DNA Damage Provokes Cohesin Sumoylation in *Saccharomyces cerevisiae*

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Declaration

The work contained in this thesis is all my own, except where indicated.

In this thesis I shall be describing results in *Saccharomyces cerevisiae* except where specified otherwise.

Abstract

Cohesin tethers sister chromatids together from their creation during S-phase until their separation at the metaphase to anaphase transition. Cohesion is essential for correct segregation and therefore genomic stability. Cohesin is also involved the regulation of transcription and in DNA repair. Cohesin affects these processes by its ability to hold two pieces of DNA together.

Cohesin is subject to a variety of post-translational modifications. In this study, the modification of cohesin, more specifically the Scc1 subunit, by Small ubiquitin-like modifier (SUMO) was analysed. It was found that cohesin is sumoylated during an unperturbed cell cycle, but is hyper-modified after DNA damage. To determine the function Scc1 modification by SUMO, attempts were made to identify the modified residues. Mass spectrometry techniques for SUMO site identification were developed in parallel to site-directed mutagenesis studies.

Potential sumoylation sites were identified on Scc1, but when these were mutated there was little effect on either viability or the sumoylation pattern obtained. Mutants were then constructed in which only one lysine was present. Surprisingly, several of these were viable, not sensitive to methyl methanesulfonate and were not sumoylated, suggesting that either sumoylation of Scc1 is not essential and not required after DNA damage, or that sumoylation of the rest of the cohesin complex can compensate.

To investigate this, Scc1 fusions with the SUMO E2 ligase, Ubc9, and a SUMO isopeptidase, Ulp1, to mimic constitutively sumoylated and unsumoylatable cohesin respectively, were constructed. Unexpectedly, it was found that both fusions relocalized to a double strand break, and that the Ulp1 fusion was recruited to a greater extent. Data from our laboratory suggests that the Ubc9 fusion is not competent for cohesion after damage, although it is sufficient for viability. Sumoylation may therefore be involved in cohesin unloading or turnover, which might be required after DNA damage.

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1 Introduction

1.1 Cell division and the importance of cohesin

Dividing cells pass through the cell cycle, which consists of interphase, in which cells prepare by synthesising cell components and replicating DNA, and cell division. Eukaryotic cells undergo two types of cell division: mitosis, in which two daughter cells are formed with an identical complement of chromosomes to the mother cell, and meiosis, in which a single cell gives rise to four cells, each with half the chromosome number of the parent cell.

Interphase can be divided into three stages: G1 phase, in which cell components are synthesised and the decision to enter the cell cycle is taken; S-phase, during which DNA is replicated; and G2, another growth phase which occurs prior to mitosis. Similarly, cell division can be divided into stages: prophase, during which chromosome condensation takes place and centrioles begin to separate to form a spindle; metaphase, in which the spindles form and chromosomes line up on the metaphase plate; anaphase, in which separation begins and telophase in which separation is completed. In mitosis sister chromatids are separated. In meiosis, in the first division homologous chromosomes are separated. A second division occurs, without intervening replication, during which sister chromatids are separated. Cytokinesis, in which the cytoplasm is divided and two new cells are formed then occurs.

Correct chromosome segregation during mitosis and meiosis depends on the physical connection between sister chromatids. Linking sisters from their time of synthesis during S-phase to when they are separated at anaphase enables the cell to identify pairs of sisters and ensure equal distribution of sister chromatids into daughter cells. Cohesion between sisters may also facilitate the binding of microtubules from opposite spindle poles during mitosis and meiosis II and help generate tension, satisfying the spindle checkpoint that bi-orientation has occurred.

Catenation could be responsible for holding sister chromatids together. However, studies on yeast minichromosomes have shown that catenation is not sufficient for cohesion (Guacci et al., 1994; Koshland and Hartwell, 1987). Instead, a protein complex

named cohesin has been implicated in sister chromatid cohesion. Cohesin was identified in screens for mutants that separate sister chromatids precociously during mitosis in *Saccharomyces cerevisiae (S. cerevisiae)* (Guacci et al., 1997; Michaelis et al., 1997). Cohesin can mediate cohesion in the absence of catenation, and proteolytic cleavage of cohesin is enough to convert dimeric minichromosomes to monomeric minichromosomes (Ivanov and Nasmyth, 2007).

1.2 The Cohesin complex

Cohesin is a complex of proteins belonging to the Structural Maintenance of Chromosome (SMC) family. SMC proteins are found in archaea, bacteria and eukaryotes, where they perform vital roles in chromosome metabolism (Cobbe and Heck, 2000; Cobbe and Heck, 2004; Jessberger, 2002). In eukaryotes, there are three SMC complexes, cohesin, condensin and the Smc5/6 complex.

A cartoon of the *S. cerevisiae* mitotic cohesin complex is shown in Figure 1. Homologues of proteins in *Schizosaccharomyces pombe* (*S. pombe)*, *Drosophila* and mammalian cells are shown in Table 1. The core cohesin complex consists of Smc1, Smc3, Scc1 and Scc3. Smc1 and Smc3 have a characteristic structure shared with other SMC proteins. Two coiled coils and a hinge region separate globular N and C-terminal domains. These globular domains contain motifs involved in ATP binding and hydrolysis: Walker A and Walker B motifs, and the signature sequence LSGG, which is typical of the ABC ATPase family of proteins (Holland and Blight, 1999). Each protein folds, via the hinge region, allowing the association of the globular domains. Each SMC protein dimerizes with another SMC protein via the hinge domain. The globular ATPase heads also associate.

Scc1 links the globular head domains of Smc1 and Smc3. Biochemical mapping shows that Scc1's N-terminus binds Smc3 and Scc1's C-terminus binds Smc1 (Haering et al., 2002). A crystal structure of Smc1's ATPase domain complexed with Scc1's C-terminal domain has been solved (Haering et al., 2004). However, Förster Resonance Energy Transfer (FRET) studies suggest that Scc1 binds the Smc1 and Smc3 heads in an orientation that is perpendicular to the axis that connects the two heads (Mc Intyre et al.,

2007). In any case, Smc1, Smc3 and Scc1 form a tripartite ring structure, visible by electron microscopy (Anderson et al., 2002) with an outer diameter of ~50nm (Haering et al., 2002). Although Scc1 links the two heads, the SMC heads can interact in the absence of Scc1 to form a closed ring (Weitzer et al., 2003).

Scc3 binds to Scc1. No structure for Scc3 has been described.

In addition to these core components, cohesin is associated with a number of other proteins, including: Pds5, important for the maintenance of cohesion; Wpl1, involved in cohesion establishment, maintenance and dissolution; and, in mammalian cells, Sororin, which has a role in establishment and/or maintenance of cohesion (Rankin et al., 2005).

FRET experiments indicate that Pds5 interacts with the hinge region where Smc1 and Smc3 dimerize. However, Pds5 also shows a weak FRET interaction with Scc1, and the interaction between Pds5 and the cohesin complex depends on Scc1 (Mc Intyre et al., 2007).

Wpl1 can bind Scc3 and Pds5 in *S. cerevisiae* (Rowland et al., 2009). The binding of Wap1, the mammalian homologue of Wpl1, to cohesin depends on Scc1 and SA1/SA2, and recombinant Wap1 forms a complex with Scc1 and SA1 (Gandhi et al., 2006; Kueng et al., 2006). In human cell lysates there is evidence that Wap1 forms a distinct subcomplex with Pds5 (Kueng et al., 2006).

Therefore, it seems that Pds5 can be located at the hinge region, or with Wpl1 together with Scc1 and Scc3. It is possible that during the cell cycle the localization of Pds5 may change. Alternatively, these results could indicate a head-hinge interaction.

Mammalian cells contain Sororin, which also interacts with the cohesin complex, although how it interacts is unknown.

