for bi-orientation of sister chromatids is the Ipl1 kinase, which is closely related to the Aurora B kinase found in higher eukaryotes (Adams et al., 2001; Biggins et al., 1999; Kaitna et al., 2002; Tanaka et al., 2002). Ipl1 has been proposed to resolve monopolar attachments to allow stable bipolar attachments, by promoting the detachment of incorrect kinetochore-microtubule interactions (Tanaka et al., 2002). Sister chromatid cohesion is also essential for bi-orientation by generating tension across the chromatid pair (Nicklas, 1988). This tension between sister kinetochores is critical for counteracting the pulling forces and stabilising the attachment (Pinsky and Biggins, 2005). Only when all chromosomes have reached bi-orientation, can the spindle checkpoint be inactivated, thereby allowing progression from metaphase to anaphase. This inactivation remains incompletely understood, but it involves the dissociation between the spindle checkpoint protein Mad2 and Cdc20, an essential cofactor of the anaphase-promoting complex/cyclosome (APC/C).

The way in which cohesion is dismantled varies between different model organisms. Sister chromatid separation depends on the degradation of Securin, a protein that was first identified in fission yeast, Cut2, and in budding yeast, Pds1 (Funabiki et al., 1996b; Yamamoto et al., 1996). Securin needs to be degraded by an APC/C ubiquitin-mediated proteolysis reaction to allow chromosome segregation (Cohen-Fix et al., 1996). This degradation leads to the activation of the protease separase, named Esp1 in budding yeast and Cut1 in fission yeast (Ciosk et al., 1998; Funabiki et al., 1996a). Once released from its inhibitor securin, separase initiates sister chromatid segregation by cleaving the cohesin subunit Scc1, and thereby dissociating cohesin from chromosomes (Uhlmann et al., 1999; Uhlmann et al., 2000).

In budding yeast, all cohesins dissociate from chromosomes at anaphase onset in a separase-dependent manner. Cohesins are then reloaded in late G1 when Scc1 has been re-synthesised (Tóth et al., 1999). In contrast, in vertebrates only a small fraction of Scc1 is resolved at anaphase (Waizenegger et al., 2000). Instead, the bulk of cohesin associated with the chromosome arms, but not the one from the centromeres, dissociates in early mitosis, during prophase in a separase-independent manner (Losada et al., 2000; Waizenegger et al., 2000). This 'prophase pathway'

depends on the activity of the polo like kinase (Plk1) (Sumara et al., 2002), Aurora B (Losada et al., 2002) and Wapl (Gandhi et al., 2006).

1.4 Protein acetylation and deacetylation

Acetylation and deacetylation of proteins was first determined as a posttranslational modification of histones. Histone acetylation and deacetylation are catalysed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively, which are important regulators of gene expression (Brownell et al., 1996; Ekwall, 2005; Taunton et al., 1996). The substrates for HATs and HDACs extend to non-histone targets, including transcription factors, cell signaling components, DNA repair proteins, metabolic enzymes and cytoskeletal proteins (Lin et al., 2009; Yang and Grégoire, 2007). Since the number of proteins modified by acetylation exceeds the number of known acetylases and deacetylases, these enzymes appear to have a broad target spectrum.

A genome-wide screen for acetylated proteins revealed that acetylation of non-histone proteins modulates large macromolecular complexes with key roles in diverse cellular processes (Choudhary et al., 2009). Importantly, several non-histone proteins targeted by acetylation are relevant for tumourigenesis, tumour progression and metastasis, as many of them are the product of oncogenes or tumour-suppressor genes (Glozak et al., 2005). Thus, acetylation of both histone and non-histone proteins appears to be an important mechanism of regulation in the cell. Protein acetylation is a highly abundant and evolutionary conserved posttranslational modification. However, despite the importance of this modification, the identities of the acetyltransferases and deacetylases involved, and the mechanisms underlying their action have remained in many cases largely unknown.

1.5 The biological functions of cohesin

In addition to the established role of the cohesin complex in sister chromatid cohesion, cohesin has been implicated in a wide range of other cellular processes.

These include DNA repair by homologous recombination (HR), chromosome morphogenesis, gene regulation and transcription.

1.5.1 DNA repair

As discussed in section 1.3.3.2, cohesin has a direct involvement in DSB repair. Cohesion is required for postreplicative homologous recombination, by ensuring the close proximity of the sister chromatids, so that the intact DNA can serve as the template (Kadyk and Hartwell, 1992; Losada and Hirano, 2005; Sjögren and Nasmyth, 2001). Recent studies in budding yeast showed evidence that cohesin is recruited to extended chromosome regions surrounding DNA breaks induced during G2 (Ström et al., 2004; Unal et al., 2004), and the recruitment to the DSBs is dependent on the cohesin loading factor Scc2/Scc4.

1.5.2 Chromosome Morphogenesis

Studies in budding and fission yeast have also implicated cohesin in chromosome compaction by showing that cohesin mutants cause hypo- or hypercondensation (Ding et al., 2006; Guacci et al., 1997; Hartman et al., 2000). However, studies carried out in budding yeast revealed a cohesin-independent pathway for chromosome condensation (Lavoie et al., 2004). Moreover, loss of cohesin in higher eukaryotes does not abrogate chromosome condensation (Kueng et al., 2006; Losada et al., 2002; Sonoda et al., 2001). Nevertheless, the presence of normal amounts of condensation in cohesin mutants does not rule out the hypothesis that compaction is less ordered, resulting in problems in chromosome segregation. Further experiments will be important to address whether cohesin-independent condensation can support chromosome segregation.

1.5.3 Transcription

A role for cohesin as a regulator of gene expression was first observed in studies in flies and yeast. The *Drosophila* homolog of Scc2/Scc4, Nipped-B, facilitates long-range enhancer-promoter communication (Rollins et al., 2004). In budding yeast, Smc1 and Smc3 are required to limit the heterochromatin spreading from the transcriptionally

silenced HMR locus (Donze et al., 1999). Furthermore, the functions of Rad21 and Smc3 are needed for the expression of the zebrafish *Runx* gene (Horsfield et al., 2007). In humans, the homolog of Scc3, SA2, acts as a transcriptional co-activator (Lara-Pezzi et al., 2004), and it has also been implicated in mediating transcriptional insulation via its interactions with CTCF (Parelho et al., 2008; Rubio et al., 2008; Stedman et al., 2008; Wendt et al., 2008). Therefore, evidence from different organisms suggests that cohesin transcriptional functions can be mediated either by cohesin alone, or by interactions between cohesin and other chromatin regulators.

1.6 Relevance of cohesion establishment to human disease

1.6.1 Cohesinopathies

Although the cohesin complex is best known for its role in sister chromatid cohesion, various human diseases have been associated with mutations in genes associated with the cohesin network. The Cornelia de Lange Sndrome (CdLS) is a developmental disorder with mild symptoms caused by mutations in *SMC1* and *SMC3* and more severe symptoms caused by mutations in *NIPBL*, the human ortholog of Scc2 (Deardorff et al., 2007; Krantz et al., 2004). CdLS is characterised by distinctive craniofacial features, small stature, learning disability, gastrointestinal abnormalities and limb deficiencies (Dorsett, 2007). A similar syndrome, Roberts/SC phocomelia syndrome, has phenotypic overlap with CdLS (Dorsett, 2007), but it is caused by mutation of both alleles of *ESCO2* (Vega et al., 2005), the human orthologue of yeast Eco1. The Warsaw Breakage syndrome was also described (van der Lelij et al., 2010) and it is associated with mutations in the human *CHL1* gene. This syndrome is mainly characterised by severe growth retardation and microcephaly.

1.6.2 Sister chromatid cohesion in cancer

Results from different organisms established that problems in sister chromatid cohesion result in massive chromosome missegregation, aneuploidy and gene mis-expression, which are all hallmarks of cancer progression (Huang et al., 2005; Losada et al., 2005; Skibbens, 2005). These indicate that cohesion may play a role in cancer.

Recently, a more direct link has been made between the cohesin network and many forms of cancer. Mutations were found in several cohesin complex components in colorectal cancers (Barber et al., 2008). An upregulation of both the establishment factor ESCO2 and WAPL was observed in aggressive melanoma cells and cervical malignancies, respectively (Oikawa et al., 2004; Ryu et al., 2007) and PDS5B has also been involved in tumour suppression (Denes et al., 2010). Furthermore, mutational inactivation of STAG2, a homolog of the yeast Scc3 cohesin subunit, was also found to cause aneuploidy in human cancer (Solomon et al., 2011). Hence, a growing body of evidence is starting to emerge that links cohesion establishment pathways to a variety of cancers.

1.7 Aim and outline of this thesis

The knowledge about sister chromatid cohesion has been increasing over the past years. However, although the factors involved in cohesion have been extensively characterised, the mechanism behind establishment of cohesion is still poorly understood. The work presented in this thesis aims to gain new insights into the process of sister chromatid cohesion establishment. Using *S. cerevisiae* as a model, I focused my work on two interrelated areas:

1. The investigation of the mechanism of Smc3 deacetylation and the importance of the cohesin acetylation cycle.
2. Exploring the relationship between sister chromatid cohesion and DNA replication, by investigating the role of non-essential cohesion proteins with respect to the replication fork, and their relationship to Eco1.

Protein acetylation has gained considerable interest with the realisation that it is a posttranslational modification as widespread as phosphorylation. It has been reported that the acetyltransferase Eco1 acetylates the cohesin subunit Smc3 during the establishment of sister chromatid cohesion in S-phase (Ben-Shahar et al., 2008; Ünal et al., 2008; Zhang et al., 2008a). Despite the interest in acetylation, little was known about how this modification is removed by deacetylation. In Chapter 3, I describe budding yeast histone deacetylase Hos1 as the cohesin deacetylase. I explain how Smc3 deacetylation by Hos1 is strictly confined to anaphase. Furthermore, I demonstrate the importance of cohesin deacetylation. Only cohesin containing non-acetylated Smc3 will act as a substrate for cohesion establishment in the subsequent cell cycle. This study thereby completes the molecular description of the cohesin acetylation cycle. At the same time it provides unexpected insight into the importance of both cohesin acetylation and deacetylation.

Establishment of sister chromatid cohesion is a process thought to occur as the replication fork passes chromosomal loci bound by the cohesin complex. However, what happens at replication forks to establish sister chromatid cohesion is poorly understood. In Chapter 4 I investigate the role of replication fork-associated proteins

required for efficient cohesion establishment. In particular, using genetic and molecular assays I show that these proteins are required for efficient cohesin acetylation. Furthermore, I delineate an Eco1-independent cohesion establishment pathway, defined by Ctf4 and Chl1.

Finally, the results described in this thesis are discussed and put into perspective in Chapter 5.

Chapter 2

Materials and Methods

Chapter 2. Materials and Methods

2.1 Yeast techniques

2.1.1 Yeast growth conditions

All budding yeast strains used in this study are listed in Table II. Cells were grown in YP (Yeast Peptone) (1.1% w/v yeast extract, 2.2% w/v bacto-peptone and 0.0055% w/v adenine-HCl) supplemented with 2% w/v glucose (YPD – Yeast Peptone Dextrose) or 2% w/v raffinose/galactose (YP-Raff/Gal). For growth of strains containing Cdc20 under the control of the methionine repressible *MET3* promoter, cells were grown in synthetic YNB media (Yeast Nitrogen Base: 0.8% w/v yeast nitrogen base, and 60 µg/ml of each of the following amino acids: tyrosine, uracil, tryptophan, leucine, adenine, histidine, isoleucine and phenylalanine, 3 µg/ml arginine, 4 µg/ml lysine and 5 µg/ml threonine) supplemented with either 2% w/v glucose or 2% w/v raffinose/galactose. For the selection of transformants, YNB agar plates (as per YNB except 2.2% w/v agar) were used lacking the auxotrophic amino acid used for selection. Alternatively, for selection based on Kanamycin resistance YP-glucose plates were supplemented of the kanamycin derivative Geneticin G418 (50 µg/ml). Cells were sporulated on sporulation media (100 mM CH₃COONa, 20 mM NaCl, 25 mM KCl, 1.5 mM MgSO₄ and 1.5% w/v agar).

2.1.2 Cell synchronisation

2.1.2.1 G1 cell cycle arrest

Mating type a yeast cells were arrested in G1 with the mating pheromone α -factor. To arrest cells, an early log phase culture (OD₆₀₀ = 0.1) was treated with α -factor (provided by Peptide Synthesis Laboratory, Cancer Research UK) at a concentration of 0.5 µg/ml. The same amount of α -factor was added to the culture every hour during two hours. Mating type α yeast cells were arrested in G1 with the mating pheromone a-factor. An early log phase culture was treated with a-factor (provided by Peptide Synthesis Laboratory, Cancer Research UK) at a concentration of 0.05 µg/ml. The same amount of a-factor was added to the culture every hour during two hours. Arrests were generally complete within two hours. Cell cycle arrest was

determined both cytologically by the appearance of a pear-shaped “schmoo” and by FACS (Fluorescence-activated cell sorting) analysis of DNA content. G1 arrested cells were released by filtration within media devoid of α -factor. For filtration, cells were collected on a membrane filter (Schleicher & Schuell, ME28, 1.2 μ m) using a filtration apparatus from Millipore. Cells were extensively washed with YP before release into YP media supplemented with the appropriate source of sugar.

2.1.2.2 HU-induced early S phase arrest

Early S phase arrest was performed by addition of hydroxyurea (HU, Sigma, a ribonucleotide reductase inhibitor) to previously G1 arrested cells at a final concentration of 100 mM. The presence of small buds, after G1 release, indicated S phase arrest.

2.1.2.3 G2 arrest

For arrest in metaphase, nocodazole (Sigma) was added at 5 μ g/ml. Cell cycle arrests were assessed cytologically by the presence of large budded cells. For arrest using *GAL*-Cdc20, cells were cultured in media containing 2% raffinose and 2% galactose before being filtered, washed and transferred to media containing raffinose as the sole sugar source. For arrest using the repression of *MET3*-Cdc20, cells were grown in YNB lacking methionine and supplemented with 2% glucose. To arrest cells, 2 mM methionine was added. Cell cycle arrests were again checked cytologically and by DNA content.

2.1.3 Protein overexpression and repression from the *GAL1* promoter

For overexpression of proteins from the *GAL1* promoter, cells were grown in YP supplemented with 2% raffinose (Sigma) until mid log phase and protein expression was induced by the addition of 2% galactose (Sigma) for 2-3 hours.

For protein repression (e.g. Smc3 or Cdc20) cultures were grown in YP containing both 2% galactose and 2% raffinose. To repress expression, the culture was filtered, extensively washed with YP, and transferred to YP media containing raffinose as the sole sugar source. Protein levels were checked by Western blotting.

2.1.4 Protein expression and repression from the *MET3* promoter

To repress Cdc20 expression from the *MET3* promoter, the endogenous Cdc20 promoter was first replaced by the *MET3* promoter by one step promoter swap PCR reaction. Positive transformants were grown in complete YNB lacking methionine media containing 2% glucose, or when expression from the *GAL1* promoter is required 2% of raffinose. To repress Cdc20, cells were transferred to complete media (YP) and methionine was added to a final concentration of 2 mM.

2.1.5 Yeast transformation

Transformation of yeast was performed using purified PCR products for epitope tagging or gene disruption as described in section 2.3.2, or with linearised plasmid DNA for integration of promoter-gene cassettes or exchange of endogenous promoters of essential genes. About 50 ml of a mid log phase culture was pelleted at 3,000 rpm for 5 minutes. The cell pellet was washed with 1 ml of de-ionised water and then with 1 ml TEL (10 mM Tris/HCl pH 7.5, 100 mM EDTA and 100 mM Lithium acetate) before resuspension in a final volume of 100 µl TEL. 1 µg of either linearised vector DNA or PCR product was mixed with 2 µl of a 10 mg/ml single stranded carrier DNA from salmon sperm and 300 µl TELP (TEL plus 40% PEG 3350 or 4000). The cell suspension was added to this mix followed by a short vortex. After incubation at 25°C for 4 hours, cells were heat shocked at 42°C for 15 minutes. The cells were then pelleted at 6,000 rpm for 2 minutes, washed in 1 ml sorbitol and plated on selective media. Transformants were checked for the correct integration of the PCR cassette by PCR, Western blot analysis or death on methionine (in the case of *MET3-CDC20*), glucose (*GAL1-SMC3*, *GAL1-CDC20*) or auxin (IAA) (*eco1-aid*) containing media.

2.1.6 Yeast mating and tetrad dissection

Mating was induced by incubation of opposite mating type yeast strains on YPD plates at 25°C for 12 hours. Diploids were selected on appropriate selective media and grown again on YPD for 24 hours. To sporulate, they were placed on a sporulation plate for 2-3 days. Spores were then resuspended in 1 M sorbitol and treated with Zymolase T-20 (MP Biomedicals) at 30 °C for 10 minutes to break the asci. The four

released spores from each ascus were dissected using a Singer-MSM micromanipulator and incubated at 25°C until colony formation.

2.2 Biochemistry

2.2.1 Immunoprecipitation

Usually 100 ml of mid log phase culture ($OD_{600} = 0.3$) were pelleted at 3000 rpm for 10 minutes. Cell pellets were then resuspended in 400 μ l extraction buffer (50 mM Hepes/KOH pH 8, 0.1 M KCl, 2.5 mM $MgCl_2$, 10% glycerol, 0.1% SDS, 0.25% Triton X-100, 1 mM DTT, 10 mM sodium butyrate, protease inhibitors). Approximately 200 μ l of 0.5 mm glass beads (Biospec Products, Inc) were added and the cells were lysed using a FastPrep FP120 cell breaker (Bio101). Extracts were clarified by centrifugation, and further cleared by incubation with IgG sepharose beads (GE Healthcare) on a rotating wheel for one hour. The lysate was then incubated with 3 μ g of either anti-Pk (SV5-Pk1, Serotec) or HA (ICRF, 12CA5) antibodies for one hour and immunocomplexes were isolated by adsorption to protein A sepharose beads (GE Healthcare) for two hours. Beads were washed extensively in extraction buffer, and immunocomplexes were recovered by incubation with elution buffer (50 mM Tris/HCl pH 8, 10 mM EDTA, 1% SDS,) for 15 min at 65°C. An aliquot of this material was then added to an equal volume of 2x SDS loading buffer and analysed by Western blotting.

2.2.2 Chromatin fractionation

To analyse protein binding to chromatin, 50-100 ml of mid log phase culture ($OD_{600} = 0.5$) were pelleted at 3000 rpm for 5 minutes. Cell pellet was then resuspended in 3 ml PIPES/KOH buffer (100 mM PIPES/KOH pH 9.4, 10 mM DTT, 0.1% Na-Azide) for 10 minutes at room temperature. Cells were then pelleted at 2000 rpm for 2 minutes and resuspended in 2 ml Kpi/Sorbitol buffer (50 mM K_2HPO_4/KH_2PO_4 , 0.6 M Sorbitol, 10 mM DTT). The cells were then spheroplasted by the addition of Zymolase T-100 (MP Biomedicals) in the above buffer to reach a final concentration of 40 μ g/ml for 10 minutes at 37 °C. The cells were then collected by centrifugation (4000 rpm for 1 minute), washed with ice-cold Spheroplast wash buffer (50 mM HEPES/KOH pH 7.5, 100 mM KCl, 2.5 mM $MgCl_2$, 0.4 M sorbitol), and subsequently resuspended in

20 ml ice-cold EB buffer (50 mM HEPES/KOH pH 7.5, 100 mM KCL, 2.5 mM MgCl₂, 0.2 mM ZnCl₂) supplemented with protease inhibitors (1 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 2 µg/ml pepstatin, 200 µg/ml bacitracin, 2 mM benzamidine, plus complete mini-EDTA free protease inhibitor tablets from Roche. This cell suspension was then lysed by the addition of Triton-X-100 at 0.25% for 3 minutes, with occasionally vortexing. The cell lysate was now carefully layered onto a sucrose cushion of EBXS (EB, plus 30% sucrose) and centrifuged at 4°C for 10 minutes at 12,000 rpm to separate soluble and insoluble fractions. After centrifugation, the soluble extract consists of an upper yellow layer above the sucrose. The soluble extract on the top layer was separated and eventually mixed with an equal volume 2x SDS loading buffer for Western blotting. The remaining chromatin pellet was resuspended in 200 µl EBX. An aliquot of this material was then added to an equal volume of 2x SDS loading buffer as before. As controls for chromatin bound and soluble proteins we used antibodies against Hmo1 (Abcam) and Tubulin (Sigma), respectively.

2.2.3 ChIP on chip analysis

2.2.3.1 Formaldehyde fixation

100 ml log phase culture (OD₆₀₀ = 0.3 – 0.4) cells grown in YPD medium were fixed by the addition of formaldehyde (final concentration of 1% v/v). Cells were mildly shaken for 30 minutes at room temperature followed by overnight incubation on a rotating wheel at 4°C. Cells were pelleted at 4 °C and subsequently washed 3 times in 60 ml of ice-cold TBS (Tris-buffered saline) (200 mM Tris-HCl pH 7.5, 1.5 M NaCl).

2.2.3.2 Cell breakage

The cell pellets were resuspended in 400 µl of lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA pH 8.0, 1% Triton-X-100, 0.1% Na-deoxycholate, 1 mM PMSF). 1.2 ml of acid-washed glass beads (Sigma) were added and the cells were broken on a multi-bead shocker (MB400U, Yasui Kikai, Osaka, Japan), which is able to keep the extract below 4 °C therefore limiting protein degradation.

2.2.3.3 Sonication and chromatin immunoprecipitation (ChIP)

The soluble extract was then sonicated (Sanyo, Soniprep 150) to obtain DNA fragments of between 400 – 800 bp. The extract was then incubated with anti-Pk (clone SV5-Pk1, Serotec) coupled Protein-A Dynabeads (Dyna) on a rotating wheel at 4°C for 4 hours. After washing the beads with lysis buffer, the immunoprecipitates were eluted using elution buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) at 65°C for 10 minutes. The presence of immunoprecipitated protein was confirmed by Western blotting.

2.2.3.4 Reversal of cross-linking, proteinase K and RNase A treatment

To one volume of the eluate was now added three volumes of TES (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% SDS) and this mixture was incubated overnight at 65 °C to reverse the crosslinks. The immunoprecipitate was incubated with Proteinase K to remove proteins as follows: to 160 µl of the reaction was added 140 µl TE pH 8.0, 3 µl Glycogen (10 mg/ml) and 7.5 µl Proteinase K (20 mg/ml). This mixture was then incubated at 37°C for at least two hours. The DNA was extracted two times using phenol/chlorophorm/isoamylalcohol, and subsequently ethanol precipitated, dried in a speed-vac (Savant) and diluted in a final volume of 30 µl TE. This sample was then treated with RNase A (0.3 µg/µl) at 37°C for 1 hour to remove any contaminating RNA before amplification. The DNA was further purified over QIAprep spin columns (Qiagen). The volume was reduced by a further round of ethanol precipitation, dried, and resuspended in a final volume of 7 µl.

2.2.3.5 DNA amplification, DNase digestion and DNA end-labelling

This DNA was amplified by PCR after random priming (Iyer et al., 2001). Approximately 10 µg of amplified DNA was digested with DNase I to an average size of 100 bp. After DNase I inactivation at 95°C, these DNA fragments were subsequently end-labelled with biotin-N6-ddATP (Enzo Life Sciences) as previously described (Winzeler et al., 1998).

2.2.3.6 Hybridisation to oligonucleotide microarrays

Each sample was prepared in a 250 µl reaction containing 6x SSPE-T (0.9M NaCl, 60 mM Na₂HPO₄, 6 mM EDTA, 0.005% Triton X-100, pH 7.6), 15 µg denatured salmon sperm DNA (Gibco-BRL), and 1 nmol control oligo B2 that hybridises to specific regions of the chip to facilitate alignment. The samples were boiled at 100 °C for 10 minutes before cooling on ice and hybridised to the microarray in a hybridisation oven (GeneChip hybrid oven 320, Affymetrix, CA) at 42 °C for 16 hours. Washing and scanning procedures were performed automatically on the affymetrix fluidics station (GeneChip fluidics station 400, Affymetrix). Scanning of the microarray was carried out on a HP GeneArray Scanner (Affymetrix).

The chromosome VI CHIP was produced by the Affymetrix custom express service (rikDAF, P/N 510636, Affymetrix). Briefly, the oligonucleotide array contains sixteen 25 mer probes per every 300 bp partially tiled over each other to fully cover both coding and non-coding sequences. To distinguish between positive and negative signals we compared the ChIP fraction to a 'SUP' sample representing whole genome DNA, using the criteria as set out in (Katou et al., 2003).

2.2.4 Preparation of yeast extracts

Yeast extracts were prepared using the TCA method. 10 ml mid log phase culture ($OD_{600} = 0.3$) was collected by centrifugation at 3,000 rpm for 5 minutes, resuspended in 1 ml 20% trichloroacetic acid (TCA) and kept on ice for 15 minutes or until the end of the time-course experiment. The cell pellets were then washed with 1 ml of 1 M Tris-Base, and after centrifugation at 13,000 rpm for 1 minute resuspended in 100 µl 2x SDS buffer (100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% SDS, 0.2% Bromophenol blue, 20% glycerol). 100 µl of 0.5 mm glass (Biospec Products, Inc) were added and the cells were lysed using a FastPrep FP120 cell breaker (Bio101). Extracts were then spun down to separate them from the glass beads. Collected supernatant was transferred to a new tube, boiled at 95 °C for 5 minutes and cleared by centrifugation at 13,000 rpm for 5 minutes.

2.2.5 SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting

Protein samples were resolved on acrylamide/bis-acrylamide (37.5:1, Amresco) 375 mM Tris-HCl pH 8.8 and 0.1% SDS. Small proteins of less than 30 kDa were typically resolved on 10% and larger proteins over 100 kDa on 8% gels. A stacking gel was used on top of the separating gel and was composed of 125 mM Tris-HCl pH 6.8, 5% bis-acrylamide and 0.1% SDS.

Proteins were allowed to migrate at 50 mA using SDS-PAGE running buffer (25 mM Tris, 250 mM glycine and 0.1% SDS) in electrophoresis tanks from CBS scientific, CA. To monitor the position of the proteins in the gel and subsequently on the membrane, a broad range pre-stained protein marker (New England Biolabs) was used.

Separated proteins were transferred onto pre-equilibrated nitrocellulose membranes (Schleicher & Schuell) using either a semi-dry transfer apparatus (Hoefer) or a wet-transfer tank (Biorad). Semi-dry transfer buffer contained 14.4 g/l glycine, 3 g/l Tris base, 0.02% SDS and 10% v/v methanol. Wet transfer buffer contained 3.03 g/l Tris base, 14.1 g/l glycine, 0.05% SDS and 20% v/v methanol. Semi-dry transfer was performed at 1.2 mA/cm² for 3 hours. Transfer was carried out at 5.3 mA/cm² for 40 minutes for the wet transfer protocol. The efficiency of transfer was then checked with Ponceau S solution (Sigma).

The membrane was blocked with a 5% skimmed milk solution (Marvel) in PBST (Phosphate Buffered Saline Tween) (170 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 0.01% tween 20) for 1 hour at room temperature. Membranes were then incubated with primary antibodies diluted in PBST containing 5% milk for 1 hour at room temperature. The antibodies concentrations were as following: α -HA (12CA5, ICRF 1:5000), α -Pk (clone SV5-Pk1, Serotec, 1:5000), α -acetyl lysine (ST1027, Calbiochem, 1:1000), α -Clb2 (y-180, Santa Cruz Biotechnology, 1:2000), α -Cdc28 (sc-53, Santa Cruz Biotechnology, 1:1000), α -beta actin (ab8227, Abcam, 1:5000), α -Swi6 (a gift from K. Nasmyth; (Taba et al., 1991), 1:10,000), α -Hmo1 (Abcam, 1:2000), α -tubulin (YOL 1/34, Sigma, 1:1000). For the analysis of Smc3 acetylation two different α -acetyl-Smc3 antibodies were raised against a deacetylated peptide spanning the two Smc3 acetylation sites at amino acids 112 and 113: a polyclonal rabbit α -acetyl-Smc3 and a mouse monoclonal α -acetyl-Smc3 (a gift from K. Shirahige, 1:1000). Membranes were then washed in an excess of PBST three times for ten minutes. Horseradish peroxidase (HRP) coupled secondary antibodies α -mouse or α -rabbit

(Amersham, 1:5000) or α -rat (Chemicon, 1:5000) were then incubated with the membrane in PBST containing 5% milk for a further hour. Membranes were washed a further three times before developing with ECL (Amersham) according to the manufacturer's instructions.

2.2.6 Analysis of *in vitro* Smc3 deacetylation

The analysis of *in vitro* Smc3 deacetylation was performed in whole cell extracts prepared by spheroplast lysis as described in section 2.2.2. 2 U TEV protease (AcTEV, Invitrogen) or 1 mM CaCl_2 and 300 U micrococcal nuclease (Fermentas), or either buffer only, were added to 50 μl of extract. The reactions were incubated at 25°C, with the aliquots retrieved at the indicated time points, and stopped by boiling in SDS-PAGE loading buffer for analysis of Smc3 acetylation by Western blotting or by addition of 1% SDS. The latter samples were processed by digestion with 10 μl of proteinase K (10 mg/ml) at 65 °C overnight. The DNA was then extracted two times using phenol/chlorophorm/isoamylalcohol, and subsequently ethanol precipitated, dried in a speed-vac (Savant) and diluted in a final volume of 50 μl TE. This sample was then treated with RNase A (0.3 $\mu\text{g}/\mu\text{l}$) at 37°C for 30 minutes to remove any contaminating RNA before DNA analysis by TAE agarose gel electrophoresis.

2.2.7 Eco1 autoacetylation activity assay

The assay of Eco1 autoacetylation activity was based on a published protocol (Ünal et al., 2008). 10^9 cells were spun down and the cell pellet was stored at -80°C overnight. Approximately 200 μl of 0.5 mm glass beads (Biospec Products, Inc) and 300 μl IPH150 buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM DTT, Roche protease inhibitor cocktail, 10 mM sodium butyrate) were added to the cell extract. The cells were then lysed using a FastPrep FP120 cell breaker (Bio101). The soluble fraction was separated from the insoluble fraction by 2 consecutive centrifugations, each at 15,000 rpm, 4°C and for 10 min. To pull down Eco1-HA, 50 μl of α -HA affinity matrix (Roche) was added to the extract and further incubated for 2 hours at 4°C on a rotating wheel. After immunoprecipitation, the anti-HA beads were washed 4x 5 min each with IPH150 buffer, 2x 5 min with IPH50 buffer (50 mM Tris pH 8, 50 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1mM DTT, Roche protease inhibitor

cocktail, 10 mM sodium butyrate). Beads were then resuspended in 50 μ l IPH50 supplemented with Acetyl-CoA (400 μ M final, Roche) and BSA (0.1 mg/ml, New England Biolabs), vortexed and incubated at 30°C for 20 min. Eco1 was released from the anti-HA beads by adding 30 μ l 2 X Laemmli buffer and boiling for 5 min.

2.2.8 Mass Spectrometry

Mass spectrometric analysis of Smc3 was carried out essentially as described previously (Charlwood et al., 2002; White et al., 2007). The Smc3-containing gel slice was processed for mass spectrometry using the Janus liquid handling system (PerkinElmer). Briefly, the excised protein gel piece was placed in a well of a 96-well microtitre plate and destained with 50% v/v acetonitrile and 50 mM ammonium bicarbonate, reduced with 10 mM DTT, and alkylated with 55 mM iodoacetamide. After alkylation, Smc3 was digested with 6 ng/ μ L Arg-C overnight at 37°C, which cleaves peptide bonds specifically at the carboxyl side of arginine residues. The resulting peptides were extracted in 1% v/v formic acid, 2% v/v acetonitrile. The digest was analysed by nano-scale capillary LC/MS/MS using a nanoAcquity UPLC (Waters) to deliver a flow of approximately 300 nl/min. A C18 Symmetry 5 μ m, 180 μ m x 20 mm μ Precolumn (Waters) trapped the peptides prior to separation on a C18 BEH130 1.7 μ m, 75 μ m x 100 mm analytical UPLC column (Waters). Peptides were eluted with a gradient of acetonitrile. The analytical column outlet was directly interfaced with a Triversa nanomate microfluidic chip for mass spectrometric analysis (Advion). Mass spectrometric information was obtained using an orthogonal acceleration quadrupole-Tof mass spectrometer (SYNAPT HDMS, Waters). Data-dependent analysis was carried out, where automatic MS/MS was acquired on the 8 most intense, multiply charged precursor ions in the m/z range 400–1500. MS/MS data were acquired over the m/z range 50–1995. LC/MS/MS data were then searched against a protein database (UniProt 12.4) using the Mascot search engine (Matrix Science) (Perkins et al., 1999). The fragmentation spectrum generated from a $[M+2H]^{2+}$ ion at m/z 874.924 showed a continuous series of y-ions, allowing confident assignment of the peptide containing Smc3K112 and K113. The acetylated nature of these lysines is suggested by the 170 Da gap between consecutive b- and y-ions at these positions. The presence of acetylated lysine is confirmed by the occurrence of the diagnostic acetyl lysine immonium ion derivative at m/z 126.095 (Trelle and Jensen, 2008).

2.3 Molecular Biology

2.3.1 Genomic DNA preparation

Yeast genomic DNA for PCR genotyping was prepared from fresh patches of the strain of interest. A toothpick of cells was incubated in 200 µl SCE/ME/Zymolase solution (1 M sorbitol, 0.1 M sodium citrate pH 7.0, 60 mM EDTA, 8 µl/ml β-mercaptoethanol, 2 mg/ml Zymolase T-20) for 45 minutes at 37°C on a shaking thermomixer. 200 µl of SDS solution (100 mM Tris-HCl pH 9.0, 50 mM EDTA, 2% SDS) was added and the mix was heated up for 5 minutes at 65°C. 200 µl of 5 M KAc was added at room temperature. After 10 minutes centrifugation at 14,000 rpm, the supernatant was treated with 800 µl ethanol. After a further centrifugation at 6000 rpm for 2 minutes, the pellet was washed with 500 µl of 70% ethanol and dissolved in 200 µl of de-ionised water. For PCR, 0.5-1 µl of the genomic DNA was used.

2.3.2 Polymerase Chain Reaction (PCR)

PCR reactions were carried out in 50 or 100 µl reactions containing Taq (Qiagen) or Expand High Fidelity (Roche) polymerases with buffers supplied by the manufacturers, 0.2 mM dNTPs mix and 0.5 µM of each primer. A reaction volume of 100 µl contained 5 U of enzyme. 10 ng of plasmid DNA was used as a template. All PCR were performed on a Peltier Thermal Cycler (MJ Research). PCR products were resolved by agarose gel electrophoresis to confirm the size of the fragments.

2.3.2.1 *C-terminal tagging*

Epitope tagging of endogenous genes was performed by gene targeting using polymerase chain reaction (PCR) products (Bähler et al., 1998; Knop et al., 1999). Forward primers contain approximately 50 bp of homology to the 3' end of the gene of interest before the STOP codon. This is followed by 18 mer homologous sequence to the vector used for tagging. The reverse primer again contains sequence homology to 3' UTR (untranslated region) of the gene, followed by sequence to facilitate priming to the vector. The subsequent PCR product contains flanking regions homologous to the gene of interest, thus targeting the epitope-containing cassette for in-frame fusion with the desired gene. Transformants were subsequently selected on plates either using

Geneticin G418 resistance (in the case of KanMX6), or by using auxotrophic markers. In the case of auxotrophic markers, these are derived from either *Kluyveromyces lactis* or *S. pombe* to minimize the chance of integration at the marker locus. Vectors used for one-step tagging are listed in section 2.6.

2.3.2.2 Gene disruption

All genes were disrupted using PCR generated fragments (Bähler et al., 1998). A marker gene (*LEU2*, *URA3*, *HIS3*, *TRP1*) or KanMX6 was amplified with flanking sequences corresponding to the 5' and 3' ends of the relevant genes. Fragments were transformed into a strain lacking the selective marker. Colonies were selected on plates lacking the selective marker. Vectors used for gene disruption are listed in section 2.6.

2.3.3 Restriction enzyme digest

Restriction digests were performed using New England Biolabs enzymes and buffers according to the manufacturer's instructions. Typically, approximately 1-2 µg of plasmid DNA were digested in a 20 µl reaction for 1 hour at the appropriate temperature for digestion.

2.3.4 Phosphatase treatment

To prevent re-ligation of plasmid DNA, 5' phosphatases were removed from plasmid DNA by treatment with Calf Intestinal Phosphatase (CIP, New England Biolabs) according to the manufacturers instructions. This was carried out immediately after the restriction reaction.

2.3.5 Agarose gel electrophoresis

DNA samples were mixed with 1/5 volume of 6x loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 30% (v/v) glycerol) and loaded on a 1-2% agarose gel (depending on the size of the DNA). Agarose gels were prepared in 1x Tris-acetate EDTA buffer (TAE) (40 mM Tris base pH 7.5, 1 mM EDTA pH 8.0, 0.115%

v/v acetic acid). After boiling and cooling to below 60 °C, ethidium bromide was added to a final concentration of 0.5 µg/ml. The electrophoresis was carried out in TAE at 80-120 V for the time necessary to achieve a good fragment resolution (depending on DNA fragment size) in electrophoresis tanks from Anachem Biosciences. The size of the DNA was monitored by comparison with a DNA marker (Novagene) sample run in parallel. The resolved DNA was visualised under a UV transilluminator (BioDoc-It).

2.3.6 Okazaki fragment assay

2.3.6.1 DNA purification

Yeast strains carrying doxycycline-repressible alleles of *CDC9* and a galactose-inducible *UBR1* allele were grown at 30°C in YP supplemented with 2% raffinose. At OD₆₀₀=0.4, galactose and doxycycline were added to final concentrations of 2% and 40 mg/l, respectively, and the culture shaken at 37°C for 2.5 h. 50 ml cultures were used for labelling experiments.

Genomic DNA was prepared from spheroplasts as described in (Murakami et al., 2009). Following ligase repression, cells were collected by centrifugation, washed in SCE buffer (1 M sorbitol, 100 mM sodium citrate, 60 mM EDTA, pH 7.0) and spheroplasted for 3 min with 5 mg zymolyase 20T per 50 ml culture. Spheroplasts were washed with SCE, and resuspended in 480 µl lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 100 mM NaCl, 1.5% sarkosyl) containing 150 µg proteinase K. Digestion was carried out for 2–16 h at 37°C. After digestion, residual proteins and peptides were precipitated by adding 200 µl 5 M KAc and spinning at 13,000 rpm for 30 min at 4°C. Nucleic acids were precipitated from the supernatant by addition of 500 µl isopropanol and centrifugation at 16,000 g for 10 min. Pellets were washed twice with 500 µl 70% ethanol, resuspended in 200 µl STE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl) and digested with 25 µg RNase A (Sigma) and/or 10 U Riboshredder RNase blend (Epicentre) at 37°C for 30 min. Genomic DNA was precipitated by addition of 20 µl NaOAc, pH 5.5 and 800 µl ethanol followed by centrifugation at 5,000 g for 10 min at room temperature. Pellets were washed with 70% ethanol and resuspended in 1 µl TE (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA) per ml original culture volume. DNA was stored at 4°C and never frozen.

2.3.6.2 DNA labelling

Two microlitres of DNA (corresponding to the genomic DNA content of 2 ml cultured cells) was used in 20 µl labelling reactions containing 5 U Klenow (exo-) polymerase (NEB) and [α -³²P]-dCTP (Perkin Elmer) at a final concentration of 33 nM. Free label was removed using Illustra microspin G-50 columns (GE healthcare). Labelled DNA was separated in 1.3% denaturing agarose gels (50 mM NaOH, 1 mM EDTA). After electrophoresis, the gel was neutralised and DNA transferred to an uncharged nitrocellulose membrane (Hybond-N; GE healthcare) via capillary transfer. Membranes were exposed to phosphor screens or film (GE Healthcare).

2.3.7 Retrieval of DNA fragments from agarose gels

DNA resolved in an agarose gel was visualised under a long wave ultraviolet (UV) transillumination and the band of interest excised with a clean scalpel. The QIAquick gel extraction kit (Qiagen) was used according to the manufacturer's protocol to extract the DNA from the agarose.

2.3.8 Quantification of DNA

A NanoDrop ND1000 spectrophotometer (Labtech International) was used to determine the concentration of DNA solutions using the OD₂₆₀ reading. DNA purity was assessed using the OD₂₆₀/OD₂₈₀ ratio, with a ratio between 1.8 and 2.0 being ideal.

2.3.9 DNA ligation

DNA fragments and linearised vector, both with sticky ends, in a molar ratio of 3:1 were ligated in a reaction volume of 20 µl in the presence of 1 U of T4 DNA ligase (New England Biolabs) in 1x T4 DNA ligase buffer (New England Biolabs). The ligation reaction was incubated overnight at 16°C.

2.3.9.1 Transformation of competent bacteria with plasmid DNA

Chemically competent *Escherichia coli* (DH5 α) cells were thawed on ice and 10 μ l of the DNA ligation reaction was added to the competent cells. The mix was incubated on ice for 30 min and then heat shocked for 90 seconds at 42°C. After 5 minutes incubation on ice, 1 ml LB media (10% w/v Bacto-Tryptone, 5% w/v Yeast extract and 170 mM NaCl) was added. The cells were allowed to recover in a shaking incubator at 37°C for 1 h. The cells were plated on LB agar plates (LB plus 1.5% w/v agar) containing the appropriate antibiotic and left to incubate overnight at 37°C.

2.3.9.2 Isolation of plasmid DNA from E. Coli

Plasmid DNA was purified using the Qiagen miniprep kits according to the manufacturer's instructions. Minipreps were prepared from 5 ml *E. coli* cultures and were used for screening plasmids.

2.3.9.3 DNA sequence analysis

Each sequencing reaction was prepared with 200-500 ng DNA, 3.2 pmol of sequencing primer and 8 μ l of BigDye Terminator Cycle Sequencing Ready Reaction Mix, made up to 20 μ l with water. Following thermal cycling, unincorporated nucleotides were removed by ethanol precipitation and sequencing reactions were loaded on automated sequencing machines (Applied Biosystems) at the sequencing service of CR-UK.

2.4 Cell biology and microscopy

2.4.1 Cell cycle analysis using flow cytometry

To determine cell cycle progression by DNA content, 1 ml of a mid log phase culture (OD_{600} = 0.4) was pelleted and fixed in 70% ethanol on ice for 2 hours. Cells were then RNase treated in 1 ml 200 mM Tris-HCl pH 7.5 containing 0.1 mg/ml RNase A overnight at 37°C. After pelleting, DNA was now stained using 400 μ l of a propidium iodide containing solution (200 mM Tris-HCl pH 7.5, 211 mM NaCl, 78 mM MgCl₂, 50

µg/ml propidium iodide). Cells were sonicated (Sanyo, Soniprep 150) before being analysed on a FACScan (Becton Dickinson). Subsequent image preparation was performed using CellQuest software.

2.4.2 *In situ* immunofluorescence (IF)

2 ml of log phase culture were resuspended in 1 ml ice-cold 1% formaldehyde buffer (IF-I buffer) (100 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, 0.5 mM MgCl_2 pH 6.4) and fixed overnight at 4°C. Then cells were first washed in the same buffer lacking formaldehyde once and then resuspended in a new sorbitol based buffer (IF-II; as IF-I but with Sorbitol 1.2 M pH 7.4). Cells were resuspended in 200 µl of spheroplasting solution (as above IF-II plus 2 µl of β-mercaptoethanol and 2 µl of 20 mg/ml Zymolase T-100 for each ml of solution) and incubated at 30°C for 20 to 40 minutes. After this point spheroplasts were delicately washed once and resuspended in IF-II. 5 µl of cells were loaded on polylysine-coated wells on 15 multi-well slides (MP Biomedical). Slides were blocked with a blocking buffer (0.5% Bovine Serum Albumine in PBS) after the fixation in methanol for 3 minutes and in acetone for 10 seconds.

Incubation with primary and secondary antibodies was carried out in the dark in a humid chamber for 1 hour each. Wells were washed with blocking buffer 3 times between antibody staining and 4 times before addition of mounting antifading media Fluoroguard with 0.1 µg/ml of DAPI. Slides were then covered with a coverslip and sealed. The antibodies used were α-HA (3F10, Roche) and α-tubulin (YOL 1/34, Serotec).

2.4.3 Sister chromatid separation assay

Sister chromatid separation was performed using the tetracycline operator/repressor GFP system as described in (Michaelis et al., 1997). Under conditions when sister chromatids are tightly cohered, the GFP coated tetracycline operator arrays appear as one dot. Upon separation of sister chromatids, two GFP dots can be seen. Two ml culture was pelleted (13,000 rpm for 1 minute) and resuspended in 1 ml ice-cold absolute ethanol. Cells were fixed on ice for 2 hours. An aliquot of the cell suspension was placed onto a thin 2% agar pad on a glass slide and covered with a coverslip. GFP dots were imaged on an Axioplan 2 microscope (Zeiss).

2.5 Table of strains

The guidelines for budding yeast gene and protein nomenclature are outlined in the *Saccharomyces* Genome Database on <http://www.yeastgenome.org/>. All strains used in this work are listed below in Table II.