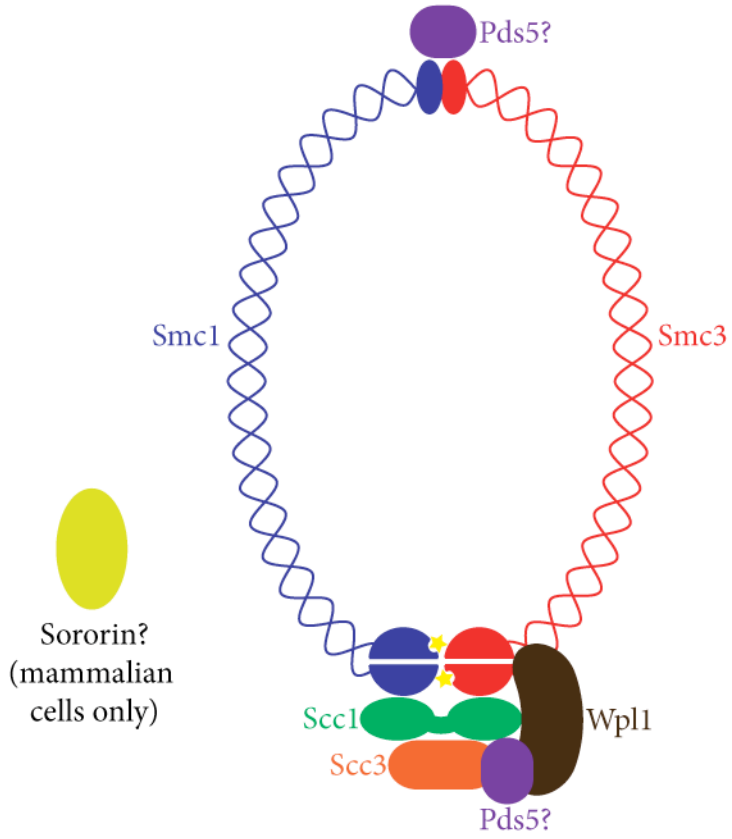


Figure 1: Cartoon of the *S. cerevisiae* mitotic cohesin complex

The subunits and their probable arrangement are shown, incorporating findings in other organisms (for subunit names in other organisms see Table 1). Two ATP molecules are depicted between the globular head domains of Smc1 and Smc3 (represented by stars). Scc1 is shown in this cartoon as linking the heads but may lie perpendicular to them. Scc3 binds Scc1. There is evidence for Pds5 association with the hinge region of the Smc1-Smc3 heterodimer (top) and with Scc1 and Scc3. Wpl1, which in mammalian cells can form a subcomplex with Pds5, can associate with Scc1. Since post-translational modification of Smc3 can influence Wpl1 activity, Wpl1 is shown contacting it as well. Sororin is present in mammalian cells only. How it contacts the cohesin complex is unknown.

	S. cerevisiae	*S. pombe*	*Drosophila*	Human
Core cohesin subunits	Smc1	Psm1	Smc1	hSmc1
	Smc3	Psm3	Cap	hSmc3
	Scc1 (Mcd1)	Rad21	Rad21	hRad21 (hScc1)
	Scc3 (Irr1)	Psc3	SA and SA2	hSA1 and hSA2
Cohesin-associated proteins	Pds5	Pds5	Pds5	hPds5A and hPds5B
	Rad61/Wpl1	Wap1	Wap1	Wap1
				Sororin

Table 1: Mitotic cohesin subunits and associated proteins in *S. cerevisiae*, *S. pombe*, *Drosophila* and humans

1.3 Cohesin's association with DNA

How cohesin interacts with the DNA to cause sister chromatid cohesion has been intensely investigated, and a number of models proposed, including the ring or embrace and a number of oligomerisation models (Huang et al., 2005). Models for cohesin's association with DNA and how they might lead to cohesion are shown in Figure 2.

It was initially thought that cohesin might bind directly to DNA as recombinant fragments of Smc3 and the cohesin core complex can bind DNA *in vitro* (Akhmedov et al., 1999; Kagansky et al., 2004; Losada and Hirano, 2001; Sakai et al., 2003). However, *in vivo* cohesin cannot be dissociated from DNA, even at high salt concentrations (Ciosk et al., 2000), and a topological model for cohesin's association with DNA is now favoured.

There is compelling evidence for a topological model. Proteolytic cleavage (by inserting tobacco etch virus (TEV) protease cleavage sites) of Scc1 or Smc3 destroys cohesin's binding to chromatin *in vivo* (Gruber et al., 2003). In addition, linearization of a minichromosome reduces the co-precipitation of minichromosomal DNA with cohesin to background levels (Ivanov and Nasmyth, 2005). DNA linearization also converts dimeric minichromosomes, held together by cohesin, to monomers (Ivanov and Nasmyth, 2007). Also, the fusion of subunits of the ring prevents the association of cohesin with DNA,

suggesting that the cohesin complex must be opened for DNA binding to occur (Gruber et al., 2006).

However, it is unclear whether both sisters are encompassed by a single ring, or whether cohesion is caused by the oligomerisation of two complexes that each bind an individual sister. There is evidence for both of these models. The most compelling evidence for a single ring causing cohesion is the finding that cross linking subunits of cohesin which form the tripartite ring (Smc1, Smc3 and Scc1) together allows them to hold sister minichromosomes together even after protein denaturation. From efficiency of crosslinking and cleavage data the authors concluded that a single ring, rather than intertwined rings, mediate cohesion (Haering et al., 2008). However, recently, evidence for a two-ring model in which each sister is encompassed by a ring, and rings between sisters are bridged by Scc3 in human cells has been published (Zhang et al., 2008b). Previously it has been reported that C-terminally tagged Scc1-18myc and Scc1-6HA do not co-immunoprecipitate in yeast (Haering et al., 2002; Ivanov and Nasmyth, 2005), and Rad21-9myc cannot immunoprecipitate endogenous Rad21 in stably transfected HeLa cells (Hauf et al., 2005). This was also used as evidence for the single ring model. However, (Zhang et al., 2008b) found that Rad21 (Scc1), Smc1 and Smc3 can co-immunoprecipitate themselves when N-terminally tagged, and Rad21 interacts with itself in a yeast two-hybrid assay. There is also strong evidence that at the *Hidden MAT Right* (*HMR)* locus each ring only embraces one sister, and this mediates cohesion (Chang et al., 2005). Cohesion is dissolved on a minichromosome consisting of the *HMR* locus excised from the genome if silencing is abrogated. However, cohesin remains bound. The authors suggest that cohesin does not embrace both sisters, and instead cohesin rings embrace a single sister and interact with the silent domain of the other chromatid. Cohesin's mode of interaction with DNA could be locus specific.

In any case, it is unlikely that cohesion can be mediated by a static ring, as cohesion can be dissolved without cohesin complex dissociation or Scc1 cleavage (discussed later, and reviewed in Guacci, 2007).

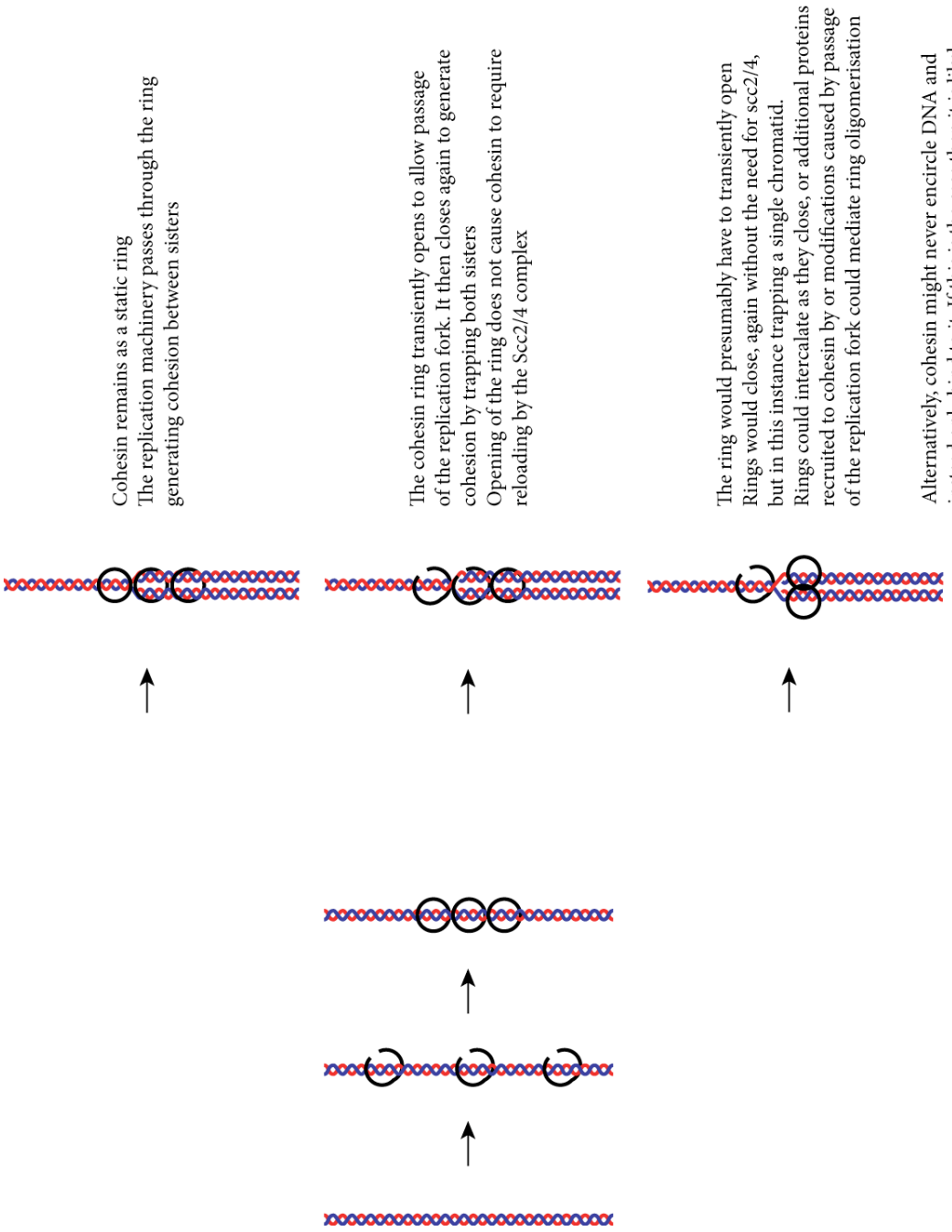


Figure 2: Models for the mode of interaction of cohesin with DNA
DNA is represented by the red and blue lines. The cohesin complex is depicted as a circle.