Table II – Strains used for functional experiments in this study

Strain	Genotype	Origin
K699	<i>MATa ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+</i>	Kim Nasmyth
K7100	<i>MATa URA3::tetOs HIS3::tetR-GFP</i>	Attila Toth
K7101	<i>MATa URA3::tetOs HIS3::tetR-GFP</i>	Attila Toth
K8869	<i>MATa SCC1-HA6::HIS3</i>	Gabriela
Y933	<i>MATa ura3::3URA3 tetO112, his3::HIS3tetR-GFP wpl1Δ::KAN</i>	Tom Rolef Ben-Shahar
Y1093	<i>MATa ctf18Δ::KANR URA3::tetOs HIS3::tetR-GFP</i>	Armelle Lengronne
Y1451	<i>MATa Ctf4-HA6::HIS</i>	Armelle Lengronne
Y2221	<i>MATa eco1-1</i>	Armelle Lengronne
Y2316	<i>MATa rad52Δ::LEU2</i>	John Mc Intyre
Y3320	<i>MATa ura3::3URA3 tetO112, his3::HIS3tetR-GFP eco1Δ::LEU2 wpl1Δ::KAN</i>	Tom Rolef Ben-Shahar
Y3413	<i>MATa URA3::tetOs HIS3::tetR-GFP eco1-1::TRP1 wpl1Δ::KANR</i>	Tom Rolef Ben-Shahar
Y3459	<i>MATa GAL1-CDC20::LEU2 SMC3-Pk3::HIS3</i>	Chris Lehane
Y3564	<i>MATa SMC3-Pk3::HIS3</i>	Tom Rolef Ben-Shahar
Y3569	<i>MATa ura3::3XURA3 tetO112, his3::HIS3tetR-GFP eco1-1::TRP</i>	Tom Rolef Ben-Shahar
Y3583	<i>MATa smc3(K113N)::TRP1 URA3::tetOs HIS3::tetR-GFP</i>	Tom Rolef Ben-Shahar
Y3612	<i>MATa Chl1-3PK::HIS</i>	Vanessa Borges
Y3615	<i>MATa Ctf4-HA6::HIS Chl1-3PK::TRP</i>	Vanessa Borges
Y3616	<i>MATa Ctf4-HA6::HIS Pol1-3PK::TRP</i>	Vanessa Borges
Y3617	<i>MATa Chl1-3PK::HIS ctf4Δ::TRP</i>	Vanessa Borges
Y3638	<i>MATa hos1Δ::TRP1 GAL1-CDC20::LEU2 SMC3-Pk3::HIS3</i>	Chris Lehane
Y3722	<i>MATa sin3Δ::TRP1 GAL1-CDC20::LEU2 SMC3-Pk3::HIS3</i>	Chris Lehane
Y3759	<i>MATa sds3Δ::TRP1 GAL1-CDC20::LEU2 SMC3-Pk3::HIS3</i>	Chris Lehane
Y3794	<i>MATa ura3::3URA3 tetO112, his3::HIS3 tetR-GFP ctf4Δ::TRP</i>	Vanessa Borges
Y3803	<i>MATa hos1Δ::LEU2 URA3::tetOs HIS3::tetR-GFP</i>	Tom Rolef Ben-Shahar
Y3804	<i>MATa eco1-1::TRP1 hos1Δ::TRP1 URA3::tetOs HIS3::tetR-GFP</i>	Tom Rolef Ben-Shahar
Y3805	<i>MATa ura3::3URA3 tetO112 his3::HIS3 tetR-GFP eco1Δ::LEU2 wpl1Δ::KAN chl1Δ::TRP</i>	Vanessa Borges
Y3806	<i>MATa ura3::3URA3 tetO112 his3::HIS3 tetR-GFP eco1Δ::LEU2 wpl1Δ::KAN mrc1Δ::TRP</i>	Vanessa Borges

Y3830	<i>MATa ura3::3URA3 tetO112 his3::HIS3 tetR-GFP eco1Δ::LEU2 wpl1Δ::KAN ctf18Δ::TRP</i>	Vanessa Borges
Y3832	<i>MATa ura3::3URA3 tetO112 his3::HIS3 tetR-GFP eco1Δ::LEU2 wpl1Δ::KAN tof1Δ::TRP</i>	Vanessa Borges
Y3833	<i>MATa ura3::3URA3 tetO112 his3::HIS3 tetR-GFP eco1Δ::LEU2 wpl1Δ::KAN csm3Δ::TRP</i>	Vanessa Borges
Y3853	<i>MATa ura3::3URA3 tetO112, his3::HIS3 tetR-GFP ctf18Δ::TRP</i>	Vanessa Borges
Y3854	<i>MATa ura3::3URA3 tetO112, his3::HIS3 tetR-GFP tof1Δ::TRP</i>	Vanessa Borges
Y3855	<i>MATa ura3::3URA3 tetO112, his3::HIS3 tetR-GFP csm3Δ::TRP</i>	Vanessa Borges
Y3856	<i>MATa ura3::3URA3 tetO112, his3::HIS3 tetR-GFP chl1Δ::TRP</i>	Vanessa Borges
Y3857	<i>MATa ura3::3URA3 tetO112, his3::HIS3 tetR-GFP mrc1Δ::TRP</i>	Vanessa Borges
Y3858	<i>MATa hos1Δ::TRP1 SMC3-Pk3::HIS3</i>	Vanessa Borges
Y3870	<i>MATa ctf7Δ::HIS3 LEU2::CTF7 (S288C background)</i>	Robert Skibbens
Y3871	<i>MATa ctf7Δ::HIS3 LEU2::ctf7-203 (S288C background)</i>	Robert Skibbens
Y3885	<i>MATa HOS1-HA6::HIS3</i>	Vanessa Borges
Y3886	<i>MATa smc3(K113N)::TRP1 hos1Δ::KANR URA3::tetOs HIS3::tetR-GFP</i>	Vanessa Borges
Y3892	<i>MATa sir2Δ::TRP1 GAL1-CDC20::LEU2 SMC3-Pk3::HIS3</i>	Chris Lehane
Y3906	<i>MATa hos1Δ::TRP1</i>	Vanessa Borges
Y3907	<i>MATa ctf7Δ::HIS3 LEU2::CTF7 hos1Δ::TRP1 (S288C background)</i>	Vanessa Borges
Y3908	<i>MATa eco1-1 hos1Δ::TRP1</i>	Vanessa Borges
Y3909	<i>MATa ctf7Δ::HIS3 LEU2::ctf7-203 hos1Δ::TRP1 (S288C background)</i>	Vanessa Borges
Y3912	<i>MATa SMC3-Pk3::HIS3 ctf4Δ::TRP</i>	Vanessa Borges
Y3913	<i>MATa SMC3-Pk3::HIS3 ctf18Δ::TRP</i>	Vanessa Borges
Y3914	<i>MATa SMC3-Pk3::HIS3 tof1Δ::TRP</i>	Vanessa Borges
Y3915	<i>MATa SMC3-Pk3::HIS3 csm3Δ::TRP</i>	Vanessa Borges
Y3916	<i>MATa SMC3-Pk3::HIS3 chl1Δ::TRP</i>	Vanessa Borges
Y3917	<i>MATa SMC3-Pk3::HIS3 mrc1Δ::TRP</i>	Vanessa Borges
Y3931	<i>MATa LEU2::GAL1-SCC1(R180D,R268D)-HA3 SMC3-PK3::HIS3</i>	Vanessa Borges
Y3959	<i>MATa MET3-HA3-CDC20::TRP1 TRP1::GAL1-NLS-myc9-TEVprotease-NLS2 SCC1-TEV268-HA6::URA3 SMC3-PK3::HIS3</i>	Vanessa Borges
Y3971	<i>MATa LEU2::GAL1-ECO1-HA3 URA3::tetOs HIS3::tetR-GFP</i>	Vanessa Borges
Y3973	<i>MATa LEU2::GAL1-ECO1-HA3 SMC3-Pk3::HIS3</i>	Vanessa Borges
Y3981	<i>MATa SMC3-Pk3::HIS3 URA3::tetOs LEU2::tetR-GFP</i>	Chris Lehane
Y3985	<i>MATa/MATa smc3(K112,113N)-Pk3::TRP1::HIS3/SMC3 URA3::tetOs/URA3::tetOs LEU2::tetR-GFP/LEU2::tetR-GFP</i>	Chris Lehane
Y3991	<i>MATa hos1Δ::KANR SMC3-Pk3::HIS3 URA3::tetOs LEU2::tetR-GFP</i>	Chris Lehane
Y4000	<i>MATa LEU2::GAL1-ECO1-HA3 SMC3-Pk3::TRP1</i>	Vanessa Borges

	<i>URA3::tetOs HIS3::tetR-GFP</i>	
Y4019	<i>MATa SMC3-Pk3::HIS3 ECO1-HA3::LEU2</i>	Vanessa Borges
Y4049	<i>MATa MET3-HA3-CDC20::TRP1 TRP1::GAL1-NLS-myc9-TEVprotease-NLS2 SCC1-TEV268-HA6::URA3 SMC3-PK3::HIS3 hos1Δ::LEU2</i>	Vanessa Borges
Y4063	<i>MATa GAL1-SMC3-Pk3::TRP1::HIS3 URA3::tetOs LEU2::tetR-GFP</i>	Frank Uhlmann
Y4064	<i>MATa hos1Δ::KANR GAL1-SMC3-Pk3::TRP1::HIS3 URA3::tetOs LEU2::tetR-GFP</i>	Frank Uhlmann
Y4151	<i>MATa ura3::3URA3 tetO112, his3::HIS3tetR-GFP wpl1Δ::KAN ctf4Δ::TRP</i>	Vanessa Borges
Y4152	<i>MATa ura3::3URA3 tetO112, his3::HIS3tetR-GFP wpl1Δ::KAN ctf18Δ::TRP</i>	Vanessa Borges
Y4153	<i>MATa ura3::3URA3 tetO112, his3::HIS3tetR-GFP wpl1Δ::KAN tof1Δ::TRP</i>	Vanessa Borges
Y4154	<i>MATa ura3::3URA3 tetO112, his3::HIS3tetR-GFP wpl1Δ::KAN csm3Δ::TRP</i>	Vanessa Borges
Y4155	<i>MATa ura3::3URA3 tetO112, his3::HIS3tetR-GFP wpl1Δ::KAN chl1Δ::TRP</i>	Vanessa Borges
Y4156	<i>MATa ura3::3URA3 tetO112, his3::HIS3tetR-GFP wpl1Δ::KAN mrc1Δ::TRP</i>	Vanessa Borges
Y4157	<i>MATa ura3::3URA3 tetO112 his3::HIS3 tetR-GFP eco1Δ::LEU2 GAL-wpl1-PK9::KAN;TRP</i>	Vanessa Borges
Y4161	<i>MATa ura3::3XURA3 tetO112, his3::HIS3tetR-GFP ctf4Δ::TRP</i>	Vanessa Borges
Y4165	<i>MATa ura3::3URA3 tetO112, his3::HIS3 tetR-GFP ctf4Δ::LEU</i>	Vanessa Borges
Y4169	<i>MATa ura3::3URA3 tetO112, his3::HIS3 tetR-GFP chl1Δ::LEU</i>	Vanessa Borges
Y4184	<i>MATa ura3::3URA3 tetO112, his3::HIS3 tetR-GFP fen1Δ::TRP</i>	Vanessa Borges
Y4189	<i>MATa ura3::3URA3 tetO112, his3::HIS3 tetR-GFP dna2-2::LEU chl1Δ::TRP</i>	Vanessa Borges
Y4193	<i>MATa ura3::3URA3 tetO112, his3::HIS3 tetR-GFP dna2-2::LEU</i>	Vanessa Borges
Y4227	<i>MATa ura3::3XURA3 tetO112, his3::HIS3tetR-GFP ctf4Δ::TRP chl1Δ::LEU</i>	Vanessa Borges
Y4298	<i>MATa tor1-1 fpr1::NAT, RPL13A-2xFKBP12::TRP1, Scc1-FRB-GFP::HIS3MX6, ctf4Δ::LEU</i>	Vanessa Borges
Y4299	<i>MATa tor1-1 fpr1::NAT, RPL13A-2xFKBP12::TRP1, Scc1-FRB-GFP::HIS3MX6, chl1Δ::LEU</i>	Vanessa Borges
Y4300	<i>MATa tor1-1 fpr1::NAT, RPL13A-2xFKBP12::TRP1, Scc1-FRB-GFP::HIS3MX6, ctf18Δ::LEU</i>	Vanessa Borges
Y4336	<i>MATa tor1-1 fpr1::NAT, RPL13A-2xFKBP12::TRP1, Scc1-FRB-GFP::HIS3MX6, ADH1-OsTIR1-9Myc::URA3</i>	Vanessa Borges
Y4340	<i>MATa tor1-1 fpr1::NAT, RPL13A-2xFKBP12::TRP1, Scc1-FRB-GFP::HIS3MX6, ADH1-OsTIR1-9Myc::URA3, Eco1-AID::KanMX6</i>	Vanessa Borges
Y4342	<i>MATa tor1-1 fpr1::NAT, RPL13A-2xFKBP12::TRP1, Scc1-FRB-GFP::HIS3MX6</i>	Lidia Lopez-Serra
yIW310	<i>MATa cdc9::tetO7-CDC9 cmv_Laci-NAT</i>	Iestyn Whitehouse
yIW312	<i>MATa cdc9::tetO7-CDC9 cmv_Laci-NAT ctf4Δ::TRP</i>	Iestyn Whitehouse

yIW313	<i>MATa cdc9::tetO7-CDC9 cmv_Laci-NAT chl1Δ::TRP</i>	lestyn Whitehouse
yIW314	<i>MATa cdc9::tetO7-CDC9 cmv_Laci-NAT ctf18Δ::TRP</i>	lestyn Whitehouse

2.6 Table of DNA vectors

Table III – DNA vectors used in this work

Basic vectors for integration in yeast

Number	Name	Description	Origin
32	pBS-KI <i>TRP1</i>	<i>K. lactis TRP1</i> complementing <i>S. cerevisiae TRP1</i> in pBluescript	Matt Sullivan
73	Ylp128 GAL-SCC1(R180D,R268D)-HA3	Construct to replace the endogenous Scc1 with SCC1(R180D,R268D) (<i>LEU2</i> marker)	Frank Uhlmann
210	pBS-KI <i>URA3</i>	pBluescript was digested with Xho1/Hind3 and 1.45kb <i>URA3</i> gene (<i>K. lactis</i>) inserted	Matt Sullivan
211	pBS-KI <i>LEU2</i>	pBluescript was digested with EcoR1/Hind3 and 2kb <i>LEU2</i> gene (<i>K. lactis</i>) inserted	Matt Sullivan
212	pBS-Sp <i>HIS3</i>	pBluescript was digested with EcoR1/Hind3 and 1.4kb <i>HIS3</i> gene (<i>S. pombe</i>) inserted	Matt Sullivan
213	pBS-kanMX4	pBluescript was digested with EcoR1/BamH1 and 1.55kb KANMX4 gene inserted	Matt Sullivan
808	Ylplac128 GAL-ECO1-HA3	Construct to replace the endogenous Eco1 with GAL-Eco1-HA3 (<i>LEU2</i> marker)	Tom Rolef Ben-Shahar
992	Ylp204-Smc3K112R/113N- <i>TRP1</i>	Construct to replace the endogenous Smc3 with Smc3K112R/113N (<i>TRP1</i> marker)	Chris Lehane
993	Ylp204-Smc3K112N/113R- <i>TRP1</i>	Construct to replace the endogenous Smc3 with Smc3K112N/113R (<i>TRP1</i> marker)	Chris Lehane
1054	Ylplac211-pADH1-OsTIR1-9myc	Ylplac was digested with StuI for integration of pADH1-OsTIR1-9myc at <i>URA3</i> locus	M. Kanemaki

Vectors for epitope tagging in yeast

Number	Name	Description	Origin
36	pUC19-HA6- <i>HIS5</i>	One-step C-terminal HA tagging vector (<i>S. pombe HIS5</i> marker)	Gustav Ammerer
554	pUC19-3Pk- <i>TRP1</i>	One-step C-terminal Pk tagging vector (<i>K. lactis TRP1</i> marker)	Wolfgang Zachariae
555	pUC19-3Pk- <i>HIS3</i>	One-step C-terminal Pk tagging vector (<i>K. lactis HIS3</i> marker)	Wolfgang Zachariae
562	pUC19-HA3- <i>LEU2</i>	One-step C-terminal HA tagging vector (<i>K. lactis LEU2</i> marker)	Chris Lehane
563	pUC19-3Pk- <i>LEU2</i>	One-step C-terminal Pk tagging vector (<i>K. lactis LEU2</i> marker)	Chris Lehane

773	pUC19-HA6- <i>URA3</i>	One-step C-terminal HA tagging vector (<i>K. lactis</i> <i>URA3</i> marker)	Toru Higuchi
776	pUC19-myc18- <i>LEU2</i>	One-step C-terminal myc18 tagging vector (<i>K. lactis</i> <i>LEU2</i> marker)	Toru Higuchi
1053	linker-IAA17-KANMX	One-step C-terminal AID tagging vector (KanMX marker)	M. Kanemaki

Integrative vectors for one step promoter swapping in yeast

Number	Name	Description	Origin
49	pBS- <i>GAL1</i>	Construct for promoter swapping with the <i>GAL1</i> inducible promoter (<i>K. lactis</i> Trp1 marker)	Frank Uhlmann
453	Ylp22 <i>GAL1</i> - <i>CDC20</i>	Construct to replace the endogenous <i>CDC20</i> promoter with the <i>GAL1</i> inducible promoter (<i>LEU2</i> marker)	Armelle Lengronne

Chapter 3

Hos1 and the Cohesin Acetylation Cycle

Chapter 3. **Hos1 and the Cohesin Acetylation Cycle**

Smc3 acetylation is restricted to chromosome-bound cohesin and occurs during DNA replication in S-phase (Ben-Shahar et al., 2008; Ünal et al., 2008; Zhang et al., 2008a). This Eco1-mediated cohesin acetylation is a fundamental mechanism for the establishment of cohesion. Acetylation is maintained throughout G2 and metaphase, but at the time of anaphase Smc3 loses its acetylation. The importance of this and the enzyme responsible for Smc3 deacetylation during anaphase and its regulation were unknown. To address the importance of deacetylation during mitosis and to understand whether deacetylated Smc3 fulfils its own specific function during G1 that is distinct from that of acetylated Smc3 in G2, I set out to identify Smc3's deacetylase.

3.1 Hos1 is required for Smc3 deacetylation

3.1.1 Increased acetyl-Smc3 levels in cells lacking Hos1

The search for the deacetylase responsible for Smc3 deacetylation started from the idea that, in its absence, Smc3 would no longer be deacetylated during anaphase. This would lead to detectable acetyl-Smc3 in G1, when acetylation is normally absent and would also result in increased overall acetyl-Smc3 levels in asynchronously proliferating cultures. Therefore, a screen to find the Smc3 deacetylase was performed using a set of strains from the *Saccharomyces* genome deletion project (Giaever et al., 2002), where several deacetylases (*HOS1*, *HOS2*, *HOS3*, *HOS4*, *HDA1*, *RPD3*, *SIR2*, *HST1*, *HST2*, *HST3* and *HST4*) and also key deacetylase complex components (*SET3*, *HDA2*, *HDA3*, *SIN3* and *SDS3*) were deleted. Cell extracts were prepared from wild type cells and from the deletion mutant strains, either from asynchronous cultures or from cultures arrested in G1 by addition of α -factor (Figure 3.1). A polyclonal antibody raised against an Smc3 peptide covering acetylated lysine residues K112 and K113, and that recognises both acetylated lysines K112 and K113 (Figure 3.2A), was used to probe the Western blot. An increase in the levels of acetyl-Smc3 was observed in asynchronous *hos1* Δ cells. Furthermore, Smc3 acetylation was absent in G1-arrested wild type cells, but it was clearly detectable in *hos1* Δ cells. It was also detectable in *sin3* Δ , *sds3* Δ and *sir2* Δ , but in the latter cases it was likely because these cells did not

respond to α -factor treatment. This result suggests that Hos1 is responsible for Smc3 deacetylation.

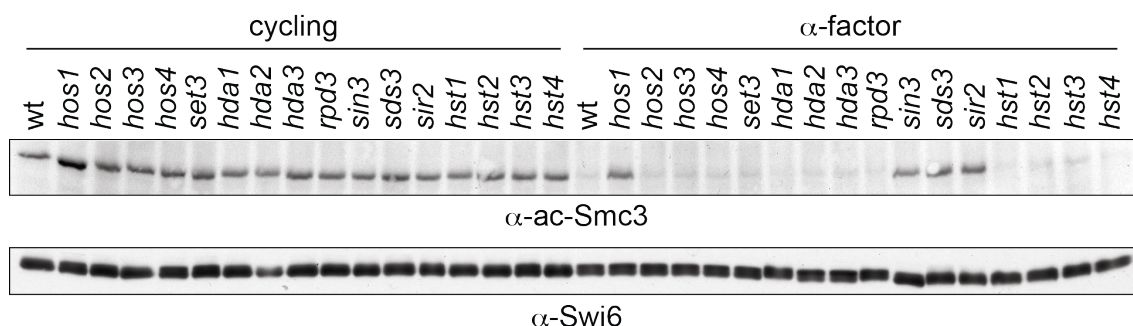


Figure 3.1 – Identification of Hos1 as the Smc3 deacetylase.

Cell extracts were prepared from either asynchronously or G1 arrested cells of strains from the *S. cerevisiae* genome deletion project (Table II), in which the indicated genes were deleted. The level of acetylated Smc3 was evaluated by Western blot by using a polyclonal α -acetyl-Smc3 antibody. Swi6 serves as a loading control. This experiment was carried out by Tom Rolef Ben-Shahar from the Chromosome Segregation Laboratory.

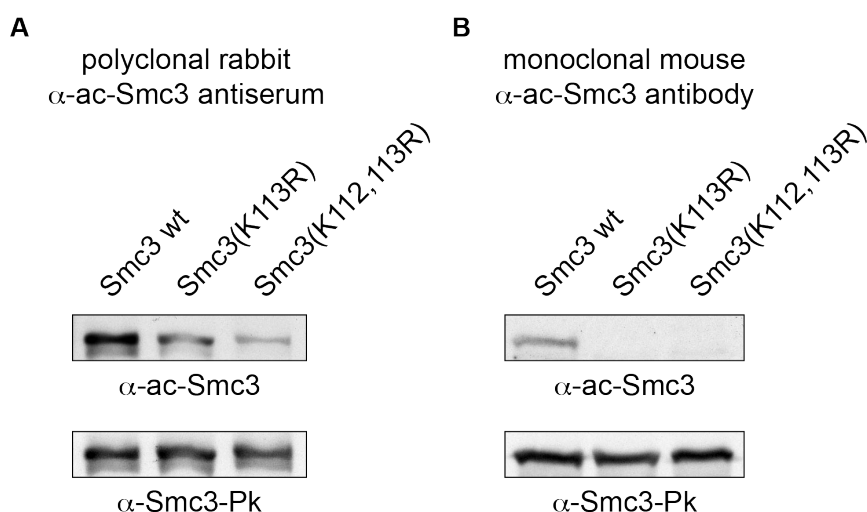


Figure 3.2 – Specificity of the acetyl-Smc3 antibodies used in this study.

Smc3 was immunopurified from cell extracts containing the indicated Smc3 variants and analysed by Western blotting using a (A) polyclonal or (B) monoclonal rabbit α -ac-Smc3 antiserum raised against a peptide covering acetylated lysines K112 and K113. Total Smc3 levels were visualised using an α -Pk antibody that recognises an epitope tag fused to Smc3.

3.1.2 Confirmation of Hos1 as the main Smc3 deacetylase

As Smc3 acetylation was also detectable in *sin3Δ*, *sds3Δ* and *sir2Δ* in G1-arrested cells from the previous experiment, the reconstruction of *HOS1*, *SIN3*, *SDS3* and *SIR2* deletions was performed in the W303 strain background. The cells were arrested in metaphase by Cdc20 depletion, which was under the control of the *GAL1* promoter, and released to progress in the cell cycle by inducing Cdc20 expression. The synchronous progression of the cells was monitored by FACS analysis of DNA content stained with propidium iodide (Figure 3.3). Cell extracts were prepared from the wild type and the mutant strains, and the Smc3 acetylation was analysed by Western blot using a polyclonal α -acetyl-Smc3 antibody. The result showed that Hos1, but not Sin3, Sds3 or Sir2, was required for Smc3 deacetylation at the time of anaphase, confirming the role of Hos1 as the main Smc3 deacetylase in budding yeast (Figure 3.3).

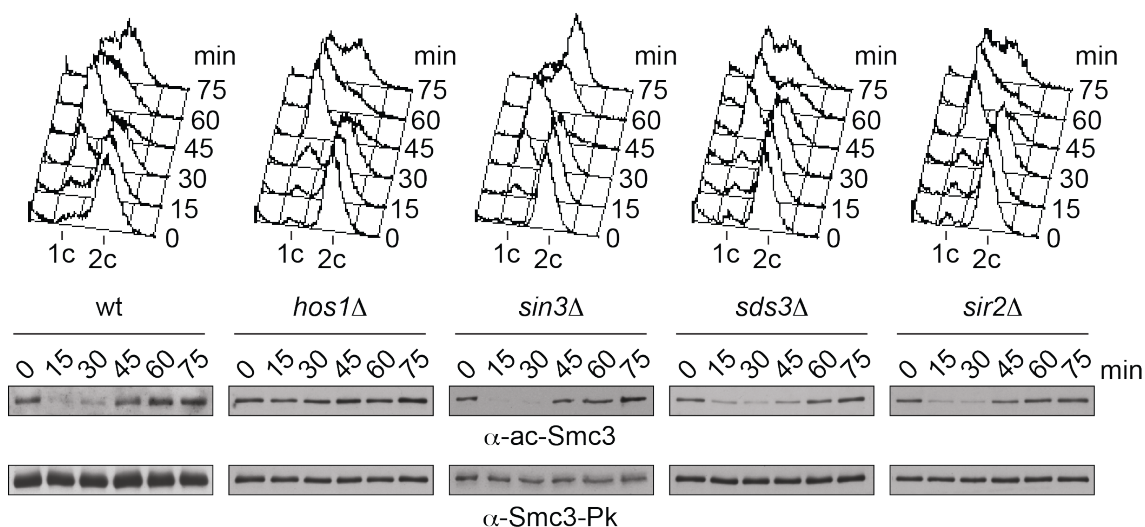


Figure 3.3 – Confirmation of Hos1 as the main Smc3 deacetylase.

Smc3-Pk was immunopurified from extracts of cells with the indicated genotypes, arrested in metaphase and released by Cdc20 depletion and re-addition under control of the *GAL1* promoter. The Smc3 acetylation status was analysed by Western blotting by using a polyclonal α -acetyl-Smc3 antibody. Cell cycle progression was monitored by FACS analysis of DNA content. This experiment was carried out by Chris Lehane from the Chromosome Segregation Laboratory.

3.2 Persistent Smc3 acetylation in the absence of Hos1

3.2.1 Analysis of Smc3 acetylation during the cell cycle

To further investigate the role of Hos1 as the Smc3 deacetylase, I compared the pattern of Smc3 acetylation throughout the cell cycle of wild type and *hos1* Δ cells. Cells were synchronised in G1 by α -factor treatment, released from the pheromone-induced arrest and then re-arrested in G1. The synchronous progression of the cells was monitored by FACS analysis of DNA content as before, and the time of anaphase was microscopically determined by the counting of binucleate cells. As previously reported (Ben-Shahar et al., 2008), in the wild type cells Smc3 acetylation was hardly detectable in G1, but increased at the time of S phase. After remaining strong throughout the period between S phase and M (G2), acetylation diminished again when cells entered anaphase (Figure 3.4A). In contrast to the wild type, Smc3 acetylation in the *hos1* Δ cells was detectable in G1 and persisted throughout the cell cycle (Figure 3.4B).

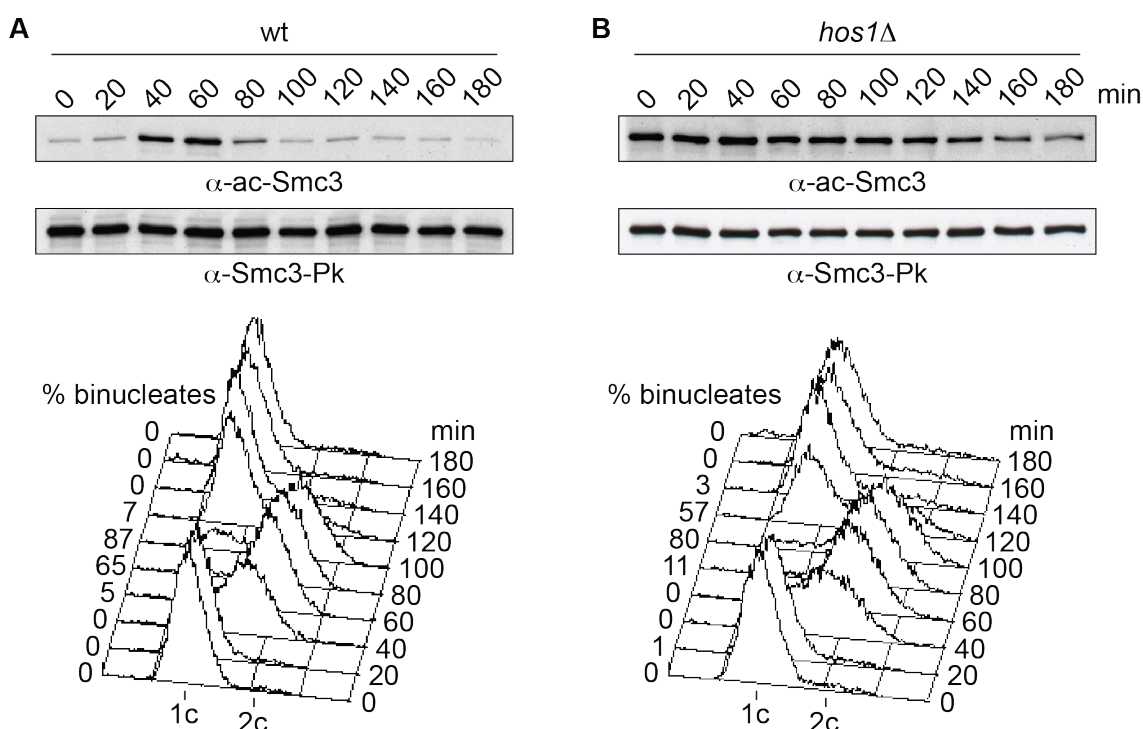


Figure 3.4 – Persistent Smc3 acetylation throughout the cell cycle in *hos1* Δ .

Wild type (A) and *hos1* Δ (B) cells were synchronised using α -factor block and release. Smc3-Pk was immunopurified from cell extracts, and the Smc3 acetylation analysed by Western blot using a polyclonal α -acetyl-Smc3 antibody. Cell cycle progression was monitored by FACS analysis of DNA content, and the time of anaphase was microscopically determined by the counting of binucleate cells.

3.2.2 Mass spectrometric analysis confirms persistent Smc3 K112 and K113 acetylation in *hos1* Δ cells

As previously reported, Eco1 acetylates Smc3 in 2 lysines residues: K112 and K113 (Ben-Shahar et al., 2008). The acetyl-Smc3 antibody used in the experiment to check persistent Smc3 acetylation throughout the cell cycle in *hos1* Δ (Figure 3.4) recognises both acetylated lysines K112 and K113 (Figure 3.2A), which means that the persisting Smc3 acetylation in the *hos1* Δ cells resulted from either one or both lysines being acetylated. To test this, I arrested *hos1* Δ cells in G1, immunopurified Smc3 and subjected it to mass spectrometric analysis (Figure 3.5). The fragmentation spectrum showed that the two residues are acetylated, suggesting that Hos1 is the deacetylase for both K112 and K113.

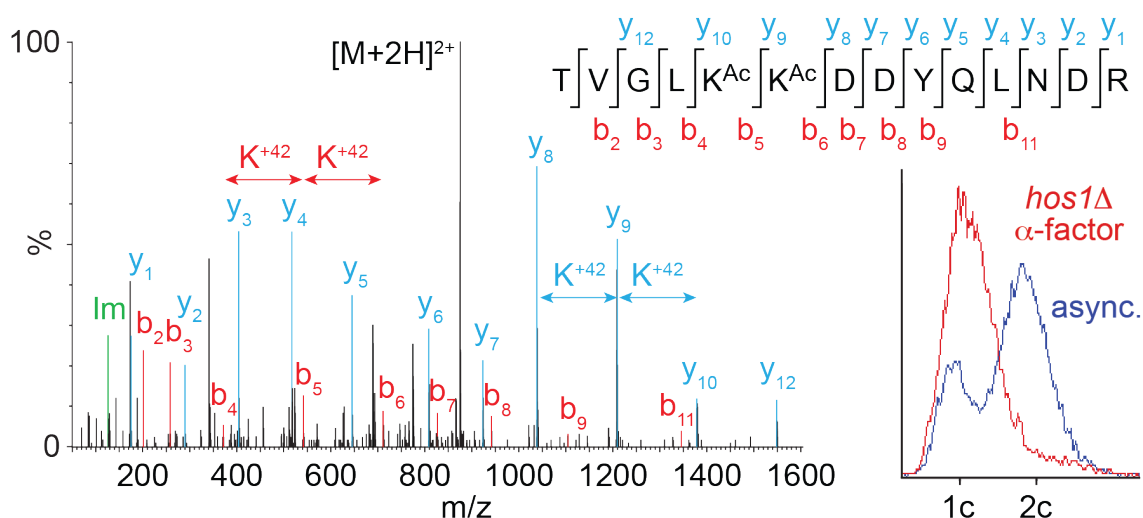


Figure 3.5 – K112 and K113 acetylation in *hos1* Δ .

Smc3-Pk was immunopurified from *hos1* Δ cells arrested in G1 and subjected to mass spectrometric analysis (Helen Flynn and Mark Skehel, Protein Analysis and Proteomics Laboratory, Clare Hall). The characteristic mass difference of acetyl lysines 112 and 113 in the y fragment ion series, and a diagnostic immonium ion derivative (Im) is shown. The G1 arrest was confirmed by FACS analysis (red) compared to asynchronous cells (blue).

3.3 Hos1 levels and localisation are constant during the cell cycle

3.3.1 Hos1 levels and migration remain unchanged during the cell cycle

As Smc3 deacetylation coincides with anaphase, I wondered whether the timing of deacetylation correlated with a regulation in the levels of Hos1. To analyse this I used a strain with an HA epitope tag fused to the endogenous gene locus of Hos1 and synchronised the cells in G1 using α -factor block and release and analysed the protein levels by Western blotting. The result revealed constant levels of *Hos1* throughout the cell cycle (Figure 3.6), suggesting that Hos1 does not undergo cell cycle-regulated synthesis or degradation. The migration pattern of Hos1 did not noticeably change during cell cycle progression, which might have been indicative of a posttranslational modification that regulates Hos1 activity. This observation does not exclude modifications that do not result in an accompanying mobility shift or modification of putative Hos1 interactors.

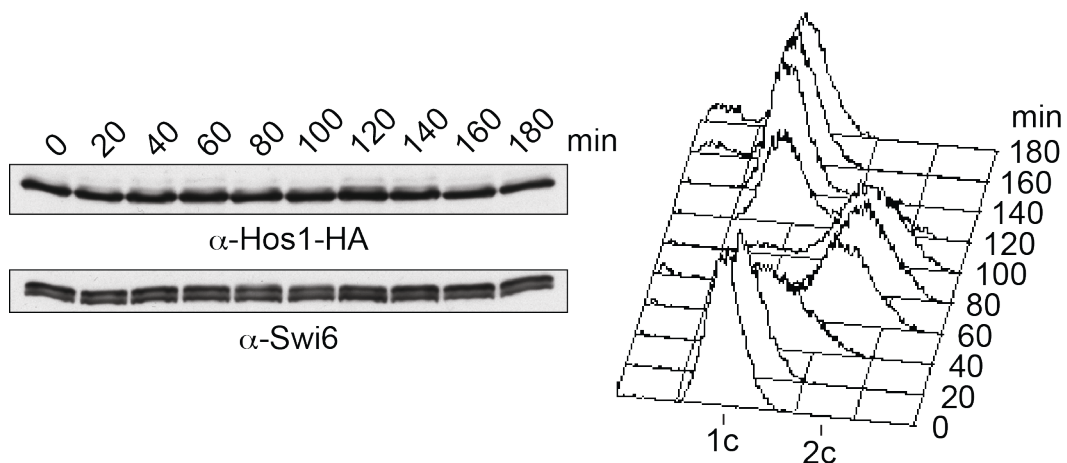


Figure 3.6 – Constant Hos1 levels during the cell cycle.

Western blotting against a C-terminal HA epitope tag fused to the endogenous Hos1 was performed to analyse the pattern of Hos1 throughout the cell cycle, by α -factor block and release. Swi6 serves as a loading control. Cell cycle progression was monitored by FACS analysis.

3.3.2 Unchanged, diffuse localisation of Hos1 throughout the cell cycle

The fact that Hos1 protein levels did not appear to change during the cell cycle does not exclude the hypothesis that its subcellular localisation may change. I therefore investigated the localisation of Hos1 in different stages of the cell cycle by indirect immunofluorescence. However, the Hos1-HA signal appeared very weak and diffuse and did not seem to change during the cell cycle (Figure 3.7). The cohesin subunit Scc1 was used as a control for cell cycle regulated nuclear accumulation, confirming its nuclear accumulation from late G1 until anaphase (Michaelis et al., 1997). Thus, I could not find evidence for Hos1 regulation by changes to its subcellular localisation.

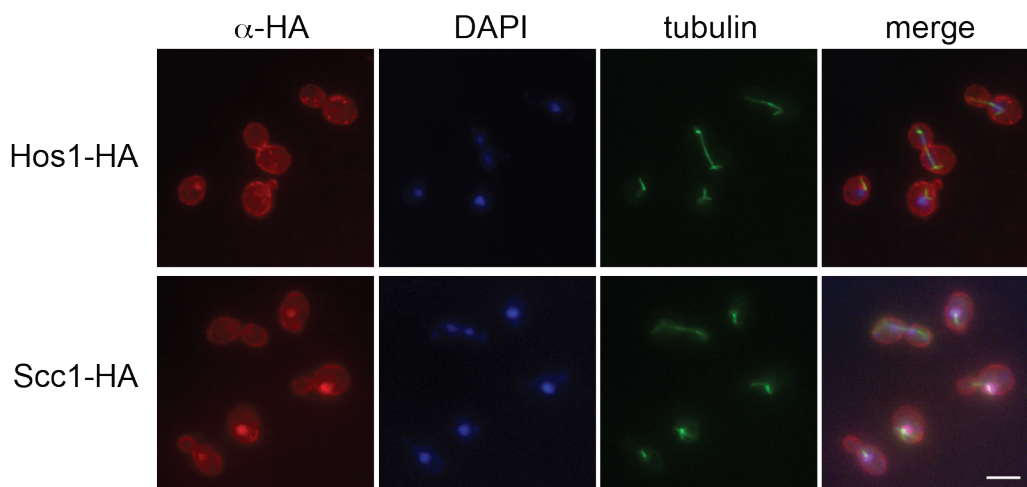


Figure 3.7 – Constant Hos1 localisation throughout the cell cycle.

Subcellular localisation of Hos1, stained for its HA epitope tag (red), was analysed at various cell cycle stages by indirect immunofluorescence. DAPI staining for cellular DNA (blue) and tubulin staining (green) are shown. Scc1-HA was included as a control. Size bar, 5 μ m.

3.4 Fluctuating Eco1 levels do not explain the Smc3 acetylation pattern

In the absence of evidence for Hos1 cell cycle regulation, I decided to investigate whether a possible cell cycle regulation of Eco1 contributes to anaphase specificity of Smc3 deacetylation. After Smc3 acetylation during S-phase, maintenance of acetylation might require continued Eco1 activity (Rowland et al., 2009). I therefore used a strain with an HA epitope tag fused to the endogenous gene locus of *Eco1*, synchronised the cells in G1 using α -factor block and release and analysed the protein levels by Western blotting (Figure 3.8). I observed an increase of Eco1 protein levels at the time of S-phase, which follows the pattern of ECO1 mRNA expression that also peaks at this time (Spellman et al., 1998), while during G2 and mitosis the levels decreased again. Although the timing of the increase of Eco1 correlates with the increase of Smc3 acetylation, the protein levels start to decrease before deacetylation occurs (Figure 3.8).

Nevertheless, to test directly if variation in Eco1 protein levels was somehow responsible for deacetylation, I used a strain overexpressing Eco1 under the control of the strong inducible *GAL1* promoter. I repeated the same experiment with this strain and analysed synchronised cells during the cell cycle. The result showed that even with increased levels of Eco1 the pattern of acetylation/deacetylation remained unchanged (Figure 3.8). It is therefore unlikely that reduced Eco1 levels explain Smc3 deacetylation during anaphase. As a control, I checked that the Eco1 overexpression did not cause any detectable defect in cell proliferation or sister chromatid cohesion (Figure 3.9). This further confirms the tight control over Smc3 acetylation independently of Eco1 protein levels.

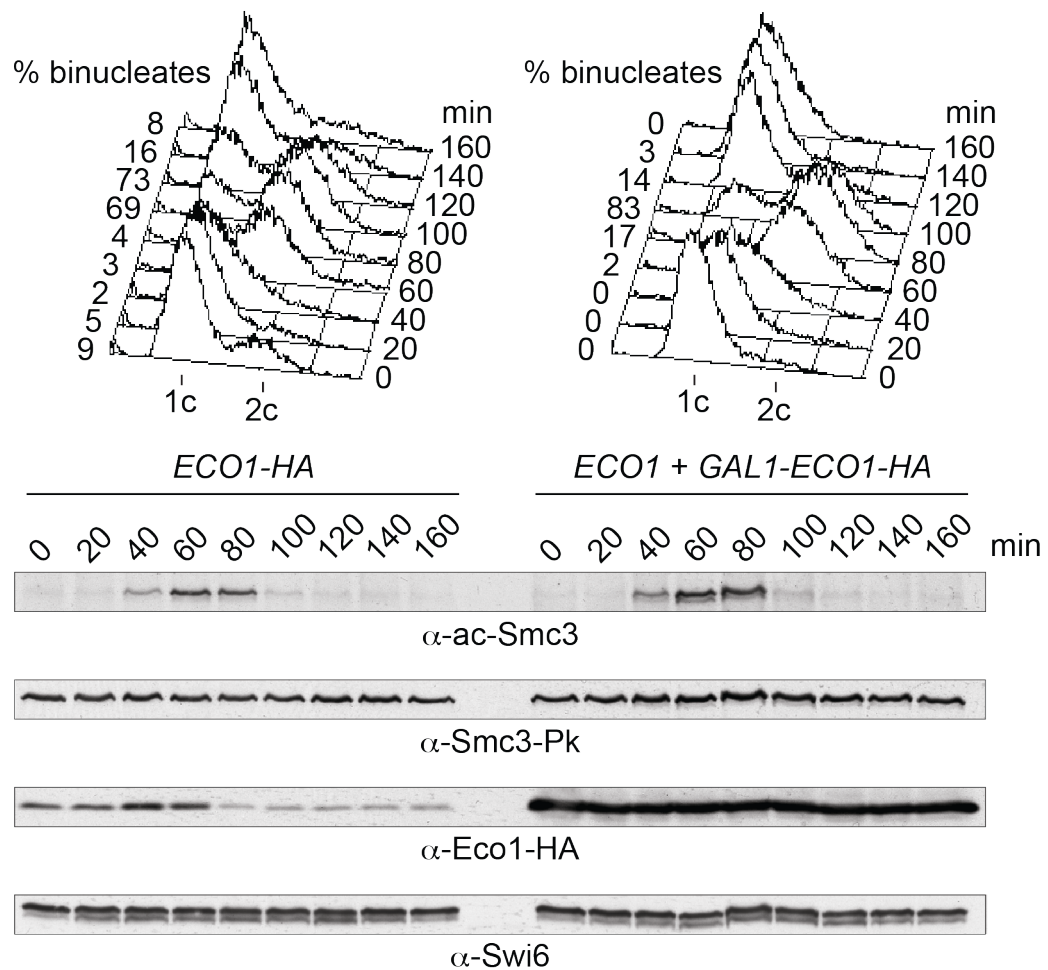


Figure 3.8 – Eco1 levels do not affect Smc3 acetylation.

Eco1 levels and Smc3 acetylation were analysed by Western blotting from cell extracts of synchronised cells, expressing endogenous levels or overexpressing Eco1 under the control of the *GAL1* promoter. Swi6 serves as a loading control. FACS analysis was used to monitor cell cycle progression and the time of anaphase was microscopically determined by the counting of binucleate cells.

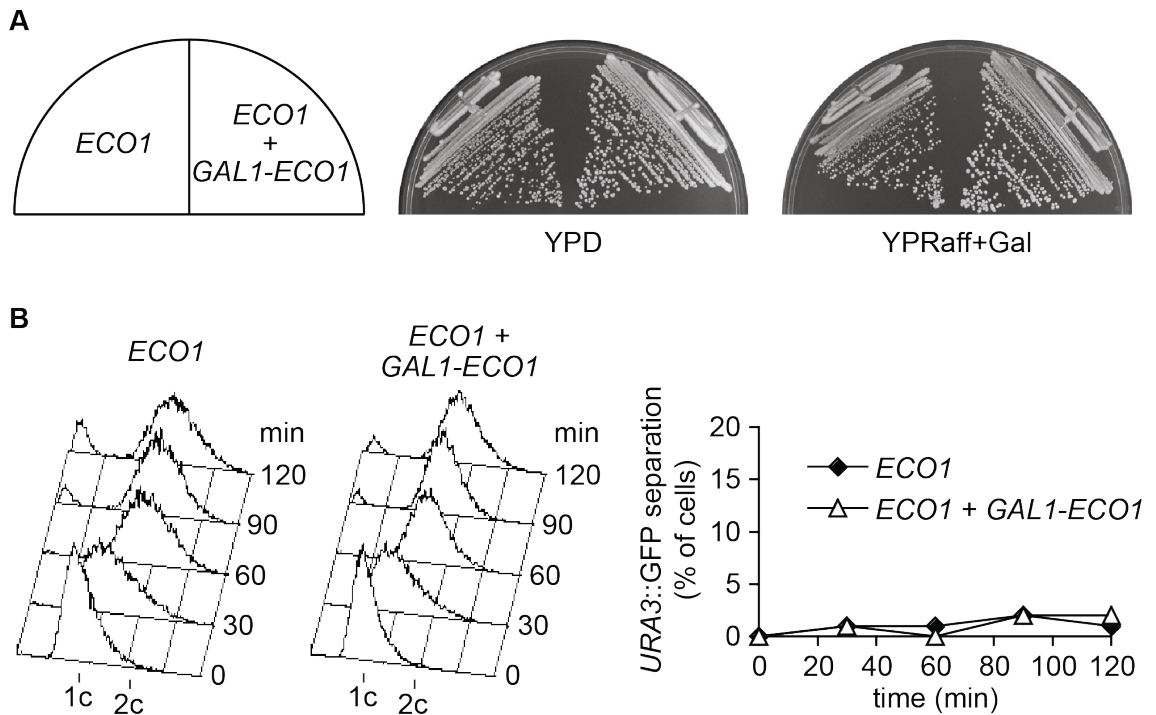


Figure 3.9 – Cell growth and sister chromatid cohesion are unaffected by *Eco1* overexpression.

(A) Strains without or with an extra copy of *ECO1* under control of the *GAL1* promoter were grown on medium to repress (YPD) or induce (YPRaff+Gal) ectopic *Eco1* expression. Plates were incubated for 2-3 days at 25°C. **(B)** Cultures of the strains in (A) were grown in YPRaff+Gal medium to induce *Eco1* expression and synchronised by α -factor block and release. Sister chromatid cohesion at the *URA3* locus was monitored using the tetOs/tetR-GFP system while cells progressed into nocodazole-imposed mitotic arrest. Cell cycle progression was monitored by FACS analysis of DNA content.