1.4 Cohesin associated regions

In *S. cerevisiae* cohesin is found enriched at discrete sites along chromosome arms, named Cohesin Associated Regions (CARs), and at centromeres (Blat and Kleckner, 1999; Megee et al., 1999; Tanaka et al., 1999). CARs are approximately 0.8kb in size and 10kb apart (Laloraya et al., 2000), have a high A/T content (Blat and Kleckner, 1999) and are localized at points of convergent transcription (Glynn et al., 2004; Lengronne et al., 2004).

The observation that cohesin is located at points of convergent transcription in *S. cerevisiae*, and that transcription alters chromosomal localization of cohesin has led to the model in which transcription determines cohesin localization (Bausch et al., 2007; Lengronne et al., 2004). However, cohesin is also enriched at peri-centromeric regions and silent chromatin (Glynn et al., 2004). Enrichment in these regions cannot be due to transcription, suggesting that cohesin is localized by another mechanism. In addition, binding sites for cohesin are different in different organisms. For example, in *Drosophila*, cohesin binds transcribed regions and in mammalian cells co-localizes with CTCF (Parelho et al., 2008; Rubio et al., 2008; Stedman et al., 2008; Wendt et al., 2008).

Cohesin might be localized by other chromatin bound proteins, or by chromatin structure. For example, in *S. cerevisiae*, cohesin binding to centromeres requires the proteins Cse4, Mif2, Ndc10 (Tanaka et al., 1999), and enrichment at silent domains requires Sir2, Sir3 and Sir4 (Chang et al., 2005). Binding of cohesin to the outer centromeric repeats of *S. pombe* requires Swi6 (HP1) and Clr4, a H3K9 methyltransferase (Bernard et al., 2001b; Nonaka et al., 2002), and Swi6 may physically interact with cohesin (Nonaka et al., 2002). CTCF influences cohesin binding sites in mammalian cells (Parelho et al., 2008; Wendt et al., 2008). A chromatin modification, γH2AX, is required for cohesin enrichment after a double strand break (DSB) (Unal et al., 2004), discussed in subsequent sections. Chromatin remodellers are also important for cohesin binding and establishment of cohesion. The Rsc2-remodelling complex facilitates cohesion of *HMR* in *S. cerevisiae* (Baetz et al., 2004; Chang et al., 2005; Huang et al., 2004) and ISWI (SNF2h) has a role in cohesin loading in human cells (Hakimi et al., 2002). It is possible that transcription also alters cohesin localization by this mechanism.

1.5 The mitotic cohesin cycle

A Schematic of the mitotic cohesin cycle is shown in Figure 3.

1.5.1 Loading of cohesin

In *S. cerevisiae*, cohesin loading begins in G1, although in higher eukaryotes cohesin is reloaded in telophase, following the re-formation of the nuclear envelope. Loading requires another complex composed of Scc2 and Scc4 (Bernard et al., 2006; Ciosk et al., 2000; Furuya et al., 1998; Rollins et al., 2004; Seitan et al., 2006; Toth et al., 1999). Cohesin first associates with Scc2/4 sites. A small fraction of Scc2 can be immunoprecipitated with Scc3 (Toth et al., 1999) and mass spectrometry analysis of a Scc2-TAP purification identified Smc1, Smc3 and Scc1 (Arumugam et al., 2003). However, once loaded cohesin moves to CARs, and in *S. cerevisiae* Scc2 and cohesin do not co-localize. Cohesin loading also requires ATP hydrolysis, as Smc1 and Smc3 hydrolysis mutants do not associate with chromatin (Arumugam et al., 2003).

How the Scc2/4 complex promotes loading of cohesin is unknown, although it has been hypothesised that it may stimulate the ATPase activity of the SMC heads and how the cohesin ring opens to encompass the DNA, if it does so, is also unclear. Scc2/4 is not required for formation of the cohesin complex, as in its absence soluble cohesin complexes form (Ciosk et al., 2000; Losada et al., 1998; Sumara et al., 2000; Toth et al., 1999). It was originally thought that loading might occur via opening of the SMC heads, possibly with the dissociation of Scc1. However, it now appears that cohesin is loaded via opening of the hinges, as mutants with fused hinges cannot load onto DNA (Gruber et al., 2006). ATP hydrolysis might open the hinges or the hinges might fold back to directly contact ATPase domains, as atomic force microscopy reveals that SMC complexes can exist in different conformations, including a compact form in which the hinge region folds back upon the globular heads (Yoshimura et al., 2002).

Binding of Scc1 stimulates the ATPase activity of the SMC heads (Arumugam et al., 2006). Cohesin's ATPase activity is low, suggesting that ATP hydrolysis possibly drives a conformational switch that occurs infrequently. Arumugam et al. (2006) suggest that if

ATP hydrolysis mediates hinge dissociation, then its dependence on Scc1 would ensure that the SMC subunits remain linked.

In *S. cerevisiae*, during early G1, Esp1 activity cleaves Scc1, preventing the stable association of Scc1 with chromatin. Non-cleavable Scc1 (named RRDD due to two amino-acid changes) is able to bind to chromosomes during early G1, demonstrating that stable Scc1 is sufficient for cohesin association with chromosomes. In mammalian cells, Wap1 also controls the dynamics of cohesin interaction with DNA during interphase. If Wap1 is depleted, more cohesin binds and Fluorescence Recovery After Photobleaching (FRAP) experiments show that its residence time is increased (Kueng et al., 2006). A similar role has been ascribed to the *S. pombe* homologue Wpl1 (Bernard et al., 2008) and has been suggested for the *S. cerevisiae* homologue (Ben-Shahar et al., 2008) during G1. In early S-phase cohesin binding becomes more stable (Bernard et al., 2008).

Despite Scc2/4 being essential, there is some evidence for an Scc2/4 independent mechanism of cohesin loading after centromere breathing (Ocampo-Hafalla et al., 2007) and after dissociation due to transcription (Bausch et al., 2007).

1.5.2 Establishment of cohesion

Cohesion is established during S-phase. In an unperturbed cell cycle, for cohesion generation to occur, cohesin must be loaded prior to replication (Lengronne et al., 2006). Cohesin can be loaded onto chromosomes later during the cell cycle, for example during G2/M (Ciosk et al., 2000). This cohesin accumulates at the same sites as G1 loaded cohesin, and also requires the Scc2/4 complex for loading. However, it is not competent for cohesion (Lengronne et al., 2006). Similarly, wild-type (WT) Smc1 expressed in G2/M cannot complement for temperature sensitive *Smc1* and uncleavable Scc1 expressed after S phase does not affect separation of sisters (Haering et al., 2004; Strom et al., 2004).

Direct interactions between Smc1 and replication proteins are detected if a tagged version of Smc1 is transiently overexpressed in cultured mammalian cells (Ryu et al., 2006). It is possible that the replication machinery passes through cohesin rings, and this is how cohesion is generated. If cohesin dissociates to allow replication fork passage, re-

association and cohesion formation is independent of Scc2/4 and the ATPase activity of Smc1 and Smc3 (Lengronne et al., 2006). Alternatively, cohesin molecules could become linked to generate cohesion after replication fork passage. However cohesion is generated, coupling cohesion generation to replication ensures that sister chromatids are cohesed together.

Eco1/Ctf7 is essential for cohesion establishment and hence viability (Skibbens et al., 1999; Toth et al., 1999). It is required during S-phase (Skibbens et al., 1999; Toth et al., 1999). Although it is not required for replication, Eco1 genetically and physically interacts with PCNA and RF-C (Kenna and Skibbens, 2003; Moldovan et al., 2006), suggesting that it travels with the replication fork. Interestingly, Eso1, the orthologue of Eco1 in *S. pombe* exists as a fusion to Polη, again linking Eco1 and cohesion establishment to the replication fork.