Although Eco1 protein levels during the cell cycle do not explain the timing for Smc3 deacetylation, I hypothesised that Eco1's acetyltransferase activity could be subjected to cell cycle regulation. As Eco1 possesses autoacetylation activity (Ivanov et al., 2002), I used this as readout for its catalytic activity. I analysed Eco1's autoacetyltransferase activity using an acetyl-lysine specific antibody to probe for *GAL1*-promoter expressed Eco1-HA immunoprecipitates prepared from synchronised cultures. After incubation with acetyl-CoA, I detected Eco1 autoacetylation throughout the cell cycle that was however noticeably reduced during S and G2 phases (Figure 3.10). This result was unexpected considering that this is normally when Smc3 acetylation is high. As a caveat, we do not know how far the *in vitro* autoacetyltransferase activity reflects Eco1's ability to acetylate Smc3 *in vivo*. My attempts to measure Eco1 acetyltransferase activity using a recombinant Smc3 head domain as substrate were unsuccessful, maybe due to additional requirements for Smc3 acetylation at the replication fork. Nevertheless, this result suggests that Smc3 deacetylation is not a consequence of a downregulation in Eco1's acetyltransferase activity in anaphase.

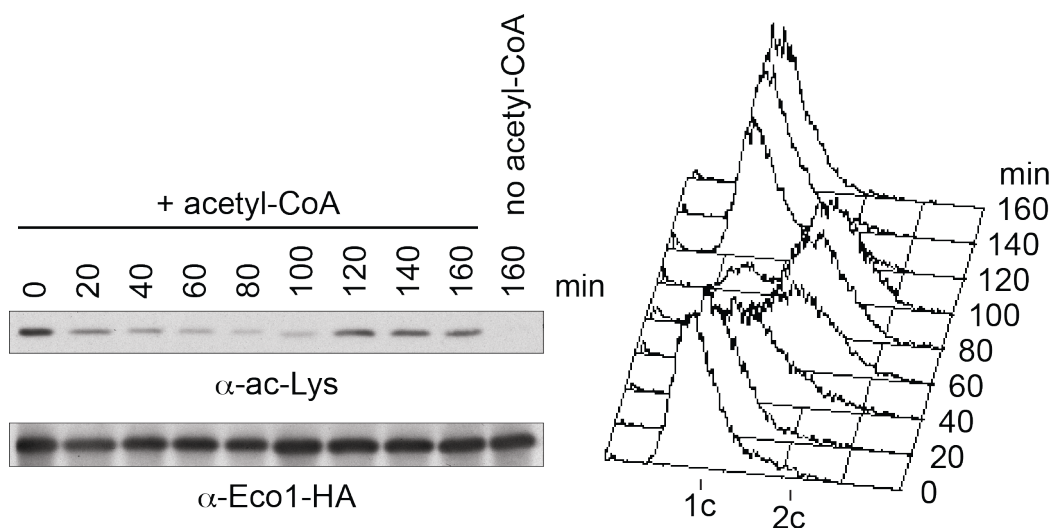


Figure 3.10 – Eco1 autoacetyltransferase activity is regulated during the cell cycle.

Overexpressed Eco1 was immunopurified from a synchronous culture and incubated for 25 min at 30 °C in the presence or absence of acetyl-CoA. The levels of autoacetylated Eco1 were evaluated by Western blot by using an α -acetyl lysine antibody. Cell cycle progression was monitored by FACS analysis.

3.5 Cohesin dissociation from chromosomes triggers Smc3 deacetylation

3.5.1 Uncleavable Scc1 prevents Smc3 deacetylation

Since the above results failed to explain how Smc3 deacetylation during anaphase is achieved, I decided to explore how changes in the cohesin complex may affect its acetylation. Cleavage of Scc1 by separase releases cohesin from chromosomes to trigger chromosome segregation at anaphase onset (Uhlmann et al., 2000). Additionally, Smc3 deacetylation occurs by the time of anaphase. This means that deacetylation is either a consequence of cohesin cleavage or simply due to cell cycle progression. To differentiate between these two possibilities I expressed from the *GAL1* promoter an uncleavable version of Scc1 in which both separase recognition sites have been mutated. In this case the cell cycle progresses normally but cohesin is not cleaved. Cells were synchronised in G1, released from the pheromone-induced arrest and then re-arrested in G1. Although the cells struggled to go through anaphase, the cell cycle is progressing mostly unaltered as evidenced by the accumulation and destruction of the mitotic cyclin Clb2 (Figure 3.11). Nevertheless, absence of Scc1 cleavage prevented Smc3 deacetylation in anaphase, when it would normally take place. A partial reduction in Smc3 acetylation that became apparent could be due to cleavage of cohesin complexes containing endogenous wild type Scc1 that is also present in these cells. This suggests that Scc1 cleavage is necessary for Smc3 deacetylation.

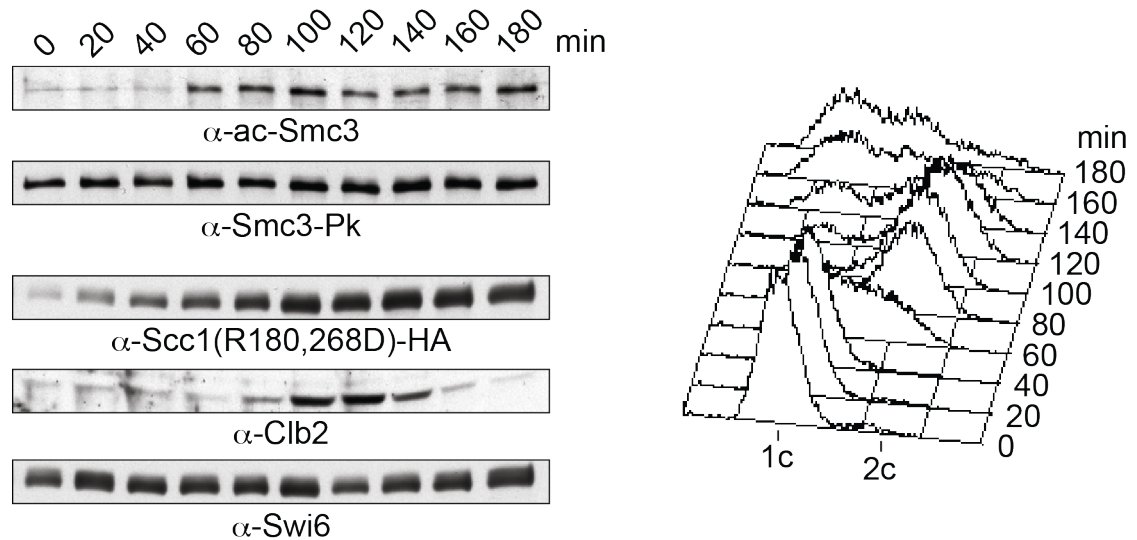


Figure 3.11 – Uncleavable Scc1 prevents Smc3 deacetylation.

Cells expressing uncleavable Scc1 (R180, 268D) under the control of the *GAL1* promoter were synchronised by α -factor block and release. The *GAL1* promoter was induced upon cell release. The acetylation level of Smc3 was monitored by Western blot using a polyclonal α -acetyl-Smc3 antibody. FACS analysis of DNA content and accumulation and degradation of the mitotic cyclin Clb2 were both used to monitor cell cycle progression.

3.5.2 Scc1 cleavage triggers Smc3 deacetylation

I next tested whether Scc1 cleavage was sufficient to trigger Smc3 deacetylation. In this case I used a strain in which one of the two separate cleavage sites were replaced by the recognition sequence of the TEV protease. Cells were arrested in metaphase by depletion of Cdc20 and TEV protease was expressed from the *GAL1* promoter. Cells did not progress further through the cell cycle, as shown by the persistence of the mitotic cyclin Clb2. However, even though cells remained arrested in metaphase, Scc1 cleavage triggered Smc3 deacetylation (Figure 3.12). This result indicates that Hos1 is already active in metaphase and it is the cleavage of Scc1 rather than a change in Hos1 that is responsible for Smc3 deacetylation.

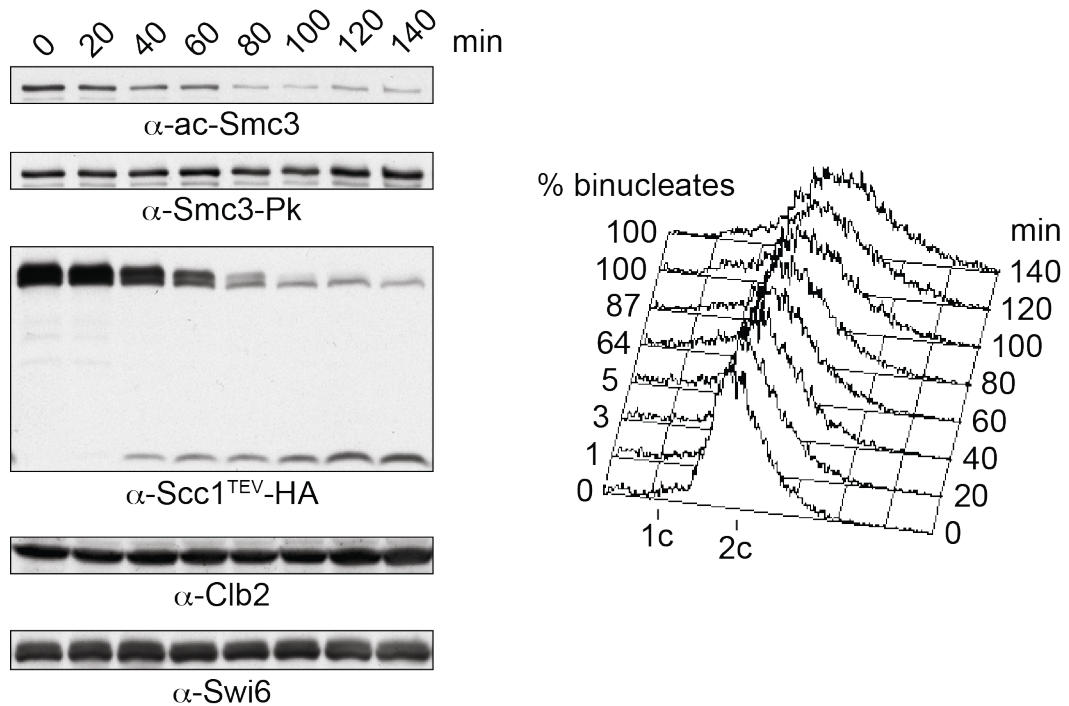


Figure 3.12 – Scc1 cleavage triggers Smc3 deacetylation.

Cells containing TEV-cleavable Scc1 were arrested in metaphase by depletion of Cdc20. Scc1 cleavage was induced by expression of TEV protease under the control of *GAL* promoter. The acetylation level of Smc3 was monitored by Western blot using a polyclonal α -acetyl-Smc3 antibody. FACS analysis of DNA content and accumulation of the mitotic cyclin Clb2 were both used to confirm the mitotic arrest. The time of anaphase was microscopically determined by the counting of binucleate cells.

3.5.3 Hos1-dependent *in vitro* Smc3 deacetylation in response to chromosome fragmentation

The above findings showed that Smc3 deacetylation occurs as a consequence of Scc1 cleavage. This cleavage leads to two different events: (1) a conformational change in the cohesin complex and (2) the dissociation of cohesin from chromosomes. To distinguish which of these events is responsible for Smc3 deacetylation I established an *in vitro* assay to analyse Smc3 deacetylation in response to cohesin cleavage or chromosome fragmentation. Extracts were prepared from metaphase arrested wild type or *hos1* Δ cells and incubated at 25 °C either with TEV protease or

micrococcal nuclease. As expected, incubation with TEV protease resulted in Scc1 cleavage and consequent decrease in Smc3 acetylation (Figure 3.13).

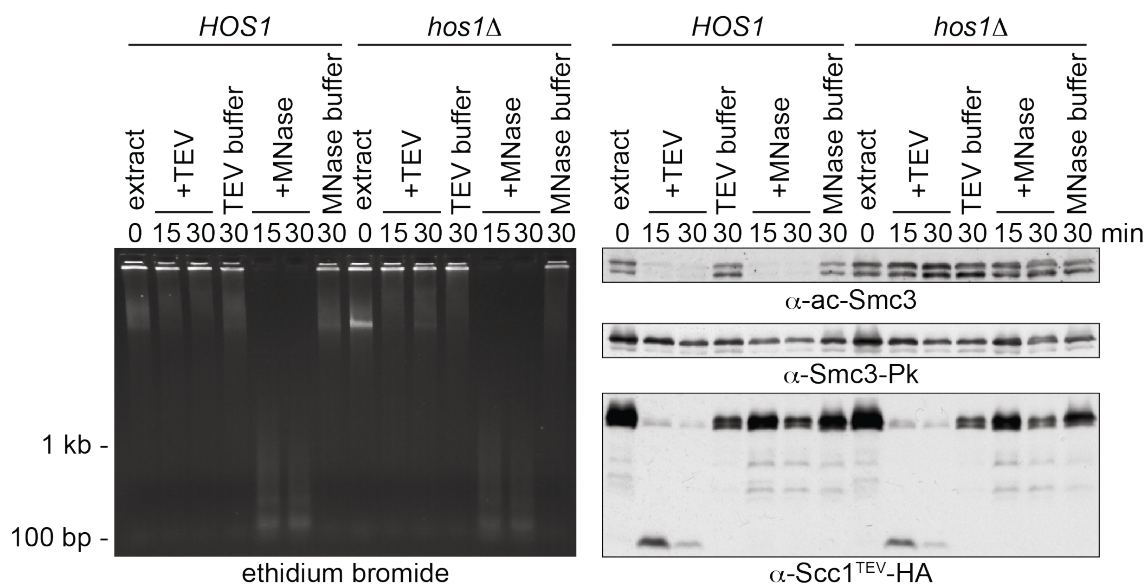


Figure 3.13 – Chromosome fragmentation triggers Smc3 deacetylation.

Cells containing TEV-cleavable Scc1 were arrested in metaphase by depletion of Cdc20. Cell extracts were incubated for 15 min at 25 °C with TEV protease, micrococcal nuclease or the respective buffers as control. Acetylation of Smc3 and Scc1 cleavage were analysed by Western blotting, and chromosome cleavage was monitored by ethidium bromide staining.

In addition, incubation of these cells with micrococcal nuclease resulted in chromosome fragmentation that also led to Smc3 deacetylation, although the cohesin complex remained intact. As a control, when extracts were prepared from cells lacking *HOS1* Smc3 acetylation persisted in both conditions. These results indicate that Hos1 is capable of deacetylating Smc3 when cohesin is dissociated from chromosomes, even if Scc1 is not cleaved. As long as Smc3 is part of a chromosome-bound cohesin complex it remains protected from Hos1.

3.6 Persisting Smc3 acetylation does not establish sister chromatid cohesion

3.6.1 *hos1* Δ does not rescue the defects of the *eco1-1* mutant

The above experiments have provided insight into the regulation of Smc3 deacetylation, but have not addressed its importance. Eco1-dependent Smc3 acetylation is required for cohesion establishment, so the persistence of acetyl-Smc3 in *hos1* Δ cells might partially compensate for compromised acetyltransferase activity in *eco1* mutant cells. To test this, I streaked wild-type, *eco1-1*, *hos1* Δ and *eco1-1 hos1* Δ cells, in either W303 or S288c strain background, on YPD medium and incubated at different temperatures for 2-3 days. In contrast to my expectation, the thermosensitive growth of an *eco1-1* mutant strain was not improved by *hos1* deletion even at semi-permissive temperatures (Figure 3.14A). The same observation was made using a second temperature sensitive *eco1* allele, *ctf7-203* (Skibbens et al., 1999; Tóth et al., 1999). I then tested whether the *hos1* deletion would rescue the radiation sensitivity of an *eco1-1* strain. However, the sensitivity of *eco1-1* cells to irradiation-induced double stranded DNA breaks (Ström et al., 2007; Ünal et al., 2007), persisted in the absence of Hos1 (Figure 3.14B).

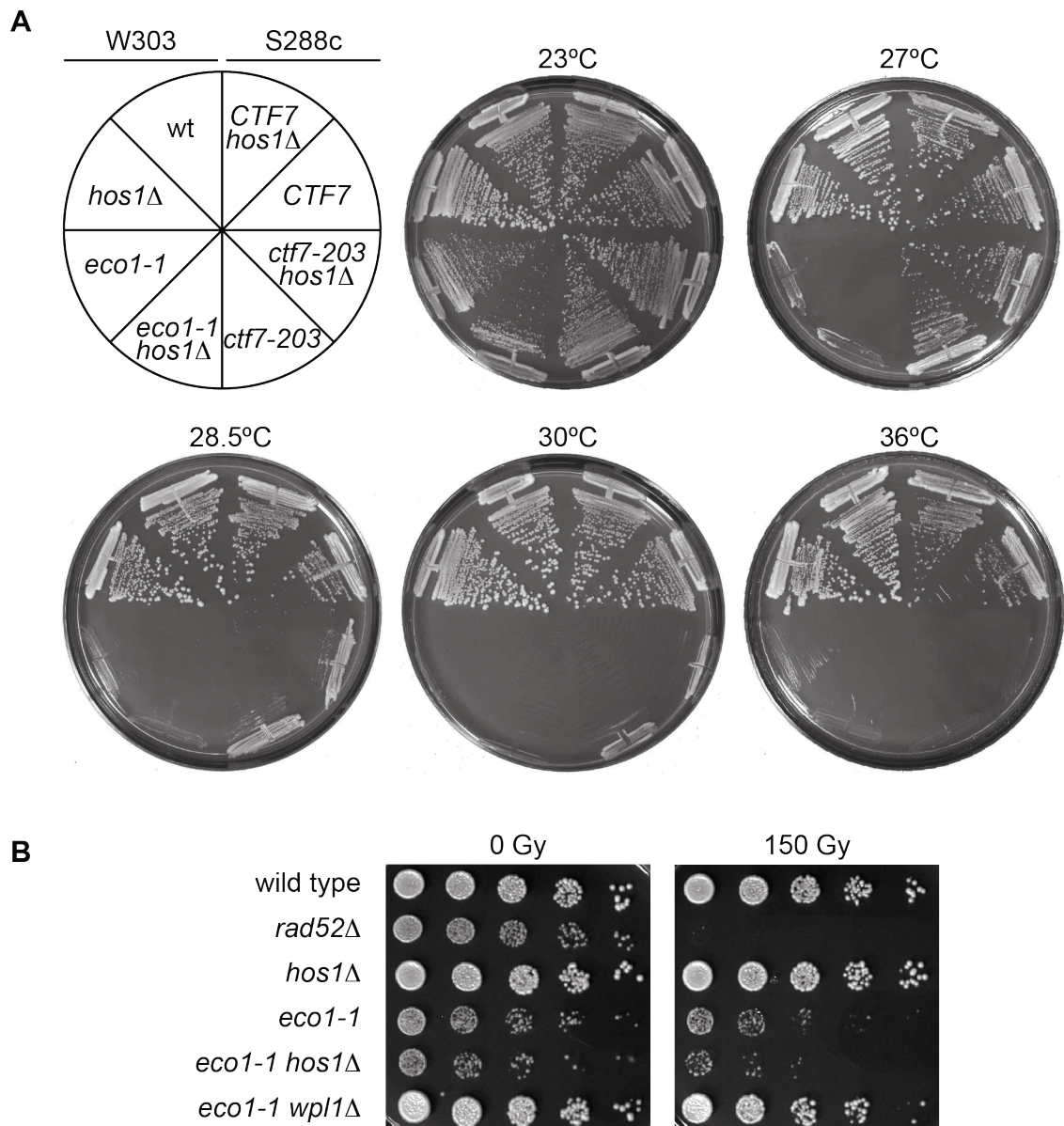


Figure 3.14 – *hos1* deletion does not rescue the temperature sensitive growth or radiation sensitivity of *eco1* mutants.

(A) Cells of the indicated genotypes, in either W303 or S288c strain background, were streaked on YPD medium and incubated at the indicated temperatures for 2-3 days. **(B)** 5-fold serial dilutions of cultures of the indicated genotypes were spotted onto YPD agar plates and incubated at 23°C without radiation or after exposure to 150 Gy g-radiation from a ¹³⁷ Cs source. *rad52Δ* cells served as a radiation sensitive control, *eco1-1 wpl1Δ* cells were included as control for the suppression of *eco1-1* radiation sensitivity (Ben-Shahar et al., 2008).

To analyse whether the persistence of acetylated Smc3 levels in *hos1* Δ mutants could compensate for the cohesion defects of the *eco1* Δ mutant cells, I analysed sister chromatid cohesion at the *URA3* locus, visualised by a tetR-GFP fusion protein bound to tet operator sequences inserted at this locus (Michaelis et al., 1997). In G2/M cells a single GFP dot visible within a cell indicates cohesion between the two chromosomes. Two GFP dots visible within a cell indicate loss of cohesion. I arrested *eco1-1* and *eco1-1 hos1* Δ in G1 at the permissive temperature, released into restrictive temperature and blocked in metaphase by nocodazole treatment. As expected, Eco1 inactivation in *eco1-1* cells compromised the establishment of cohesion (Figure 3.15). Deletion of *Hos1* did not reduce the cohesion defect, suggesting that persisting acetylated Smc3 is unable to establish sister chromatid cohesion. *De novo* acetylation of Smc3 during DNA replication appears to be essential to promote sister chromatid cohesion establishment.

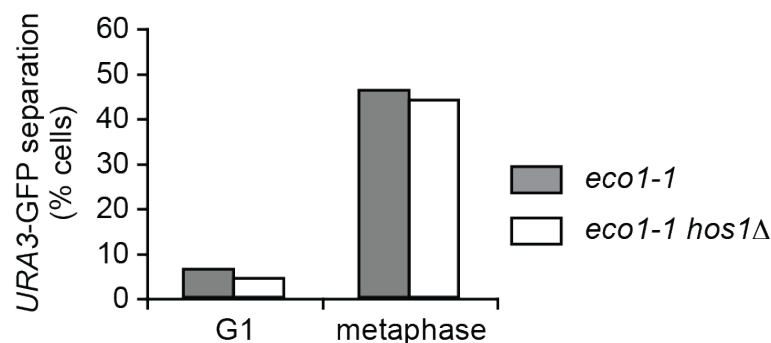


Figure 3.15 – *hos1* Δ does not rescue the *eco1-1* cohesion defect.

eco1-1 and *eco1-1 hos1* Δ cells were arrested in G1 by α -factor treatment at 21.5 °C and released at 35 °C, to inactivate Eco1, into medium containing nocodazole for metaphase arrest. Sister chromatid cohesion was analysed at the GFP-marked *URA3* locus.

3.6.2 Evidence for the importance of *de novo* Smc3 acetylation

To investigate the importance of *de novo* Smc3 acetylation, I analysed sister chromatid cohesion by the analysis of GFP-marked *URA3* locus in metaphase-arrested cells in the absence of Hos1. *hos1* Δ cells are viable, but displayed a clearly discernible defect in sister chromatid cohesion (Figure 3.16). As suggested by the above findings, this could be due to the reduced availability of non-acetylated Smc3 as substrate for cohesion establishment. It could alternatively be an indirect consequence of derepression of certain genes that affect sister chromatid cohesion under control of the Ssn6/Tup1/Hos1 histone deacetylase complex. To differentiate between these possibilities, I compared *hos1* Δ with *smc3(K113N)* cells in which Smc3 lysine K113 has been replaced with acetylation mimicking asparagine. Despite its ability to overcome the Eco1 requirement for cohesion establishment (Ben-Shahar et al., 2008), the *smc3(K113N)* mutation caused a cohesion defect comparable to that of *hos1* Δ cells. Importantly, combination of the *smc3(K113N)* and *hos1* Δ mutations hardly increased this defect. This suggests that both mutations cause defective sister chromatid cohesion for the same reason, probably the reduced availability of nonacetylated Smc3.