Other proteins involved in DNA replication, including components of the replication fork itself are important for cohesion generation. These include the DNA polymerase Trf4 (Wang et al., 2000), components of an alternative RF-C complex (Ctf8, Ctf18, Dcc1) (Hanna et al., 2001; Mayer et al., 2001) and Ctf4, which associates with GINS complex and DNA polα/primase (Lengronne et al., 2006). The finding of an alternative RF-C and a polymerase which have a role in cohesion generation led to the polymerase switch model, in which the alternative RF-C may catalyse a polymerase switch, which may occur prior to the replication of cohesin associated sites. This may be required for replication through cohesin associated sites or may promote the binding of Eco1. Alternatively, the alternative RF-C may have a role on the lagging strand. However these proteins promote cohesion establishment, their role is non-essential as although mutation or deletion of these complexes causes a cohesion defect, they are not essential for viability.

Mutations in replication fork proteins and Eco1, whilst impacting cohesion, do not seem to affect the binding or localization of cohesin (Lengronne et al., 2006; Noble et al., 2006). Instead, it has been recently discovered that Eco1 acetylates Smc3, and this modification is critical for cohesion establishment. Smc3 is acetylated on K112 and K113 by Eco1 in *S. cerevisiae* (Ben-Shahar et al., 2008; Rowland et al., 2009; Unal et al., 2008; Zhang et al., 2008a). When unacetylated Smc3 interferes with the establishment of cohesin and promotes anti-establishment via Pds5 and Rad61 (Wpl1) (Ben-Shahar et al.,

2008; Rowland et al., 2009; Unal et al., 2008) and Scc3 (Rowland et al., 2009). When Wpl1 is deleted, or specific mutations in Smc3, Pds5 and Scc3 are present, Eco1 is dispensable for cohesion. However, when these mutations are present in cells containing wild-type Eco1, they result in reduced sister chromatid cohesion (Rowland et al., 2009).

Although cohesion establishment occurs predominantly in S-phase, it can occur at other stages of the cell cycle, for example, after DNA damage (discussed in subsequent sections) or during centromere breathing (Ocampo-Hafalla et al., 2007).

1.5.3 Maintenance of cohesion

Cohesion between sisters is maintained until the metaphase to anaphase transition. Although acetylation levels of Smc3 are high during G2 (Ben-Shahar et al., 2008; Unal et al., 2008; Zhang et al., 2008a), neither Smc3 acetylation (Rowland et al., 2009) nor Eco1 (Skibbens et al., 1999; Toth et al., 1999) are required for the maintenance of cohesion. Instead, Pds5 and Wpl1, which have anti-establishment activities prior to S-phase are required. In the absence of Pds5 cohesion is lost even though cohesin remains chromatin bound (Dorsett et al., 2005; Hartman et al., 2000; Losada et al., 2005; Stead et al., 2003; Tanaka et al., 2001). Inactivation of Wpl1 (Rad61) compromises sister chromatid cohesion (Warren et al., 2004) and Wpl1 is needed for the maintenance of cohesion (Sutani et al., 2009).

Rowland et al. (2009) suggest that Pds5-Wpl1 act by hindering the opening of the ring, which could be necessary to allow rings that have trapped a single chromatid to entrap both sisters or to allow simultaneous entrapment, thereby hindering establishment of cohesion. The same activity, the prevention of ring opening, would then promote maintenance of cohesion. Acetylation somehow blocks anti-establishment without affecting maintenance. Alternatively, Pds5-Wpl1 could affect the dimerization of rings. In either case, acetylation, or lack of acetylation, does not affect the association of cohesin with a single chromatid, just the establishment of cohesion.

Pds5 has also been reported to be associated with both the hinge region of the SMC dimer, and Scc1. It may be that the Pds5-Wpl1 complex relocates from one binding

site to another after the acetylation of Smc3, and by this mechanism can be involved in seemingly opposing processes.

1.5.4 Cohesion dissolution

At the metaphase to anaphase transition, Cdc20 binds to the Anaphase Promoting Complex (APC). The APC is an E3 ubiquitin ligase which targets proteins for degradation. As the cell enters mitosis M-Cdks cause the phosphorylation of core APC subunits, which promotes the binding of the APC activator Cdc20. APC^{Cdc20} initiates a cascade that results in loss of cohesion and sister chromatid separation. APC^{Cdc20} is kept inactive by the spindle checkpoint until correct attachment to the spindle has occurred. APC^{Cdc20} targets Pds1 (Securin) for degradation. Pds1 is the inhibitory binding partner of Esp1. Esp1 (Separase) is responsible for cleaving Scc1, abolishing cohesion and allowing segregation.

In *S. cerevisiae* Scc1 is cleaved at R268 and R180 by Esp1 (Uhlmann et al., 1999). A cleavage resistant mutant version of Scc1 (RRDD) can associate with chromosomes and establish cohesion, but it causes a transient delay in cytokinesis, after which the cells divide without separating their sisters, producing cells with abnormal DNA content, similar to *esp1-1* (Uhlmann et al., 1999).

Scc1's cleavage is enhanced and regulated by phosphorylation by Cdc5 (Polo). Although destruction of Pds1 is necessary for sister separation (Cohen-Fix et al., 1996), it is not sufficient. Pds1 is not essential in *S. cerevisiae* at low temperatures (Yamamoto et al., 1996), and Scc1 cleavage is tightly regulated in *pds1Δ* cells (Alexandru et al., 1999). Phosphorylation of Scc1 by Cdc5 (Polo) makes it a more attractive target for cleavage by Separase (Alexandru et al., 2001). This mechanism also ensures that chromatin bound Scc1 is preferentially cleaved over soluble Scc1, which may be important to ensure that the sisters separate (Hornig and Uhlmann, 2004). After cleavage, the C-terminal fragment of Scc1 is degraded by the N-rule pathway, and this degradation is essential for chromosome stability (Rao et al., 2001).

Pds5 sumoylation peaks in mitosis and it has been suggested that Pds5 sumoylation promotes the dissolution of cohesin, as overexpression of the Small

ubiquitin-like modifier (SUMO) isopeptidase SMT4 suppresses the inviability and precious sister separation of *pds5* mutants (Stead et al., 2003).

1.5.5 Prophase pathway

In *S. cerevisiae* cohesin is maintained on chromosomes until G2/M, when Scc1 is cleaved. In vertebrate cells, only a small amount (~10%) of cohesin remains on chromosomes until metaphase. Instead, during prophase the majority of cohesin, the Scc2/4 complex, CTCF, Wap1, Pds5A and Sororin are all removed, in a process that is independent of Scc1 cleavage. Cohesin remains at centromeres, and is cleaved at the metaphase to anaphase transition by Esp1 to allow sister separation (Gerlich et al., 2006; Hauf et al., 2005; Hauf et al., 2001; Hoque and Ishikawa, 2001; Losada et al., 1998; Losada et al., 2000; Sumara et al., 2000; Waizenegger et al., 2000).

Cdc5 and Wap1 play key roles in the prophase removal of cohesin. CDC5/POLO kinase phosphorylates SA2 (the mammalian homologue of Scc3). As mentioned previously, Cdc5 phosphorylates Scc1 to enhance Esp1 dependent cleavage. Phosphorylation of SA2 is instead required for prophase removal of cohesin (Hauf et al., 2005; Kueng et al., 2006; Sumara et al., 2002). The removal of cohesin once SA2 has been phosphorylated requires Wap1 (Gandhi et al., 2006; Kueng et al., 2006). Wap1 is the homologue of Wpl1, and therefore this protein has been implicated in the prevention of establishment of cohesion, the maintenance of cohesion, and prophase removal. Prophase removal also requires Aurora B and the condensin complex.

To maintain the identity of sisters and ensure correct segregation, cohesion at the centromere must be maintained during prophase removal. SgoI is essential for this process (Kitajima et al., 2005; McGuinness et al., 2005; Salic et al., 2004; Tang et al., 2004). SgoI is localized to centromeres by Bub1, HP1α, and Aurora B (Dai et al., 2006; Kitajima et al., 2005; Kueng et al., 2006; Lipp et al., 2007; Resnick et al., 2006; Tang et al., 2004; Yamagishi et al., 2008). There, in combination with PP2A, it is thought to dephosphorylate SA2 and therefore protect cohesin from Wap1 mediated removal (Kitajima et al., 2006; McGuinness et al., 2005). Sororin may also have a role in

protection of cohesin from the prophase pathway (Diaz-Martinez et al., 2007; Rankin et al., 2005).