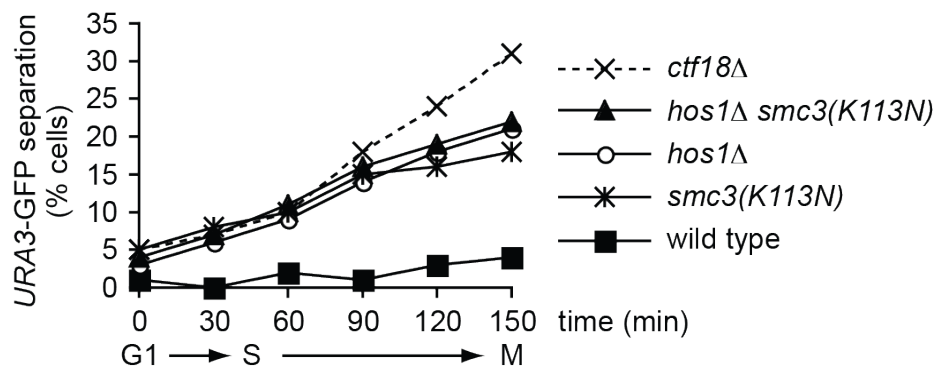


Figure 3.16 – Cohesion defect in *hos1* Δ cells.

Cells of the indicated genotypes were arrested in G1 by α -factor treatment and released into nocodazole. Sister chromatid cohesion was analysed at the GFP-marked *URA3* locus.

Smc3 transcription is upregulated at the G1/S boundary (Spellman et al., 1998), so that even *hos1* Δ cells are partly replenished with newly synthesised, non-acetylated Smc3 before cohesion establishment. The importance of newly synthesised Smc3 for *hos1* Δ cells was determined by placing Smc3 expression under control of the galactose-regulated *GAL1* promoter and analysing sister chromatid cohesion at the GFP-marked *URA3* locus (Figure 3.17). The increased expression of non-acetylated Smc3 made Hos1 dispensable for sister chromatid cohesion, as compared to cells expressing endogenous Smc3 levels. The cohesion defect of *hos1* Δ cells expressing endogenous Smc3 levels was lower in this experiment, compared to Figure 3.16, maybe because of the slower rate of proliferation of these cultures in YPRaff+Gal medium. At reduced Smc3 levels, sister chromatid cohesion became markedly compromised only in the absence of Hos1. These findings are consistent with the idea that *hos1* Δ cells rely on newly synthesised, non-acetylated Smc3 as the substrate for *de novo* acetylation during cohesion establishment.

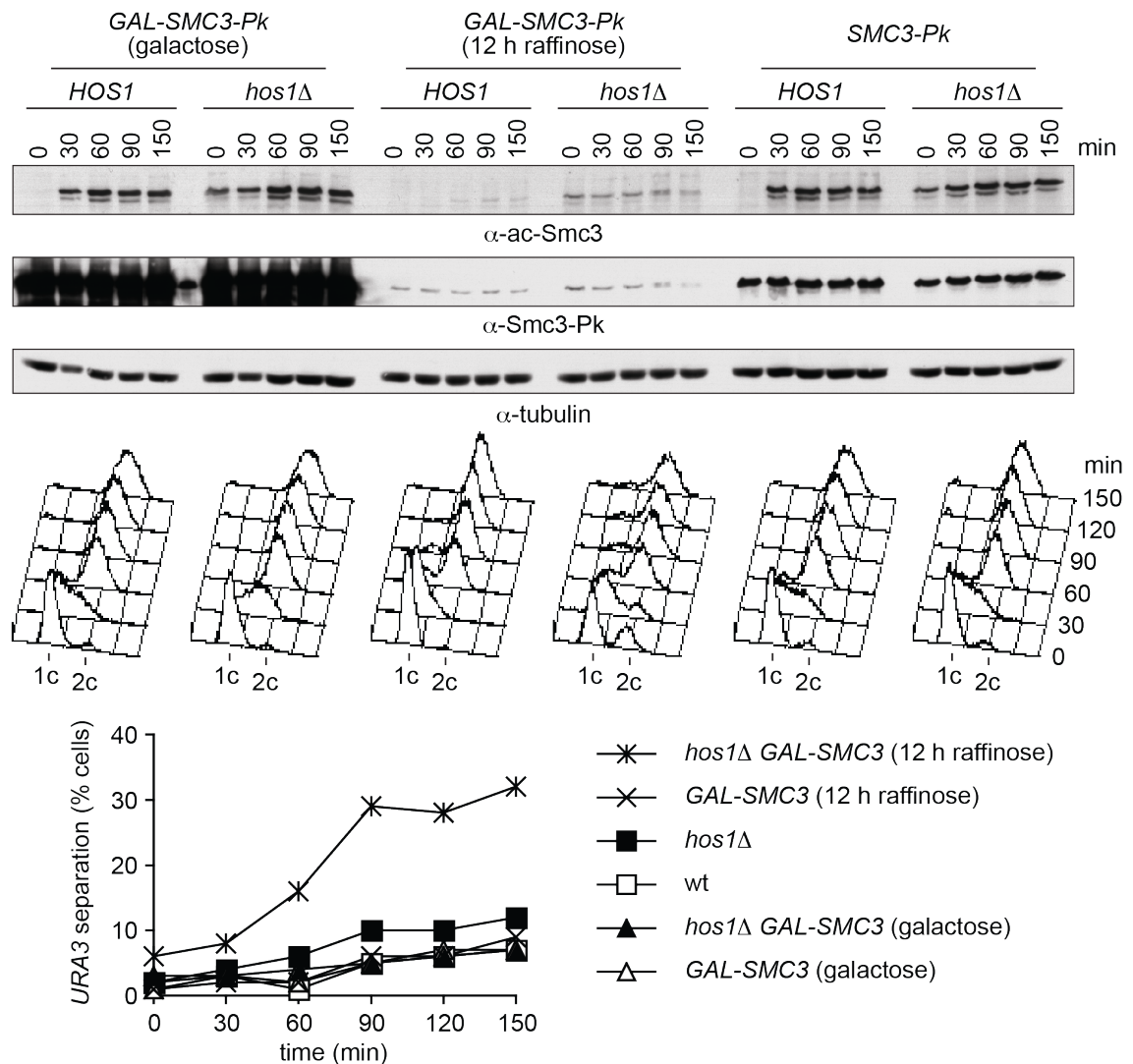


Figure 3.17 – Increased Hos1 requirement for sister chromatid cohesion at reduced Smc3 levels.

Expression of Smc3 was placed under control of the *GAL1* promoter. Cells were grown under inducing conditions (YP_{Raff}+Gal medium) and half of the culture was transferred to non-inducing YP_{Raff} medium for 12 hours. Cells were then arrested in G1 by α -factor treatment and released to progress through the cell cycle and into a nocodazole-induced mitotic arrest. A culture expressing endogenous Smc3 levels was included for comparison. Levels of acetyl-Smc3 and total Smc3 were compared by Western blotting, tubulin served as loading control. This experiment was carried out by Frank Uhlmann from the Chromosome Segregation Laboratory.

Further experiments to explore the importance of non-acetylated Smc3 were also carried out during this study and can be found in (Borges et al., 2010). Together, these results identify the class I histone deacetylase family member Hos1 as the protein responsible for Smc3 deacetylation. Interestingly, it seems that as long as Smc3 is part of a chromosome-bound cohesin complex it remains protected from Hos1. Furthermore, persisting acetylated Smc3 is unable to establish sister chromatid cohesion, suggesting that *de novo* acetylation of Smc3 during DNA replication is essential to establish sister chromatid cohesion.

Chapter 4

An Eco1-Independent Cohesion Establishment Pathway

Chapter 4. An Eco1-Independent Cohesion Establishment Pathway

Establishment of sister chromatid cohesion is a process thought to occur as the replication fork passes chromosomal loci bound by the cohesin complex. However, what happens at replication forks to establish sister chromatid cohesion is poorly understood. I therefore decided to investigate the role of replication fork-associated proteins required for efficient cohesion establishment. In particular, I explored the role of non-essential cohesion proteins with respect to the replication fork, and their relationship to Eco1.

4.1 Additional “cohesion establishment factors” contribute to cohesin acetylation

4.1.1 Decreased acetyl-Smc3 levels in all of the “cohesion establishment factors” mutants

A previous study (Xu et al., 2007) divided cohesion establishment factors into two genetic epistasis groups, one containing the Tof1/Csm3 complex, Ctf4 and Chl1, and the second containing the Ctf18-RFC complex and Mrc1. However, it is not known whether and in which of these pathways the acetyltransferase Eco1 is involved. The action of these proteins could either be to support Eco1 in acetylating cohesin, or they could contribute to an Eco1-independent cohesion establishment reaction. As Eco1's major role is to acetylate Smc3 (Ben-Shahar et al., 2008; Borges et al., 2010; Ünal et al., 2008) I investigated the contribution of each protein to Smc3 acetylation during the cell cycle. I synchronised wild type cells and the cohesion establishment factors mutants (*ctf4* Δ , *ctf18* Δ , *tof1* Δ , *csm3* Δ , *chl1* Δ and *mrc1* Δ) in G1 using α -factor block and released them into a nocodazole-imposed mitotic arrest. As expected, Western blotting using an antibody against acetyl-Smc3 revealed that in wild type cells Smc3 acetylation was hardly detectable in the prereplicative phase (G1) and increased upon DNA replication, remaining stable in M phase (G2). In contrast, the absence of each of these proteins caused a measurable decrease in Smc3 acetylation levels (Figure 4.1A).

To test for significant differences in protein levels between each cohesion establishment factor mutant, I quantified the acetyl-Smc3 bands using the ImageQuant LAS4000 software. Protein quantification showed that although there is a decrease of acetyl-Smc3 levels in all mutants, *CTF4* and *CHL1* had the biggest impact, with less than 30% Smc3 acetylation relative to the wild type (Figure 4.1B).

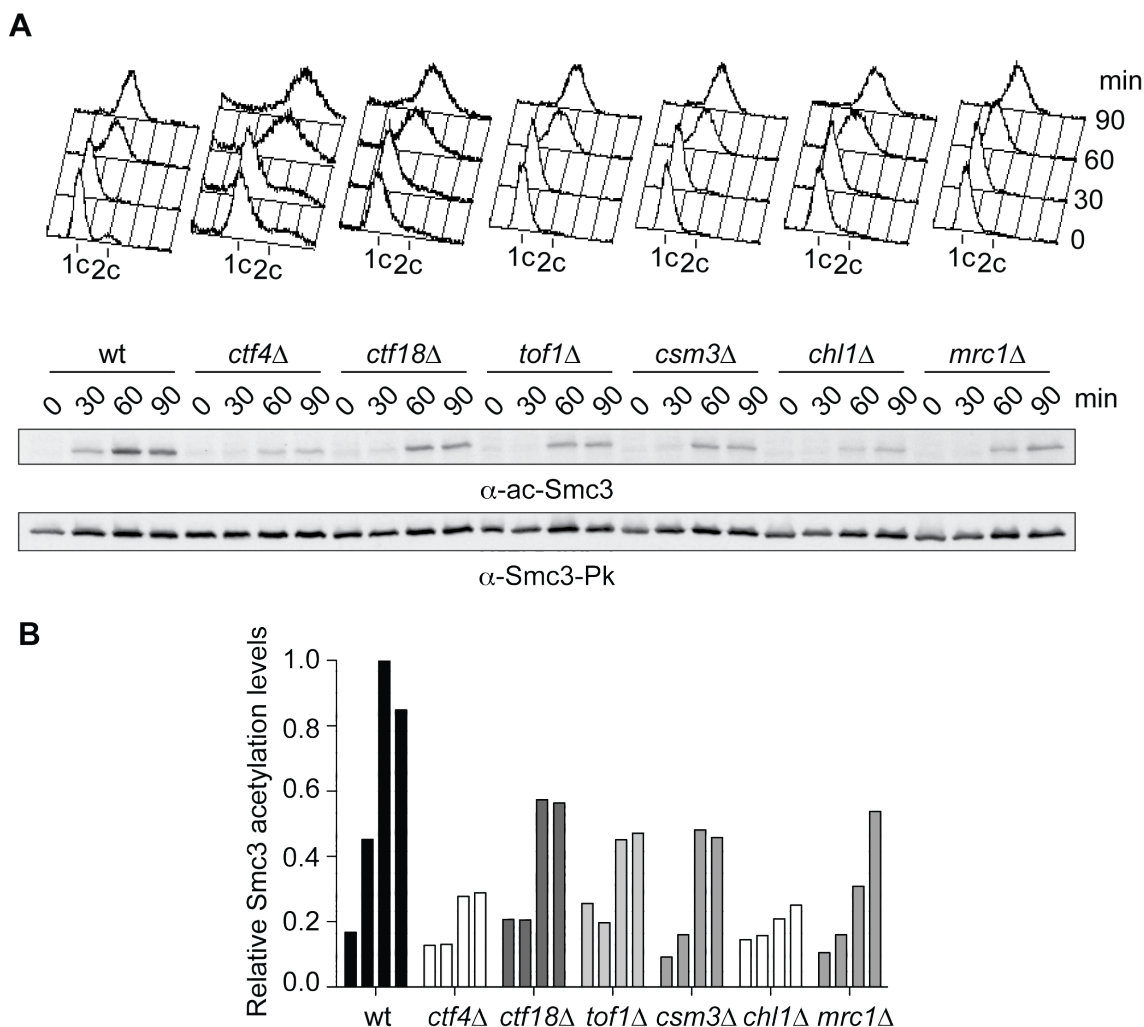


Figure 4.1 – Additional “cohesion establishment factors” contribute to cohesin acetylation.

(A) Decreased acetyl-Smc3 levels in ‘cohesion establishment factors’ mutants. Cells of the indicated strains were synchronised in G1 by α -factor treatment and released into nocodazole-imposed mitotic arrest. The acetylation status of Smc3 was analysed by Western blotting using a monoclonal α -acetyl-Smc3 antibody. FACS analysis monitored cell cycle progression. **(B)** Quantification of the relative abundance of Smc3 acetylation in the different mutants using the ImageQuant LAS4000 software.

Cohesion establishment is thought to be tightly coupled with the passage of the replication fork (Lengronne et al., 2006). In particular, both Ctf4 and Chl1 have been closely associated with events happening at the fork passage (Farina et al., 2008; Formosa and Nittis, 1999; Miles and Formosa, 1992; Zhou and Wang, 2004). These results are therefore consistent with the idea that Smc3 acetylation and replication fork progression are intimately related.

4.1.2 Reduced cohesin acetylation in *ctf4* Δ and *chl1* Δ may not explain their cohesion defects

Although Ctf4 and Chl1 are not essential for the cell, they both contribute to efficient sister chromatid cohesion (Hanna et al., 2001; Petronczki et al., 2004). Mutant forms of these nonessential cohesion genes lead to cohesion defects (30% premature sister chromatid separation) at both chromosome arms and centromere regions (Xu et al., 2007). To understand the importance of the reduced Smc3 acetylation in *ctf4* Δ and *chl1* Δ mutants I addressed whether this reduction could explain their cohesion defects by comparing them to *eco1-1* cells. This temperature sensitive mutant is lethal at the restrictive temperature, exhibiting high levels of cohesion defects (Ben-Shahar et al., 2008; Ünal et al., 2008). I synchronised the cells in G1 at the permissive temperature for the *eco1-1* allele. Cells were then released to progress through the cell cycle and arrested in mitosis by nocodazole treatment (Figure 4.2A). Acetyl-Smc3 levels in the *eco1-1* cells remained low throughout the cell cycle, even at the permissive temperature. As before, *ctf4* Δ and *chl1* Δ showed decreased levels of acetyl-Smc3 compared to the wild type, but substantially higher than the *eco1-1* allele. I next analysed sister chromatid cohesion at the *URA3* locus, visualised by a tetR-GFP fusion protein bound to tet operator sequences inserted at this locus (Michaelis et al., 1997). As expected, *eco1-1* cells compromised establishment of cohesion, showing high cohesion defects (Figure 4.2B). If there were a correlation between acetyl-Smc3 levels and the establishment of cohesion in these cells, I would expect lower levels of cohesion defects in *ctf4* Δ and *chl1* Δ , as they showed higher levels of Smc3 acetylation compared to the *eco1-1* cells. Interestingly, both mutants showed similar levels of cohesion defects when compared to the *eco1-1* allele, suggesting that the decrease in Smc3 acetylation does not correlate with an increase of cohesion defects. Thus, there

may be other factors responsible for the cohesion defects in these mutants, other than reduced cohesin acetylation.

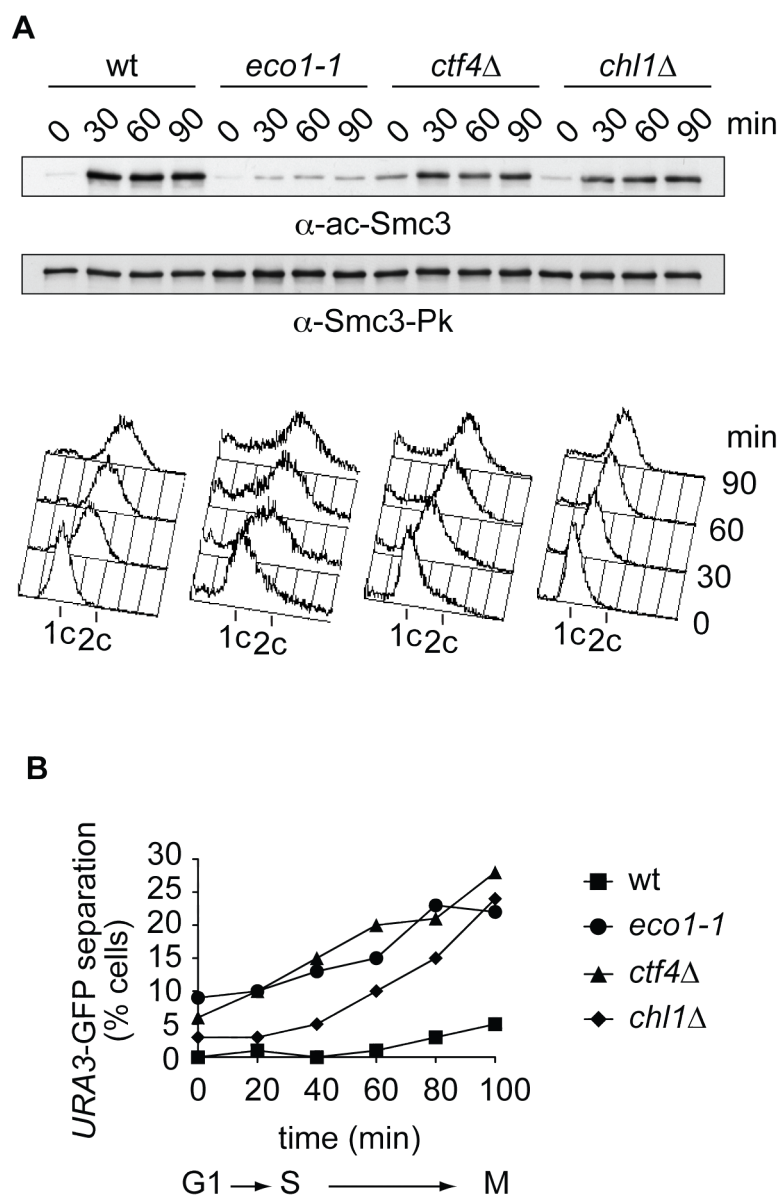


Figure 4.2 – Reduced cohesin acetylation in *ctf4Δ* and *chl1Δ* may not explain their cohesion defects.

(A) Smc3-Pk was immunopurified from extracts of the indicated cells progressing through a synchronous cell cycle following α -factor block and release into nocodazole-imposed mitotic arrest. The Smc3 acetylation status was analysed by Western blotting using a monoclonal α -acetyl-Smc3 antibody. FACS analysis monitored cell cycle progression. **(B)** Cells of the indicated genotypes were synchronised in G1 using α -factor and released into nocodazole-imposed mitotic arrest. Sister chromatid cohesion at the GFP-marked *URA3* locus was analysed.

4.2 Ctf4 and Chl1 define a subset of Eco1-independent cohesion establishment factors

4.2.1 Pronounced synthetic interactions of Ctf4 and Chl1 with Eco1

To investigate the relationship between Eco1 and the two parallel sister chromatid cohesion pathways (Xu et al., 2007), I next assessed the genetic interactions between these proteins. The genetic data could reflect redundancy between Eco1 and one of the pathways, or define an additional cohesion pathway. To test this, triple mutants were constructed between mutants in each pathway and *eco1Δwpl1Δ*, as *eco1Δ* is not viable. Cells were arrested in G1 using α -factor block and released into a nocodazole-imposed mitotic arrest. The strains were then assayed for defects in sister-chromatid cohesion seen by split GFP signals at the *URA3* locus (Michaelis et al., 1997). If indeed Eco1 defines an additional pathway, I would expect an increase in the cohesion defects when combined with the nonessential mutants. However, co-deletion of Eco1 with either mutant did not show any additional defects (Figure 4.3). Deletion of the nonessential genes (CTF4, CTF18, TOF1, CSM3, CHL1 and MRC1) caused mild to high cohesion defects (~20-30%), as reported previously (Mayer et al., 2004; Xu et al., 2004). In contrast, co-deletion with *ECO1* and *WPL1* exhibited a higher level of cohesion defects relative to the relevant single mutants, but similar to the *eco1Δwpl1Δ* mutant (~30%).

To assess cell viability I next restreaked the single and the triple mutants in YPD medium and incubated the plates at different temperatures. The result showed that co-deletion of *ECO1* with *CHL1* slightly, but reproducibly, decreased cell viability, in contrast to all other mutants (Figure 4.4).

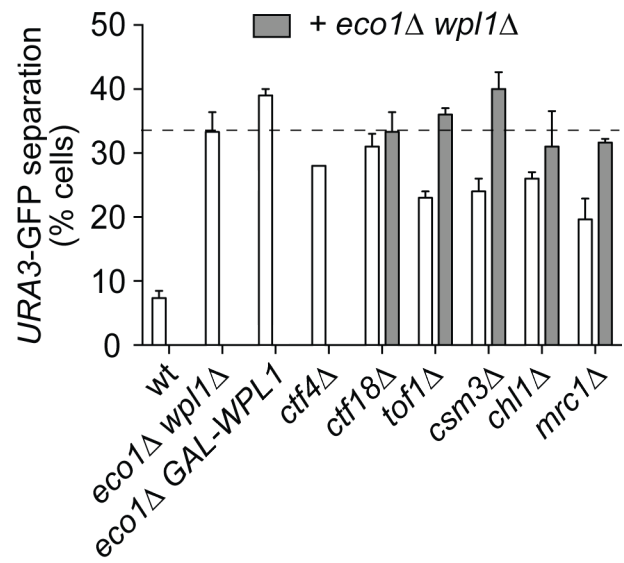


Figure 4.3 – Co-deletion of Eco1 with the ‘cohesion establishment factors’ mutants does not show any additional defects.

Strains of the indicated genotypes were synchronised in G1 by α -factor block and released into nocodazole-imposed mitotic arrest. Sister chromatid cohesion at the GFP-marked *URA3* locus from three independent experiments is indicated.