At the metaphase to anaphase transition, centromeric cohesion is cleaved via the same mechanism described earlier for yeast. Despite the fact that most cohesin is removed during prophase, the cleavage of this remaining cohesin is essential, and expression of noncleavable Scc1 mutants or depletion of Separase (the Esp1 homologue) causes defects in chromosome segregation (Hauf et al., 2001; Kumada et al., 2006; Wirth et al., 2006).

If the prophase pathway is inactivated, chromosome segregation can still occur as Separase can compensate (Hauf et al., 2005). Prophase removal may reduce the amount of cohesin that needs to be cleaved. This may result in increased fidelity of separation, by reducing the possibility of unwanted linkages. There is strong evidence that cohesin has a role in gene expression, discussed later, and high levels of intact cohesin may be essential for correct gene regulation and to allow rapid cohesin reassociation in telophase in mammalian cells.

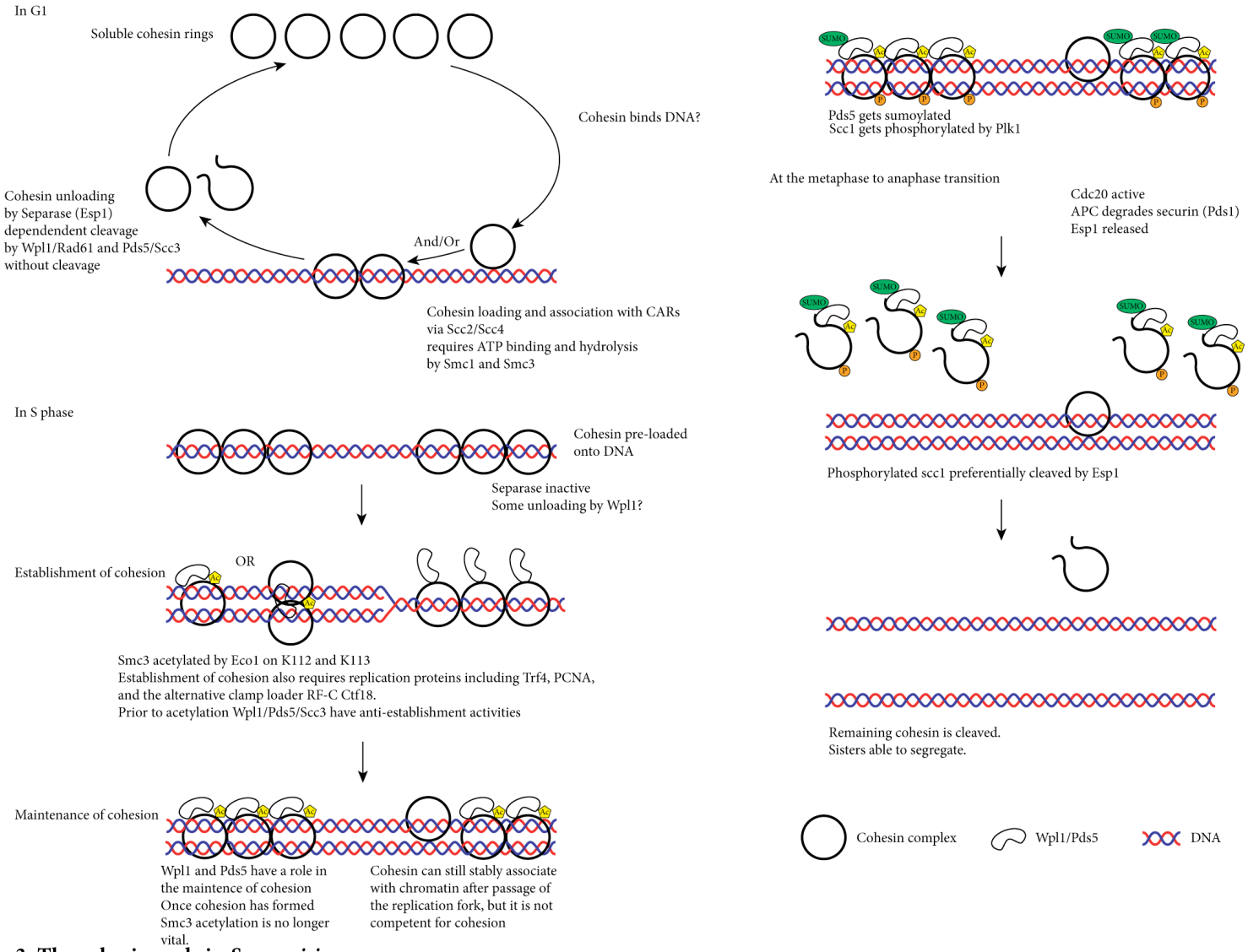


Figure 3: The cohesin cycle in *S. cerevisiae*

Cohesion is depicted to be due to a single ring encircling the two sisters.

1.6 Cohesin and Meiosis

Meiosis is a specialized form of cell division in which two rounds of segregation follow a single round of DNA replication, producing haploid cells from diploids. During the first round of segregation, homologous chromosomes are separated. In the second round of segregation, sisters are separated. Cohesin has a key role in meiosis as well as in mitosis. In addition to cohesion between sisters, Rec8, the meiotic Scc1 homologue, has a role in recombination, pairing and synaptonemal complex assembly (Klein et al., 1999). Cohesin's role in meiosis is outlined below, and its role in *S. cerevisiae* meiosis summarised in Figure 4.

1.6.1 Premeiotic S-phase

During meiotic DNA replication, meiosis-specific cohesin complexes are loaded (Watanabe and Nurse, 1999; Watanabe et al., 2001). In *S. cerevisiae*, Rec8 replaces Scc1 to form meiotic cohesin. Other organisms have more meiosis specific subunits, for example mammalian cells have Smc1β and SA3 in addition to hRec8 (see Table 2). It is thought that the Scc2/4 complex is responsible for the loading of meiotic cohesin.

S. cerevisiae **MITOTIC components**	***S. cerevisiae*** **MEIOIS specific components**	***S. pombe*** **MEIOIS specific components**	***Drosophila*** **MEIOIS specific components**	**Mammalian MEIOIS specific components**
Smc1				Smc1β
Smc3				
Scc1/Rad21	Rec8	Rec8	DRec8	hRec8
Scc3/Irr1		Rec11	DSA2	SA3

Table 2: Meiosis specific cohesin components in *S. cerevisiae*, *S. pombe*, *Drosophila* and humans

After replication, the first stage of meiosis is the induction of DNA DSBs by Spo11, which is important for alignment of homologous chromosomes (Klein et al., 1999). Recombination, due to repair, links homologues (reviewed in McKee, 2004). DSB hotspots are free of cohesin (Glynn et al., 2004). Cross-overs result in physical links

between homologous chromosomes (chiasmata). Rec8 containing cohesin has a role in recombination, pairing and synaptonemal complex assembly (a proteinaceous structure which forms between homologous chromosomes after they have paired and disassembles before mitosis) (Klein et al., 1999). There is also evidence that cohesin might bind chiasmata in place, preventing their movement towards chromosome ends and eventual loss (Hodges et al., 2005).

Scc1 is also present until shortly before the first meiotic division. There is evidence that Scc1 may have a role in meiotic recombination, as spore viability is reduced in *scc1* mutants (Klein et al., 1999).

1.6.2 Metaphase I

Chromosomes are then captured by the meiotic spindle. In budding yeast, a pair of sister kinetochores is captured by a single microtubule during meiosis I (Winey et al., 2005). Spo13, Ipl1, Cdc5 and Monopolin are important for this monopolar attachment in budding yeast, which allows the separation of homologues, rather than sisters as in mitosis (Clyne et al., 2003; Lee and Amon, 2003; Lee et al., 2004; Monje-Casas et al., 2007; Rabitsch et al., 2003; Toth et al., 2000). Monopolin is a kinetochore specific protein complex consisting of Mam1, Csm1, Lrs4 and Hrr25. Cdc5 and Spo13 seem to regulate the localization of monopolin (Lee et al., 2004).

In most eukaryotes, sister kinetochores are captured by multiple microtubules. Although fission yeast also has a kinetochore specific protein required for monopolar attachment, Moa1 (Yokobayashi and Watanabe, 2005), there is little similarity between Moa1 and the monopolin complex, and homologues of Moa1 or monopolin subunits (except Hrr25) have not yet been identified in other eukaryotes. The differences in requirements for additional proteins may be explained by species dependent differences in Rec8 or in centromere structure leading to differences in kinetochore geometry. For example, in fission yeast, it has been proposed that the central core region of the centromere loops out from the surrounding pericentromeric heterochromatin. During mitosis, cohesin containing Rad21 accumulates at the pericentromeric regions but not at the core region. The core regions of sister chromatids face away from each other allowing

bi-orientation. In contrast, during meiosis I, Rec8 containing cohesin complex also localizes to the core region, generating a side by side configuration and favouring mono-orientation (Yokobayashi and Watanabe, 2005).