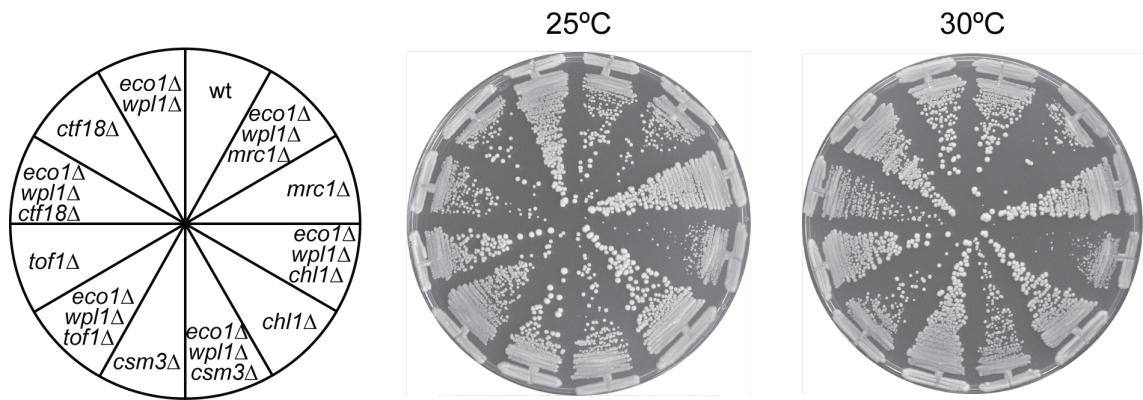


Figure 4.4 – Pronounced synthetic interactions between Eco1 and Chl1.

Cells of the indicated genotypes were streaked on YPD medium and incubated at the indicated temperatures for 2-3 days.

As it was not possible to obtain a triple mutant for Ctf4 for the above experiments, I analysed haploid progeny after sporulation of a heterozygous diploid *eco1Δ/ECO1 wpl1Δ/WPL1 ctf4Δ/CTF4* strain. The analysis revealed that the triple mutant is not able to sustain cell growth (Figure 4.5). Together, the finding that co-deletion of Eco1 with either Ctf4 or Chl1 results in cell death or decrease of cell viability, respectively, suggests that Ctf4 and Chl1 may be defining a different pathway than Eco1.



Figure 4.5 – The triple mutant *eco1Δwpl1Δctf4Δ* does not support cell growth.

An *eco1Δ/ECO1 wpl1Δ/WPL1 ctf4Δ/CTF4* heterozygous diploid was sporulated, and the genotype of the viable spores in each tetrad was analysed. Inferred genotypes of inviable spores are in gray. Asterisks (*) denote pairs of spores with either of the two indicated genotypes.

4.2.2 Removal of Wpl1 corrects the cohesion defect in all of the cohesion establishment factors mutants, except *ctf4Δ* and *chl1Δ*

Cohesion between sister chromatids is established without Eco1 when Wpl1 is absent, and loss of Wpl1 reduces the cohesion defect in *eco1-1* cells (Ben-Shahar et al., 2008). Based on this, I hypothesised that if the nonessential cohesion mutants are on the same pathway of Eco1, we should expect the same kind of rescue. To test this

possibility, double mutants were constructed between each 'cohesion establishment factor' mutant and *wpl1* Δ , and cohesion defects were scored by split GFP signals at the *URA3* locus as before. *WPL1* deletion partly rescued sister chromatid cohesion in all mutants, except for the *CTF4* and *CHL1* mutants (Figure 4.6).

The finding that deletion of *Wpl1* does not rescue the cohesion defects caused by either *ctf4* Δ or *chl1* Δ , in contrast to *eco1* Δ , is consistent with the possibility that these two genes define an additional cohesion pathway.

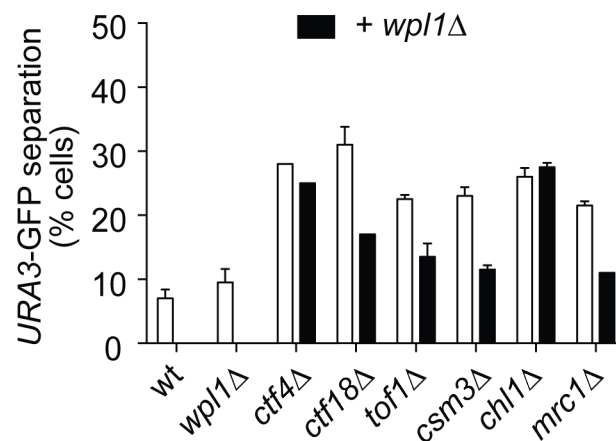


Figure 4.6 – *wpl1* Δ does not rescue cohesion defects in *ctf4* Δ and *chl1* Δ .

Strains of the indicated genotypes were synchronised in G1 by α -factor block and released into nocodazole-imposed mitotic arrest. Sister chromatid cohesion at the GFP-marked *URA3* locus from three independent experiments is indicated.

4.2.3 Cohesin stability is not affected in *ctf4* Δ and *chl1* Δ

Establishment of sister-chromatid cohesion occurs during S phase (Uhlmann and Nasmyth, 1998) and must be maintained during G2/M (Nasmyth, 1999). Smc3 acetylation by Eco1 is not only important to promote establishment of cohesion but also for the stabilisation of cohesin on chromosomes during S-phase, as described in (Lopez-Serra et al., *submitted*). I therefore investigated whether cohesin stability on chromosomes also depended on Ctf4 and Chl1. If indeed Ctf4 and Chl1 act on a different pathway than Eco1, we would expect them to have different roles in promoting sister chromatid cohesion. To investigate this, I performed an assay to assess cohesin stability on budding yeast chromosomes based on the ‘anchor-away’ technique, as previously described (Haruki et al., 2008). In this technique, a nuclear protein of interest is fused to FRB, half of a pair of rapamycin-dependent interacting protein domains. The other half, FKBP12, is attached to the ribosomal protein Rpl13a. Ribosomal proteins shuttle through the nucleus for their assembly into ribosomes that are then exported to the cytoplasm. By hitchhiking onto Rpl13a upon rapamycin addition, a freely diffusible nuclear protein is depleted from the nucleus in less than 3 minutes (Haruki et al., 2008). To visualise cohesin’s dynamic behaviour, Scc1 is fused to a tandem GFP-FRB tag. I synchronised cells in G1 using a-factor block (O’Reilly et al., 2012) and released them in metaphase by nocodazole treatment. Rapamycin was added to the arrested cells and aliquots taken in 10 minute intervals to visualise the nuclear/cytoplasmic distribution of Scc1. As Eco1 is an essential gene I inactivated it by fusing Eco1 to an auxin-inducible degron (aid) (Nishimura et al., 2009). As expected, nuclear Scc1 was no longer stable upon *ECO1* deletion. In contrast, absence of both Ctf4 and Chl1 led only to a mild decrease in the stability of nuclear Scc1, when compared to the wild type cells (Figure 4.7). Ctf18 was used as control, as it appears to be in the same cohesion-pathway as Eco1. Although its deletion resulted in a mild defect on cohesin stability when compared to the *eco1*-depleted cells, the dissociation rate was different from *ctf4* Δ and *chl1* Δ , even though the cohesion defects in the three mutants are the same. This result suggests that Ctf4 and Chl1 have a different role in promoting sister chromatid cohesion, other than stabilising cohesin on chromosomes after Smc3 acetylation. Altogether, these results indicate that Ctf4 and Chl1 define an Eco1-independent pathway to promote cohesion establishment.

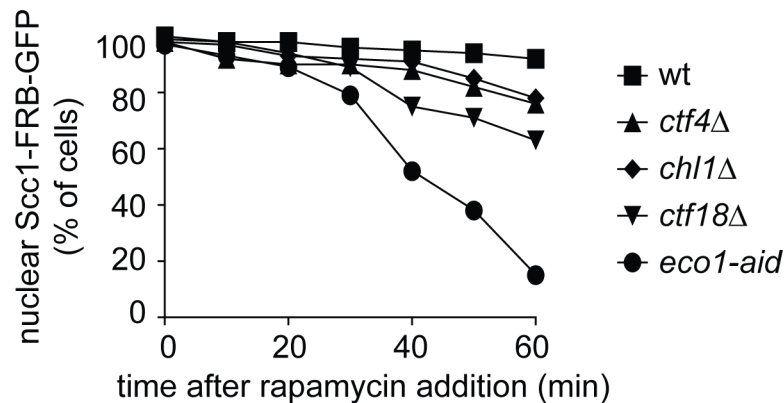


Figure 4.7 – Cohesin stability on chromosomes does not depend on Ctf4 and Chl1.

Strains of the indicated genotypes were synchronised in G1 by α -factor block. 500 μ M IAA was added to the media to inactivate the *eco1-aid* allele. Cells were released into nocodazole and rapamycin was added after 2 h to anchor-away Scc1-GFP from the nucleus. Scc1-nuclear signal is indicated.

4.3 Characterisation of Ctf4 and Chl1

Ctf4 and Chl1 were classified into the same pathway that contributes to the establishment of sister-chromatid cohesion (Xu et al., 2007). Moreover, my results suggest that they define a subset of Eco1-independent cohesion establishment factors. Hence, I decided to explore the relationship between these two proteins to understand how they promote cohesion. For this, I created a double mutant *ctf4*Δ*chl1*Δ and I assayed for defects in sister-chromatid cohesion. Cells were arrested in metaphase by nocodazole treatment after release from G1, and sister-chromatid cohesion assayed at the *URA3* locus as before. As expected, co-deletion of Ctf4 and Chl1 had no additive cohesion defect (Figure 4.8), further confirming that they are in the same pathway.

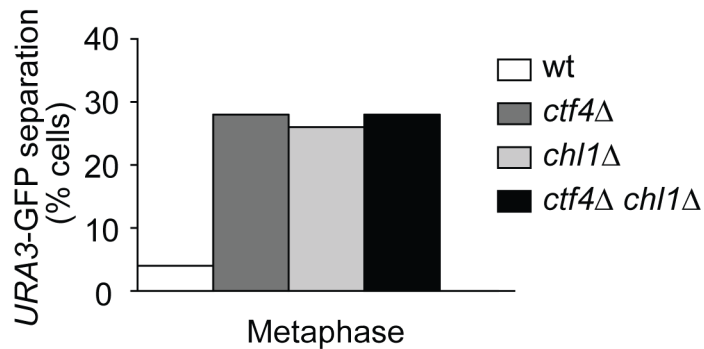


Figure 4.8 – *ctf4*Δ*chl1*Δ cells do not have additional cohesion defects.

Cells of the indicated genotypes were synchronised in G1 by a-factor treatment and arrested in metaphase by nocodazole treatment. Sister chromatid cohesion at the *URA3* locus was analysed on metaphase-arrested cells.

As members of the same pathway, one reasonable possibility is that Ctf4 and Chl1 physically interact with each other. To test this, I tagged Chl1 at the C terminus with the PK epitope in a Ctf4-HA-tagged strain. As a control, I also tagged Pol1-PK as Ctf4 binds to DNA polymerase α with high affinity (Miles and Formosa, 1992; Zhou and Wang, 2004). Immunoprecipitation of Ctf4-HA in asynchronous cultures did not co-immunoprecipitate Chl1-PK from extracts prepared from Ctf4-HA Chl1-PK (Figure 4.9), in contrast to the Pol1-PK control. Therefore, I was unable to detect physical interactions between these two proteins. It is possible that physical links among components of the same cohesion pathway are indirect or transient.

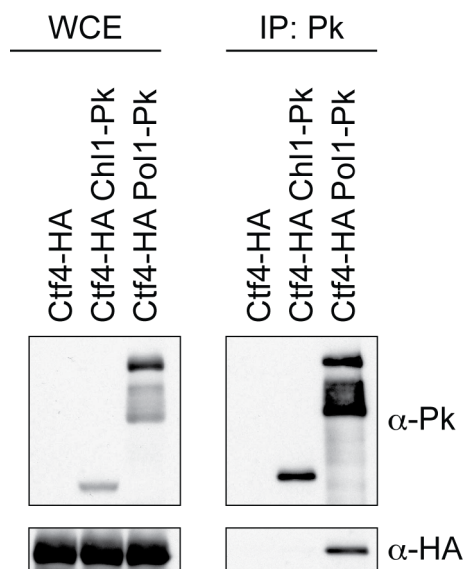


Figure 4.9 – No interaction detected between Ctf4 and Chl1.

Whole cell extract of the indicated cells was immunoprecipitated using α -Pk antibody and analysed by immunoblotting for Ctf4-HA. Pol1-3Pk was used as a positive control. Whole cell extracts (WCE) and immunoprecipitated protein (IP) are shown.

To gain insight into the mechanism of Ctf4 and Chl1 in cohesion establishment, I decided to investigate whether they are loaded to chromatin in a mutually dependent manner. For this, I separated cell extracts from wild type, *ctf4* Δ and *chl1* Δ into soluble and chromatin-bound fractions at different stages of the cell cycle and the result was analysed by Western blotting. Overall, Ctf4 binding to chromatin appeared much weaker than Chl1, and increased during S-phase (Figure 4.10). This fraction of total cellular Ctf4 bound to chromatin during S-phase decreased upon deletion of *CHL1*. In turn, Chl1 binding to chromatin remained constant in the different stages of the cell cycle, and deletion of *CTF4* did not have any effect in Chl1 binding. Therefore, Ctf4 binding to chromatin during S phase seems to be dependent of Chl1, but Chl1 binding is independent of Ctf4.

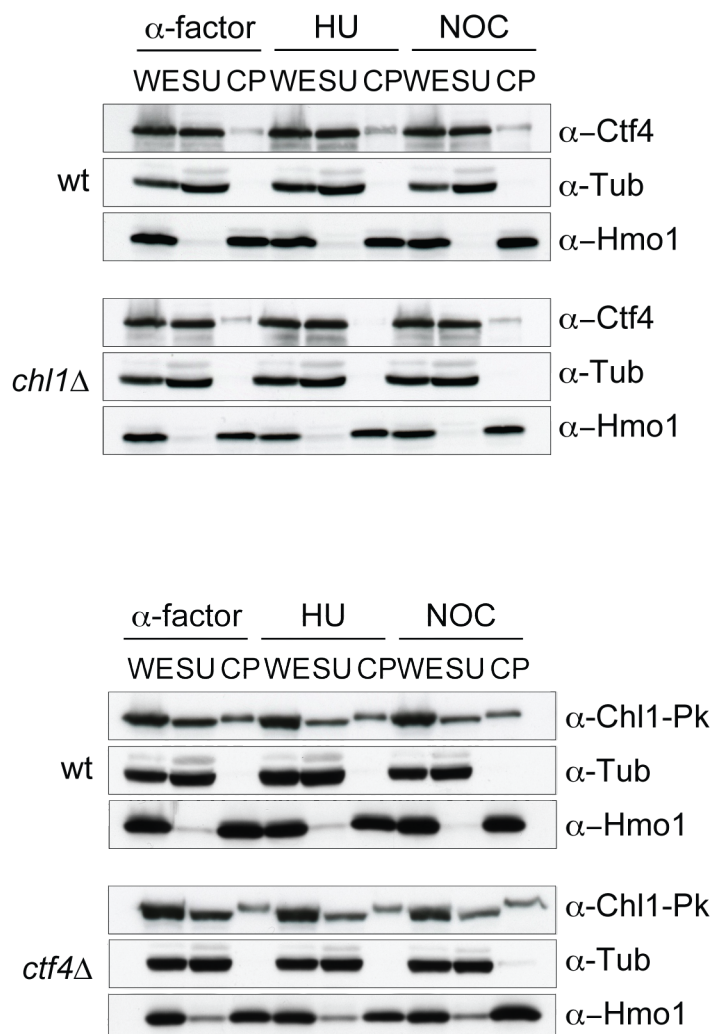


Figure 4.10 – *CHL1* deletion affects Ctf4 binding during S-phase.

Cells of the indicated genotypes were synchronised in G1 by α -factor treatment and released into either HU or nocodazole-containing media. Whole cell extracts (WE) were separated into supernatant (SU) and chromatin (CP) fractions, and α -Ctf4 or α -Pk antibodies were used to detect proteins by immunoblotting. Tubulin and Hmo1 served as loading controls for the supernatant and chromatin fractions, respectively.

4.4 Genetic interactions between Ctf4 and Chl1 and replication factors required for Okazaki fragment processing

Establishment of sister-chromatid cohesion is coupled with DNA replication during S-phase and is intimately connected to the progression of DNA replication forks (Lengronne et al., 2006). Both Ctf4 and Chl1 were previously linked to the replication fork machinery. In particular, a complex formed by Ctf4 and GINS was found to have an important role in coupling the MCM DNA helicase and the DNA polymerase alpha at the replication fork (Gambus et al., 2009). An implication of lagging strand reactions come from the finding that Ctf4 is needed to recruit the DNA primase to the replication fork (Miles and Formosa, 1992; Zhou and Wang, 2004) and its interaction with the Dna2 nuclease/helicase is essential for the processing of Okazaki fragments (Formosa and Nittis, 1999). Furthermore, hChl1 stimulates the flap endonuclease activity of Fen1 (Farina et al., 2008). I therefore decided to enhance our understanding on the mechanism of Ctf4 and Chl1 in promoting cohesion during DNA replication. To test how Ctf4 and Chl1 relate to both Fen1 and Dna2, I analysed the haploid progeny after sporulation of heterozygous diploid *fen1Δ/FEN1 ctf4Δ/CTF4* and *fen1Δ/FEN1 chl1Δ/CHL1* strains. The analysis revealed that both double mutants were not able to sustain cell growth (Figure 4.11). This synthetic lethality further supports a model of cohesion establishment being temporally coupled to lagging strand processing.

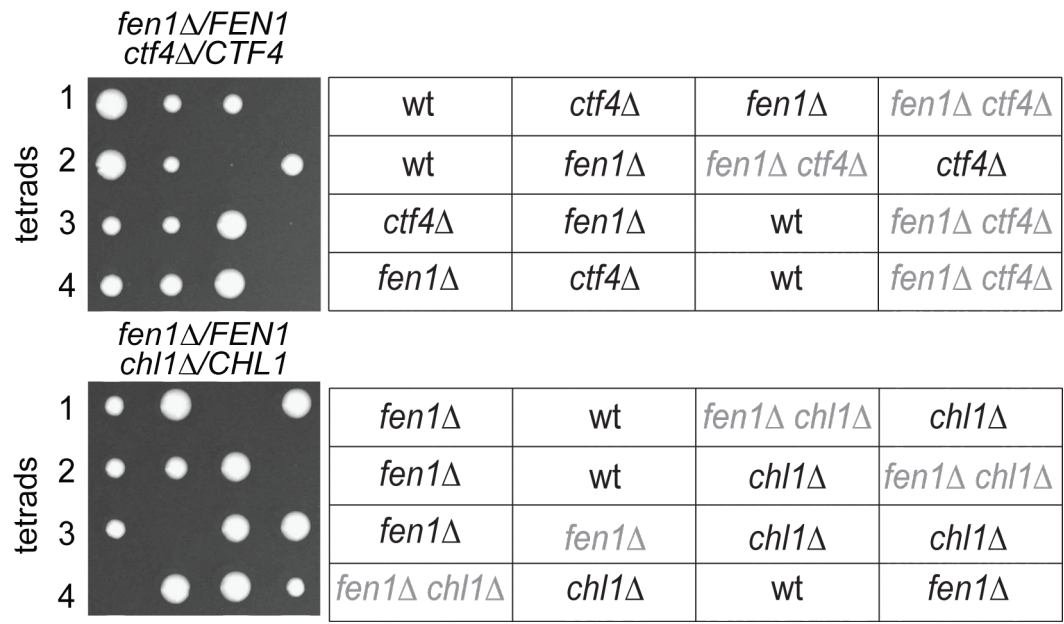


Figure 4.11 – *CTF4* and *CHL1* deletions do not support cell growth in combination with *fen1Δ*.

A *fen1Δ/FEN1 ctf4Δ/CTF4* and a *fen1Δ/FEN1 chl1Δ/CHL1* heterozygous diploid was sporulated, and the genotype of the viable spores in each tetrad was analysed. Inferred genotypes of inviable spores are in grey.

I next performed the same analysis for Dna2. As reported before, *ctf4Δ dna2-2* double mutant was synthetically lethal at the permissive temperature (Formosa and Nittis, 1999). I was able to obtain viable, but poorly growing, *chl1Δ dna2-2* spores (Figure 4.12), but no additional cohesion defects were detected in the double mutant (Figure 4.13).

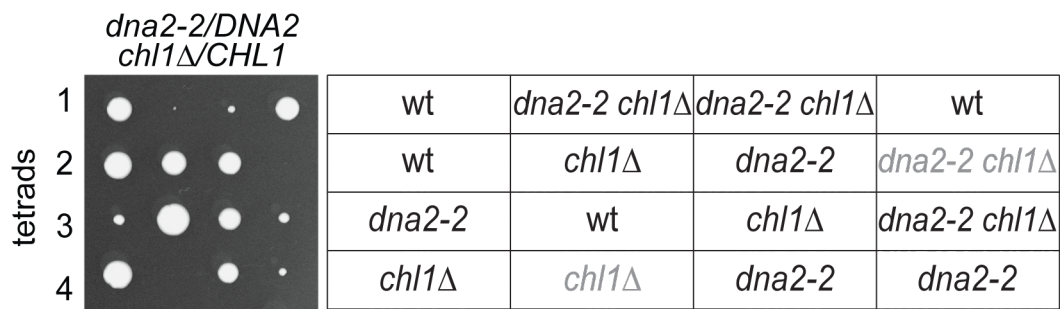


Figure 4.12 - Poor growth of *chl1Δ* in combination with the *dna2-2* allele.
Heterozygous diploid strains of the indicated genotypes were sporulated and the genotype of the viable spores in each tetrad was determined. Inferred genotypes of unviable spores are in gray.

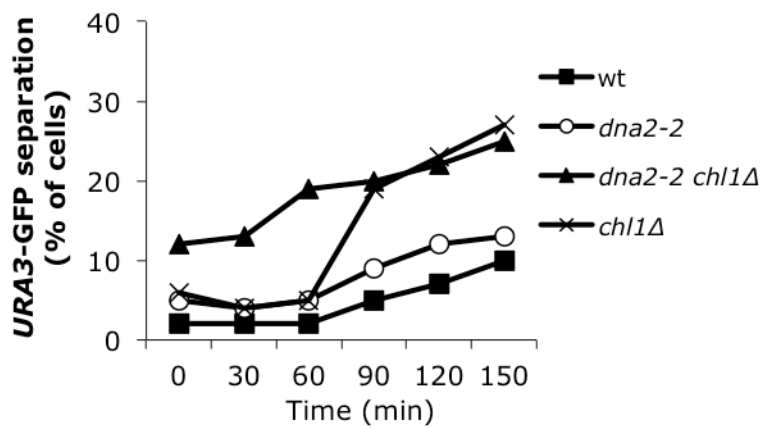


Figure 4.13 – Deletion of *CHL1* in a *dna2-2* strain does not lead to additional cohesion defects.
Strains of the indicated genotypes were synchronised in G1 by α -factor block and released into nocodazole-imposed mitotic arrest. Sister chromatid cohesion at the GFP-marked *URA3* locus is indicated.

4.5 Cohesion defects in *ctf4* Δ and *chl1* Δ are not explained by longer Okazaki fragments

Based on the 'trombone model' for Okazaki fragment synthesis (Hamdan et al., 2009), I hypothesised that the lagging strand loop could be a potential obstacle to the passage of replication forks through cohesin rings. As both Ctf4 and Chl1 have a role in the establishment of sister chromatid cohesion and are involved with lagging strand processing events, a potential role of these proteins could thus be to coordinate the passage of the replication fork through cohesin rings with Okazaki fragment processing, so that loops would be released with the encounter of cohesin. I therefore investigated whether lagging strand DNA synthesis and Okazaki fragment maturation were affected by Ctf4 and Chl1. To test this, I used a new method to analyse eukaryotic Okazaki fragments *in vivo* (Smith and Whitehouse, 2012). The inability to ligate Okazaki fragments by inactivation of the DNA ligase I (*CDC9*) resulted in the accumulation of short fragments of DNA. If Ctf4 and Chl1 were indeed coordinating the Okazaki fragment synthesis we would expect an increase of the longer DNA fragments in their absence. However, contrary to this hypothesis, *ctf4* Δ or *chl1* Δ did not have any effect on the length of these fragments (Figure 4.14). This suggests that the role of Ctf4 and Chl1 in promoting cohesion is not explained by an increase of the Okazaki fragments size, and possible entrapment of the DNA loop at the time of cohesin passage.