1.6.3 Anaphase I

During anaphase I chiasmata are resolved, and cohesin is removed from chromosome arms by Esp1, to allow the separation of homologous chromosomes. Centromeric cohesin is maintained, by the same mechanism that protects centromeric cohesin from the prophase pathway during vertebrate mitosis. Rec8 is protected from cleavage by SgoI (Kitajima et al., 2004; Marston et al., 2004; Rabitsch et al., 2004). SgoI associates with PP2A, and acts to dephosphorylate cohesin at the centromere (Riedel et al., 2006). Sgo1 also recruits Ipl1, the Aurora B kinase orthologue, which is also required for the maintenance of centromeric cohesin by maintaining the localization of PP2A (Yu and Koshland, 2007). Sgo1 localisation depends on Bub1, a component of the spindle assembly checkpoint (Bernard et al., 2001a; Huang et al., 2007; Kiburz et al., 2005; Kitajima et al., 2005; Kitajima et al., 2004; Riedel et al., 2006; Tang et al., 2004). Spo13 also acts to protect centromeric cohesin, independently of Sgo1 (Katis et al., 2004; Lee et al., 2002; Lee et al., 2004; Shonn et al., 2002).

1.6.4 Metaphase II and Anaphase II

The second meiotic division is very similar to mitosis. During metaphase II sister chromatids bi-orientate. Cells then undergo a second division, by which time SgoI has been degraded, in which sisters are separated after the remaining centromeric cohesin is cleaved by Esp1.

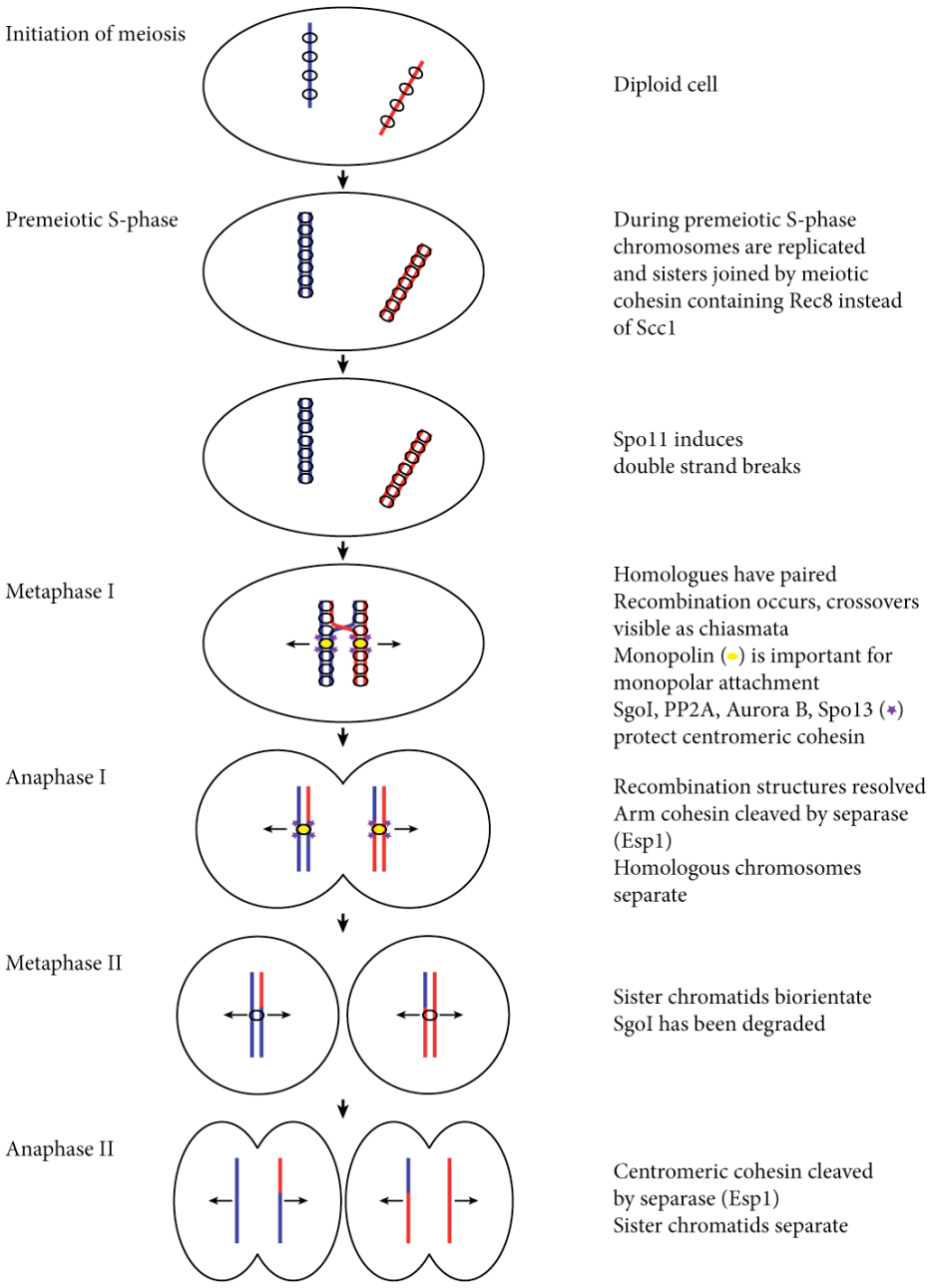


Figure 4: Cohesin in meiosis in *S. cerevisiae*

(Based on Watanabe, 2004).

1.7 Cohesin and transcription

Cohesin has a role in regulating transcription, and silent chromatin seems to have a role in cohesion. However, the interplay between cohesin and transcription appears to vary between organisms. Although cohesin is enriched at centromeric regions in all eukaryotic organisms, its site of association along chromosome arms varies. Below is a summary of the links between cohesin and transcription in *S. cerevisiae*, *S. pombe*, *Drosophila* and mammalian cells.

1.7.1 Transcription alters the location of cohesin in S. cerevisiae

As already mentioned, cohesin is located at pericentric regions and discrete sites along the chromosome arms. In *S. cerevisiae,* CARs occur at points of convergent transcription (Glynn et al., 2004; Lengronne et al., 2004). Altering the transcriptional status of genes can perturb cohesin localization (Bausch et al., 2007; Glynn et al., 2004; Lengronne et al., 2004). This led to the theory that cohesin binding was incompatible with transcription, and that the transcriptional machinery either pushes cohesin rings, referred to as the 'sliding' model, or modifies chromatin such that cohesin relocalizes from its loading site (Scc2/4) to CARs. The latter may be more plausible, as cohesin is not found at points of divergent or unidirectional transcription. Cohesin also seems to have a preference for A/T rich regions. In addition, altering transcriptional status does not always result in an accumulation of cohesin 3' to the gene, suggesting that cohesin can dissociate from chromatin (Bausch et al., 2007).

1.7.2 Silent chromatin and cohesin in S. cerevisiae

As previously mentioned, silencing aids cohesion at the *HMR* locus (Chang et al., 2005). The Sir complex is needed for the establishment and maintenance of cohesion. However, loss of cohesion is essential for the establishment of silencing at *HMR*, and loss of Scc1 allows silencing to be established early (Lau et al., 2002). Orc5, which is involved

in the establishment of cohesion, is also implicated in the establishment of silencing (Suter et al., 2004).

Cohesin also binds to a non-transcribed region in each rDNA repeat in a Sir2 dependent manner (Kobayashi et al., 2004).

The association of cohesin at repressed regions also cannot be explained by the sliding model.

The silent mating type loci are flanked by boundary elements that prevent the spread of silent chromatin established by SIR complexes. Deletion of the boundary elements or mutations in Smc1 or Smc3 allow the spreading of silencing (Donze et al., 1999).

1.7.3 Cohesin at sites of convergent transcription and centromeres in S. pombe

Like in *S. cerevisiae*, cohesin is found at centromeric regions and at points of convergent transcription in *S. pombe*. However, in this organism both a mechanism for the recruitment of cohesin to these sites and a rational for their localization have been deduced. Centromeric repeats form heterochromatin, and Swi6, which is required for the association of cohesin with heterochromatic regions is thought to recruit cohesin. Swi6 directly interacts with cohesin (Bernard et al., 2001b; Nonaka et al., 2002; Partridge et al., 2002). The recruitment of cohesin to sites of convergent transcription could occur via RNA interference (RNAi) mediated silencing. Transcriptional readthrough of convergent genes in G1 could form double-stranded RNA which activates the RNAi pathway, which in turn forms heterochromatin and recruits Swi6 and cohesin (Gullerova and Proudfoot, 2008). Some evidence exists for this model, although it remains to be determined whether it represents a general model. Cohesin is thought to then have a role in termination of transcription in G2 (Gullerova and Proudfoot, 2008).