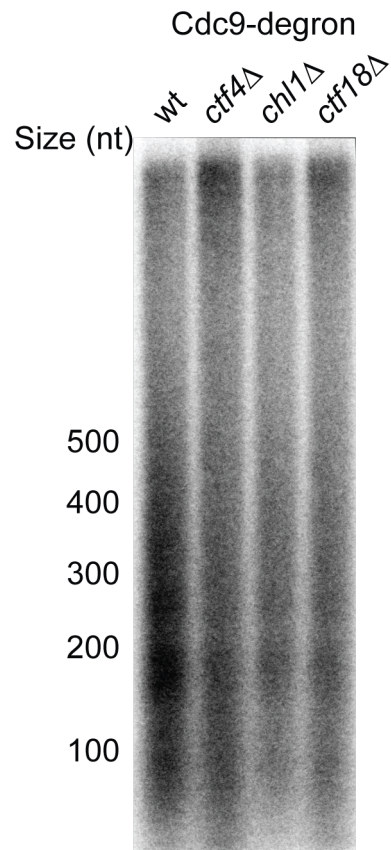


Figure 4.14 – Transcriptional repression of DNA ligase I (Cdc9) does not result in the increase of Okazaki fragment lengths in *ctf4Δ* and *chl1Δ* mutants.

Cells carrying a doxycycline-repressible allele of the *CDC9* gene were treated with doxycycline (Dox) for 2.5 h. Purified genomic DNA was labelled using exonuclease-deficient Klenow fragment and [α - 32 P]-dCTP and separated in a denaturing agarose gel. nt, nucleotides. This experiment was carried out by Duncan Smith from Dr Iestyn Whitehouse laboratory at the Sloan-Kettering Institute.

Chapter 5

Discussion and future perspectives

Chapter 5. Discussion

5.1 Hos1, a histone and Smc3 deacetylase

The coordinated processes of acetylation and deacetylation by HATs and HDACs, respectively, have been increasingly reported to affect both histone and non-histone proteins to regulate several cellular processes, including transcription, replication, DNA repair, metabolism, and cell structure (de Ruijter et al., 2003; Glozak et al., 2005; Shahbazian and Grunstein, 2007; Yang et al., 2008; Yang and Grégoire, 2007). Here, I show that the budding yeast histone deacetylase Hos1 (Hda one similar) also acts as the cohesin deacetylase. Deacetylases are classified into three main classes on the basis of sequence homology: class I includes Rpd3, Hos1 and Hos2-like enzymes, class II includes Hda1-like enzymes and Sir2-like enzymes belong to class III (Ekwall, 2005; Kurdistani and Grunstein, 2003). Although it was initially thought that the main function of type I enzymes such as Hos1 was the control of transcription by deacetylating histones, several evidences suggested that they could play other roles in the cell (Ikenoue et al., 2008; Jackman et al., 2000; Leipe and Landsman, 1997; Yao and Nyomba, 2008). The identification of Hos1 as the cohesin subunit Smc3 deacetylase provides conclusive evidence for a function for type I deacetylases, other than histone deacetylation. Recent studies also identified HDAC8, the human ortholog of Hos1, as the vertebrate SMC3 deacetylase by showing that loss of HDAC8 activity resulted in increased SMC3 acetylation and inefficient dissolution of the 'used' cohesin complex released from chromatin in both prophase and anaphase (Deardorff et al., 2012).

Hos1 was first identified to act as part of the Tup1-Ssn6 corepressor complex in *S. cerevisiae* at a diverse set of promoters (Davie et al., 2003; Watson et al., 2000). However, my findings show that Hos1's role in deacetylating Smc3 during anaphase is independent of Tup1 and Ssn6 (Borges et al., 2010). It is not known whether it acts alone or as part of another protein complex. This observation suggests that Hos1 may require different accessory proteins to perform his function as a deacetylase in different substrates. Further to its broad substrate specificity, Hos1 also act in a redundant manner with other deacetylases, as suggested by residual Smc3 deacetylation observed in the absence of Hos1 after prolonged time of arrest. Even upon deletion of Hos1's nearest relative, Hos2, a slow deacetylation could be observed (Borges et al., 2010). Therefore, Hos1 is not only a histone deacetylase, as first described (Robyr et

al., 2002), but also fulfils a role as the cohesin deacetylase and may even have other unknown non-histone substrates.

5.2 The cohesin acetylation cycle

The characterisation of Hos1 as the Smc3 deacetylase completes the molecular description of the cohesin acetylation cycle (Figure 5.1) (Beckouet et al., 2010; Borges et al., 2010). Cohesin, with nonacetylated Smc3, is loaded onto chromosomes in late G1, upon synthesis of Scc1 and assembly of the cohesin complex. Eco1-mediated Smc3 acetylation occurs only during DNA replication in S-phase, and not before. My findings that the presence of overexpressed levels of Eco1 before S-phase do not alter the kinetics of Smc3 acetylation, indicates that acetylation is independent of Eco1 levels and reinforces the idea of its interdependency with the replication fork progression, which had been previously suggested by a reduction in acetylation levels in cells entering the cell cycle without Cdc6 (Ben-Shahar et al., 2008).

During S-phase, Eco1 acetylates two evolutionary conserved lysine residues of Smc3 (K112 and K113) (Ben-Shahar et al., 2008; Ünal et al., 2008; Zhang et al., 2008a). Eco1 is thought to associate with the DNA replication fork through its physical interaction with PCNA (Lengronne et al., 2006; Moldovan et al., 2006), so the acetylation during DNA replication may occur due to close proximity of Eco1 and Smc3 as the replication fork passes or alternatively, due to some additional regulation at this time. My findings show that other factors associated with the replication fork, including Ctf4, Ctf18, Tof1, Csm3, Chl1, and Mrc1, also contribute for cohesin acetylation in as yet unknown ways. Eco1-mediated Smc3 acetylation stabilizes cohesin binding to chromatin. Although this mechanism is not completely understood, it is thought that stabilisation occurs by blocking the action of the cohesin destabilizer Wapl through the acetylation of Smc3. Intriguingly, even though after replication cohesin is no longer able to establish cohesion, a notable exception happens during G2 upon DNA damage (Ström et al., 2004; Ünal et al., 2007) when Eco1 promotes the reinforcement of sister chromatid cohesion by acetylating another cohesin subunit, Scc1 (Heidinger-Pauli et al., 2008). Similarly to what happens to the replication-fork Smc3 acetylation, Scc1 acetylation is also thought to counteract the function of Wapl.

Cohesin acetylation is then maintained throughout G2 until anaphase. The finding that deacetylation can be artificially triggered in metaphase confirms that deacetylation is tightly regulated. Even though Hos1 is already fully proficient at this

stage, its role as Smc3 deacetylase is prevented while Smc3 is part of an intact, chromosome-bound cohesin complex. Overexpression of Hos1 from the *GAL* promoter led only to a minor decrease of Smc3 acetylation, further demonstrating the efficiency of the protection mechanism (data not shown). At anaphase onset, cohesin's Scc1 subunit is cleaved by separase, which leads to cohesin dissociation from chromosomes, enabling Smc3 deacetylation by Hos1, thereby closing the Smc3 acetylation cycle.

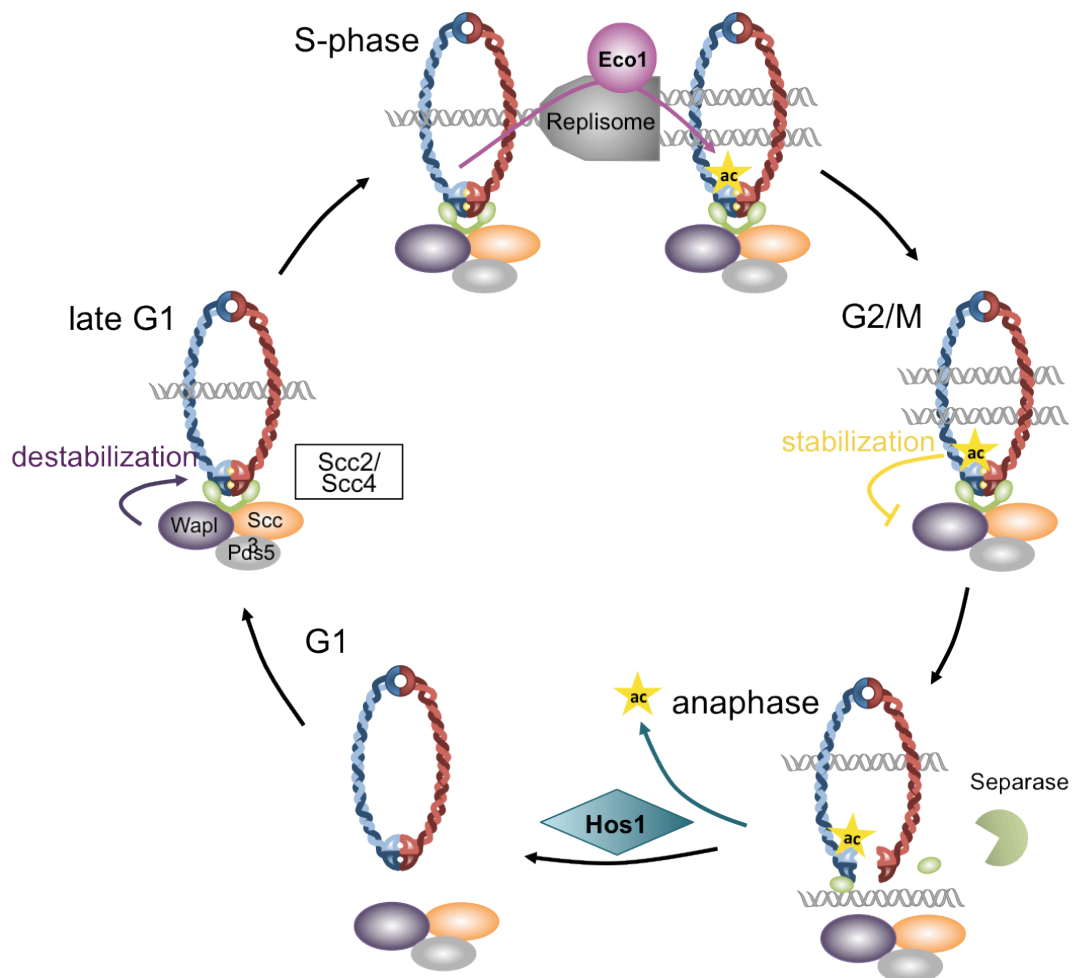


Figure 5.1 – Model for cohesin acetylation cycle.

Cohesin with nonacetylated Smc3 is loaded onto chromosomes in late G1. During S-phase, the replication fork-associated acetyltransferase Eco1 acetylates Smc3, which is required for the establishment of sister chromatid cohesion. Smc3 remains acetylated throughout G2/M phases and stabilizes cohesin by counteracting the destabilising activity of Wapl. At anaphase onset, separase cleavage of Scc1 causes cohesin dissociation from chromosomes, allowing Hos1 to deacetylate Smc3.

5.3 The importance of *de novo* Smc3 acetylation for cohesion establishment

My studies explain not only the regulation of cohesin deacetylation but also provide insight into the importance of both cohesin acetylation and deacetylation. My analysis of *hos1* Δ strains revealed that although the cells are viable, sister chromatid cohesion is compromised. Moreover, depletion of Hos1, which results in increased levels of acetylated Smc3, do not compensate for the cell viability and cohesion defects observed in *eco1-1* cells. These results indicate that Smc3 acetylation that persists from the previous cell cycle does not contribute to cohesion establishment during S-phase. Cohesin therefore needs to be *de novo* acetylated during DNA replication, implying a requirement for both the acetylated and nonacetylated state of Smc3 throughout the cell cycle. In humans, it was also demonstrated that the loss of HDAC8 activity resulted in inefficient dissolution of the 'used' cohesin complex released from chromatin in both prophase and anaphase (Deardorff et al., 2012).

The failure of promoting cohesion in *hos1* Δ cells is not due to problems in cohesin loading, as the acetylation-mimicking Smc3 mutants associate with chromosomes in a similar way than the wild-type cohesin. It would be of interest to characterize the exact timing of Smc3 acetylation by chromatin immunoprecipitation and understand the correlation between the timing of acetylation and the binding of replisome components in respect to the replication fork. This would help understanding the different requirements for acetylated and non-acetylated forms of Smc3 with respect to the replication fork. One possibility is that deacetylation is required to regenerate the cohesin ring for the next cell cycle. Alternatively, cohesin rings with acetylated Smc3 may not be capable of building cohesion during DNA replication. Perhaps the cohesin ring requires non-acetylated Smc3 in order to capture the DNA strands or to facilitate the fork passage during S-phase. Once the sister chromatids are entrapped inside the cohesin ring, the acetylated version of Smc3 may be required to then stabilize and maintain sister chromatid cohesion. If Smc3 acetylation indeed helps to stabilize cohesin rings by 'locking' them, recycled Smc3-acetylated cohesin rings from the previous cell cycle would compromise the capture of DNA, either before or during replication. This would therefore explain the requirement for an unacetylated cohesin intermediate and the importance of *de novo* acetylation to establish sister chromatid cohesion.

5.4 Cohesin acetylation and stabilisation of cohesion

The consequences of Smc3 acetylation and the mechanism by which Eco1 activity facilitates the establishment of sister chromatid cohesion are still poorly understood. Acetylation of cohesin is clearly important as evidenced by the essential nature of Eco1 and by the failure of cohesion establishment in nonacetyltable Smc3 variants. However, it is uncertain whether acetylation facilitates the entrapment of sister chromatids or whether it helps stabilising cohesion once it is established. My results suggests that Eco1, and consequently Smc3 acetylation, are responsible for the stabilisation of cohesin on chromosomes during S-phase. Interestingly, viability of *eco1Δ* cells or cells harbouring Smc3K112,113R is rescued by deletion of Wapl (Ben-Shahar et al., 2008). Therefore, a plausible model for the role of cohesin acetylation, based on these results, is that acetylation protects cohesin against a destabilising activity of Wapl. Wapl exerts this effect on cohesin both before and after DNA replication, but replication-coupled acetylation renders newly established sister chromatid cohesion Wapl-resistant. However, although the absence of Wapl improves sister chromatid cohesion in an *eco1Δ*, it is still compromised. This suggests a second role for Eco1 in cohesion establishment. According to my genetic analysis, this function seems to be also mediated via Smc3 acetylation. This could be related to its function in stabilising cohesin, possibly by modulating interactions within the cohesin ring or with other proteins, in addition to counteracting Wapl. It is interesting to note that although Smc3 acetylation seems to be essential for both establishment and stability of sister chromatid cohesion, Scc1 acetylation, and not Smc3, is thought to be required to promote *de novo* cohesion upon DNA damage in G2 cells (Heidinger-Pauli et al., 2008). The finding that the essential nature of Eco1 can be bypassed by mixed nonacetyltable/acetyl-mimicking Smc3 variants together with the fact that some eukaryotic genomes do not encode an ortholog of Eco1 (Nasmyth and Haering, 2009), suggest that Smc3 acetylation is an evolutionary addition to improve the building and stability of sister chromatid cohesion.

5.5 Ctf4 and Chl1, a subset of Eco1-independent cohesion establishment factors

In addition to Eco1, several non-essential cohesion establishment factors contribute to efficient sister-chromatid cohesion. Despite all the links between these non-essential factors with the DNA replication fork, the way that they contribute to cohesion establishment remains poorly understood. A previous study divided these cohesion establishment factors into two genetic epistasis groups (Xu et al., 2007). The definition of these two cohesion pathways raises the question of whether Eco1 and other factors important for sister chromatid cohesion belong to one of these pathways or define additional cohesion pathways.

This study now provides evidence that most of these cohesion establishment factors, Ctf4, Ctf18, Tof1, Csm3, Chl1 and Mrc1 promote the cohesin acetylation reaction during S-phase. In the case of RFC^{Ctf18}, this could be due to its activity as a PCNA loader, which in turn probably serves as a recruitment platform for Eco1 at the replication fork. How the other factors contribute to Smc3 acetylation is less clear, they could also be involved in recruiting Eco1 to replication forks, or they could modulate Eco1's activity at the fork. Once modified, the Smc3 acetylation mark remains protected from deacetylation by the protein deacetylase Hos1 while cohesin remains chromosome bound (Borges et al., 2010). The mechanism behind this protection is as yet unknown, therefore some of the cohesion establishment factors could act at the level of establishing protection of cohesin from premature deacetylation. The two cohesion establishment factors Ctf4 and Chl1 were distinct from all the others. Their deletion caused a strong synthetic growth defect in *eco1Δ wpl1Δ* cells. In addition, the cohesion defect in cells lacking Ctf4 or Chl1, unlike in cells lacking any of the other factors, was not ameliorated by removal of Wapl. This suggests that Ctf4 and Chl1 play a role in the establishment of sister chromatid cohesion that is independent of stabilising chromosome binding of the cohesin complex. The role of Ctf4 and Chl1 in promoting sister chromatid cohesion seems to be important for the establishment, but not for maintenance or stabilisation of cohesion. The reduced cohesin acetylation observed in the absence of both Ctf4 and Chl1 does not explain the cohesion defects of these mutants. Therefore, there may be other factors responsible for these defects. While Eco1 adds a lasting acetyl-mark that stabilises sister chromatid cohesion, Ctf4 and Chl1 could hold the clue as to what happens at the moment when replication forks meet cohesin rings to establish sister chromatid cohesion. It would be of interest to

explore additional components of this pathway, possible protein interactions and understand their relationship with respect to replication fork progression.

5.6 Establishment of sister chromatid cohesion during DNA replication

Cohesion establishment is thought to be tightly coupled to DNA replication during S-phase. Apart from the Timeless-Tipin protein complex (human orthologs of budding yeast Tof1 and Csm3) and the TIM-1 (a paralog of Timeless in *C. elegans*) that were shown to interact with cohesin (Chan et al., 2003; Leman et al., 2010) no other stable interactions between non-essential factors and cohesin are known. Therefore, a potential role of these proteins could thus be to coordinate the passage of the replication fork through cohesin rings with Okazaki fragment processing. In fact, different studies linked both Ctf4 and Chl1 to Okazaki fragment processing. Ctf4 is an integral component of the replisome progression complex, engaged in protein interactions between the MCM DNA helicase, the GINS complex and the DNA polymerase α /primase complex. Furthermore, studies in human cells also revealed that Ctf4/AND-1 plays an essential role in DNA replication and also stimulates the replicative DNA polymerases (Bermudez et al., 2010; Zhu et al., 2007). Chl1 in turn appears to contribute to the assembly of Ctf4 on chromosomes during S-phase. If the replisome passes through cohesin rings during DNA replication, it is conceivable that an altered replisome geometry in the absence of Ctf4 could lead to difficulties with cohesion establishment. Instead of traversing through the ring, the oncoming replication fork might displace or break cohesin.

Thus, I am interested in understanding whether Chl1 and/or Ctf4 are implicated in Okazaki fragment maturation and whether Okazaki fragment biology impinges on the establishment of sister chromatid cohesion. Given the possibility that the replication fork slides through cohesin rings to establish sister chromatid cohesion, changes to Okazaki fragment processing and concomitant change to the replication fork size and geometry could affect sister chromatid cohesion establishment. However, I could not detect problems in the maturation of the Okazaki fragments in the absence of Ctf4 or Chl1. Although the mechanism of cohesion establishment remains largely unknown, a popular model suggested that Eco1 promotes establishment of cohesion by acetylating

cohesins that reside in front of the replication fork (Sherwood et al., 2010; Tóth et al., 1999). However, recent studies also linked Eco1 to the Okazaki fragment processing machinery, and suggested a new model where cohesin loading and establishment of cohesion occur in concert with lagging strand-processing events after the two sister chromatids are synthesised (Rudra and Skibbens, 2012).

My findings also suggest that Ctf4 requires Chl1 for its assembly at the replication fork, but no direct interaction between these two proteins has been detected. It would therefore be of interest to investigate this further by exploring whether Chl1, due to its helicase activity, facilitates the loading of Ctf4 on the replication fork or if there is direct recruitment of Ctf4 by Chl1. The investigation of the mechanism by which these Eco1-independent cohesion pathway factors promote the establishment of cohesion is likely to hold important insight into understanding replication-coupled sister chromatid cohesion.

5.7 Future perspectives

The requirement of *de novo* acetylation during DNA replication implies that both acetylated and non-acetylated forms of Smc3 are important throughout the cell cycle. The characterisation of the exact timing and pattern of Smc3 acetylation, by chromatin immunoprecipitation using a specific antibody against the acetylated protein, would be important to understand the role that each form plays. Additionally, it would also be interesting to understand the correlation between the timing of acetylation and the binding of replisome components in respect to the replication fork, to further elucidate the relationship between cohesin acetylation and replication fork progression. Eco1 is thought to associate with the DNA replication fork through its physical interaction with PCNA and this interaction is essential for cohesion establishment in S-phase (Lengronne et al., 2006; Moldovan et al., 2006). It would then be interesting to see whether this is also needed for Smc3 acetylation. Studies in vertebrates showed that although Smc3 acetylation occurs independently of DNA replication, functional acetylation occurs only in association with the replication machinery (Song et al., 2012). The recruitment of Eco1 to the replication fork by PCNA could therefore be the essential step to promote Smc3 acetylation and establish cohesion. However, I cannot exclude that additional factors at the replication fork may also be required to trigger Smc3 acetylation. Thus, studies to understand the timing of cohesin acetylation and its correlation with the binding of replisome components to DNA would provide us further insight about the proteins involved in the establishment of sister chromatid cohesion. If Smc3 acetylation indeed depends on the replication machinery, it would then be interesting to examine the consequences of a delay in S-phase in cohesin acetylation and subsequent establishment.

Several lines of evidence suggest that cohesion establishment is tightly coupled to DNA replication during S-phase. Further experiments involving factors that associate with the replication fork machinery and that contribute to cohesion establishment will enhance our understanding about the mechanism underlying sister chromatid cohesion. As Ctf4 and Chl1 act in an independent pathway of Eco1 it would be of interest to delineate this Eco1-independent pathway of cohesion establishment. A genetic approach could unveil the pathways targeted by these factors during cohesion establishment. The nature of mutations that modulate the requirement of Ctf4 and Chl1 for sister chromatid cohesion establishment would be expected to carry important information as to the mechanism involved.

In addition, it would also be important to determine the molecular functions of Ctf4 and Chl1 individually. Little is known about Chl1; it is a helicase involved in the establishment but not maintenance of sister chromatid cohesion. It would be interesting to check whether the helicase activity of Chl1 is required for cohesion establishment. Moreover, it is unknown when and where Chl1 is loaded onto the replication fork, thereby chromosome-wide binding analyses could pinpoint exactly when and where Chl1 is loaded onto the replication fork. In turn, Ctf4 is a component of elongating replication forks that interacts with several replication proteins, including the GINS complex and DNA polymerase alpha/primase. It would therefore be interesting to study which of the Ctf4-mediated replisome interactions contributes to sister chromatid cohesion establishment, possibly by mapping and disrupting each interaction individually by targeted point mutations. Exploring the function of the Eco1-independent cohesion establishment pathway is likely to hold important insight to understanding replication-coupled cohesion establishment. Understanding how the sister chromatids become linked immediately after replication represents a significant step forward in our understanding of the mechanisms involved in chromosome segregation.

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