1.7.4 Nipped-B and cohesin regulate transcription in Drosophila

In *Drosophila*, cohesin is not localized between convergent genes. Instead it shows a broad distribution, with preference for transcription units, particularly intronic sequences, 5' untranslated regions, and active genes (Misulovin et al., 2008). This suggests that perhaps the relocalization of cohesin in yeast is due to a change in chromatin structure associated with transcription, rather than pushing of the cohesin rings themselves by the transcriptional machinery. Another difference to yeast is that Nipped-B, the Drosophila Scc2 homologue, co-localizes with cohesin.

Intriguingly, Nipped-B and cohesin subunits can have opposing effects on gene expression: partial reduction of cohesin subunit levels increases *cut* activation without causing cohesion defects (Rollins et al., 2004) whereas reduced Nipped-B levels result in lowered expression of *cut* (Rollins et al., 1999). This finding led to a model in which cohesin is an insulator which blocks enhancer-promoter interaction, and Nipped-B facilitates activation by removing/unloading or relocalizing cohesin (Dorsett et al., 2005). This model is supported by the finding that mutations in Wap1, which has been shown to affect cohesin stability, cause defects in position effect variegation (Verni et al., 2000). Alternatively Nipped-B could have its own role in gene activation, which is attenuated by binding cohesin. Either scenario is consistent with the finding that Nipped-B and cohesin co-localize.

The effects seen on transcription in *Drosophila* are unlikely to be due to defects in cohesion, as cohesin is found in and has a role in non-dividing cells. Rad21 (the *Drosophila* homologue of Scc1) is found in post-mitotic neurons in *Drosophila* (Pauli et al., 2008). Mutations in Smc1 and Scc3 cause defects in axon pruning, which can be reverted if the affected protein is added back (Schuldiner et al., 2008). In addition, mutations in Eco and San (*Drosophila* homologues of Eco1) do not have dosage effects on transcription (Dorsett et al., 2005).

1.7.5 Cohesin and CTCF co-localize in mammalian cells.

The cohesin cycle in mammalian cells, in which cohesin is present on chromosome arms from telophase until prophase, suggests that cohesin might be performing a function during interphase. Cohesin is expressed in a wide variety of tissues (Sumara et al., 2000), and, as in *Drosophila*, is found in post-mitotic neurons (Wendt et al., 2008; Zhang et al., 2007). In addition, mutations in NIBL (the human homologue of Scc2) (Krantz et al., 2004; Tonkin et al., 2004) or SMC1A and SMC3 (Deardorff et al., 2007; Musio et al., 2006) cause Cornelia de Lange syndrome, a developmental disorder.

In mammalian cells, cohesin is found both upstream and downstream of genes. CTCF and cohesin were found to co-localize in mammalian cells (Parelho et al., 2008; Rubio et al., 2008; Stedman et al., 2008; Wendt et al., 2008). Cohesin enrichment at CTCF sites is dependent on CTCF. In the absence of CTCF, cohesin is found at different sites on the chromosome, with no deleterious effect on cohesion but an effect on transcription (Parelho et al., 2008; Wendt et al., 2008). Genome wide expression profiling in human cells showed significant overlap between genes deregulated after knockdown of either CTCF or cohesin (Wendt et al., 2008). Knockdown of cohesin also reduced the insulator activity of CTCF in several reporter assays (Parelho et al., 2008; Wendt et al., 2008) and at the endogenous H19/IGF2 locus (Wendt et al., 2008).

Cohesin could be regulating transcription by stabilizing loops. CTCF has been shown to be required for looping by Chromosome Conformation Capture (3C) (Kurukuti et al., 2006; Splinter et al., 2006) and it may be that it performs this function by recruiting cohesin. Cohesin could stabilize loops by providing cohesion between separated sequences in a similar manner to how it provides cohesion between sister chromatids. Alternatively, cohesin could interfere directly with transcription factors or chromatin remodellers/modifying enzymes. It may function via a similar mechanism in other organisms, despite the differences in its localization.

Intriguingly, cohesin and CTCF do not co-localize in *Drosophila* (Holohan et al., 2007; Misulovin et al., 2008; Pauli et al., 2008).

1.8 Cohesin in DNA damage repair

The *S. pombe* orthologue of Scc1, Rad21, was originally identified in a genetic screen for mutants that are hypersensitive to radiation (Birkenbihl and Subramani, 1992), prior to the description of a role of cohesin in sister chromatid cohesion. Subsequently it has been found that cohesin mutants, as well as mutations affecting the loading of cohesin or cohesion establishment are hypersensitive to DNA damage and required for DNA repair (Atienza et al., 2005; Sjogren and Nasmyth, 2001; Sonoda et al., 2001; Strom et al., 2007; Strom et al., 2004; Unal et al., 2004; Unal et al., 2007). Cohesin's role in DSB repair is described below, and the pathway in *S. cerevisiae* summarised in Figure 5.

1.8.1 Cohesin is recruited to sites of DNA damage

Using site specific endonucleases, it has been possible to look at the enrichment of proteins to DSBs by Chromatin Immunoprecipitation (ChIP). Cohesin is recruited to DSBs (Strom et al., 2004; Unal et al., 2004). In *S. cerevisiae*, after induction of a DSB Mre11 is recruited and Mec1 and Tel1 phosphorylate H2AX (the predominant form of histone H2A in *S. cerevisiae*) to form γH2AX in the vicinity of the break. γH2AX is found in an ~50kb area surrounding the break (Shroff et al., 2004). This γH2AX domain is essential for the recruitment of cohesin, and defines the area in which cohesin can bind (Unal et al., 2004). Recruitment of cohesin occurs in the presence of a sister chromatid, but not in G1, when there are low levels of Scc1. The enrichment of cohesin can occur at sites other than CARs. The Scc2/4 complex is essential for this DSB induced recruitment, suggesting that *de novo* cohesin loading is required. In accordance with this, the Scc2/4 complex has been shown to be enriched at DSBs (Strom et al., 2007). The recruitment of cohesin also requires Mec1 and Tel1 (via phosphorylation of H2AX), Mre11 and Rad53 (Strom et al., 2004; Unal et al., 2004).

In vertebrate cells cohesin also localizes to sites of DNA damage, as determined by fluorescence microscopy after laser-induced damage in S and G2 in human cells (Bekker-Jensen et al., 2006; Kim et al., 2002a), and by ChIP after a site-specific DSB (Potts et al.,

2006). In human cells, the recruitment of cohesin also depends on the Smc5/6 complex, another member of the SMC family (Potts et al., 2006).

1.8.2 Making cohesin at a DSB cohesive

Strom et al. (2004) demonstrated that the G2/M loaded cohesin is functional for cohesion. Therefore, once cohesin has been loaded, it has to be made cohesive, and this can occur in the absence of replication (Strom et al., 2007; Unal et al., 2007). The residue S83 in Scc1 and the kinase Chk1 have been shown to be important for this transition, as S83A mutants in Scc1 and *chk1Δ* mutants are competent for cohesin enrichment, but this cohesin is not made cohesive. The phosphomimetic Scc1 mutant *S83D* allows cohesion generation in G2/M, without damage, in an Eco1 dependent manner, suggesting that phosphorylation of Scc1 allows Eco1 mediated cohesion generation (Heidinger-Pauli et al., 2008).

Subsequently, it has been found that mutating K84 and K210 in Scc1, two potential acetylation sites, to arginine caused a similar defect, in which cohesin can load proximal to a DSB but is unable to mediate cohesion. The acetyl-mimic mutant of Scc1, *K84QK210Q*, allowed cohesion generation in the absence of a DSB, without Eco1, and when K83 is mutated. This suggests a model in which phosphorylation of Scc1 on S83 by Chk1 allows Scc1 to be subsequently acetylated by Eco1 (Heidinger-Pauli et al., 2009). Wpl1 plays an inhibitory role in cohesion generation after a DSB as well as its anti-establishment role during mitosis (Heidinger-Pauli et al., 2009). However, Wpl1 was first identified in a screen for mutants hypersensitive to DNA damage (Game et al., 2003), suggesting that it plays a positive and negative role in cohesion generation similar to its function in cohesion in an unperturbed cell cycle. Deletion of Wpl1 bypasses the need for Eco1 mediated acetylation in G2/M (Heidinger-Pauli et al., 2009). In accordance with this, the radiation sensitivity of *eco1-1* cells is rescued by deletion of Wpl1 (Ben-Shahar et al., 2008). However, puzzlingly, it has also been found that *eco1Δwpl1Δ* mutants are sensitive to methyl methanesulfonate (MMS) and benomyl (Sutani et al., 2009).

Although there is compelling genetic evidence for the importance of the residues in Scc1 mentioned above, and enzymes have been implicated, neither phosphorylation nor acetylation of these sites in Scc1 have been detected *in vivo*.

1.8.3 Cohesion generation in G2/M is different to during S-phase

None of the residues in Scc1 implicated in cohesion generation after a DSB have any effect on S-phase cohesion. *Scc1 K84Q K210Q* (the acetyl mimic) cannot rescue the inviability of a thermosensitive *eco1* allele or *Smc3 K112R K113R*. This implies that despite using Eco1 to generate cohesion and Wpl1 to regulate cohesion, cohesion generation during G2/M acts via a different mechanism. The observations that certain *eco1* mutants that are competent for S-phase cohesion are not competent for cohesion in G2/M (Unal et al., 2007) and that Smc3 acetylation, which is crucial for the establishment of S-phase cohesion, is not required for DSB induced cohesion (Heidinger-Pauli et al., 2009) support this idea.

1.8.4 DSBs cause cohesion generation not just proximal to the DSB but genome wide.

DSBs do not just cause cohesion generation at the site of DNA damage but genome wide (Strom et al., 2007; Unal et al., 2007). It appears that the DNA damage response pathway triggers a response to the DSB that converts all chromatin bound cohesin into a cohesive state. As previously discussed, cohesin can be loaded onto chromatin throughout the cell cycle via an Scc2/4 dependent mechanism. This cohesin, if loaded after passage of the replication fork, does not normally become cohesive. However, after a DSB it does become cohesive, dependent on Mre11, Mec1, Tel1, Eco1 and Smc6 (Strom et al., 2007; Unal et al., 2007). It is unclear whether Chk1 is also required.

Overexpression of Eco1 also induces the de novo establishment of cohesion genome wide in undamaged cells in G2/M, although it is unclear whether this occurs through acetylation of Scc1 or Smc3.

1.8.5 What is the function of cohesion generation after a DSB?

Cohesin may be required to keep the damaged chromatid near its undamaged sister to allow repair by homologous recombination to occur. The kinetics of cohesin recruitment to a DSB are consistent with it having a role in recombination. Cortes-Ledesma and Aguilera found that DSBs arising from replication through a nick are repaired by cohesin-dependent sister chromatid exchange (Cortes-Ledesma and Aguilera, 2006). 5'-3' resection rates are not affected by the presence or absence of cohesin, nor is the rate of intrachromosomal gene conversion (Unal et al., 2004). Cohesin is not required for other recombinational repair events.

It is also possible that the repair process requires cohesin cleavage. Cut1/Separase, the fission yeast Esp1 homologue, has been shown to have a role in DNA damage repair by cleaving Rad21 (Nagao et al., 2004). Cohesin recruitment may be required to re-establish cohesion.

1.8.6 Why is cohesion generated genome wide?

Cohesion generation genome wide may be a side effect of cohesion generation at the DSB, or it may be a mechanism to reinforce cohesion, as suggested by Strom and Sjogren (Strom and Sjogren, 2007). During a normal cell cycle, it is likely that errors will occur during replication, leading to DNA damage. A re-activation of cohesin would allow for cohesion later in the cell cycle, when it is less likely to interfere with other cellular processes, such as replication. However, this re-activation does not appear to be essential.

1.8.7 Other modifications of cohesin in response to DNA damage

After DNA damage, other modifications of cohesin have been reported, although their place in the repair pathway has not yet been elucidated.

1.8.7.1 Phosphorylation of Smc1 in human cells

Smc1 physically interacts with Atm, the homologue of Mec1 in human cells, and is phosphorylated by it after IR on S957 and 966. After UV the same residues are phosphorylated but in an Atr (Tel1) dependent manner. Mutating S957 and S966 so they cannot be phosphorylated reduces DNA repair efficiency and cell survival both in the presence (Kim et al., 2002b; Yazdi et al., 2002) and absence of wild-type Smc1, in a similar manner to Atm inactivation. This suggests that Smc1 is a key substrate of Atm (Kitagawa et al., 2004). Expression of non-phosphorylatable Smc1 also compromises the inhibition of DNA replication normally seen after DNA damage (Kitagawa et al., 2004). In addition, phosphorylation of Smc1 is important in Atm independent pathways as well, as it is also implicated in the response to MMS (Kitagawa et al., 2004).

1.8.7.2 Phosphorylation of cohesin by Rad53

Sidorova et al., 2003 observed that Smc1, Smc3, and Scc1 contain Rad53 phosphorylation consensus sites. Scc1 is phosphorylated by Rad53 in response to DNA damage, although the exact residue modified was not determined. The Rad53 consensus sites in Scc1 are S112, S273 and S367 (Sidorova and Breeden, 2003).

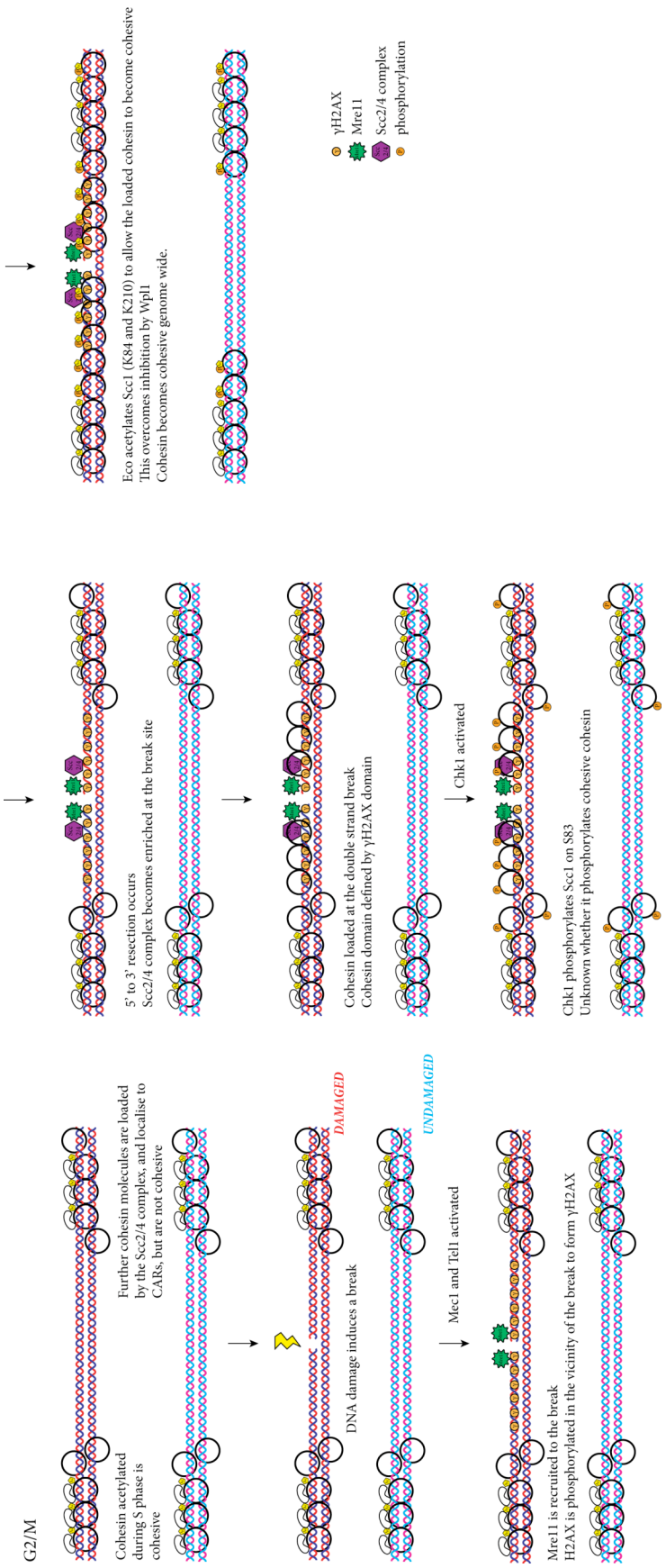


Figure 5: Cohesion generation after a double strand break in *S. cerevisiae*

1.9 Modification of the cohesin complex by SUMO

Cohesin plays roles therefore in transcription and DNA repair as well as sister chromatid cohesion. During these processes it is subject to a number of post-translational modifications, summarised in Table 3. Modification by phosphorylation and acetylation has been described during the normal cohesin cycle and in response to DNA damage. Subunits of the cohesin complex are also subject to modification by SUMO, although how this modification affects cohesin's function is unknown.

SUMO is a small protein distantly related to ubiquitin. The SUMO pathway is present in all eukaryotes, and is essential in most organisms, including *S. cerevisiae*. The exception is *S. pombe*, in which deletion of Pmt3 (SUMO) results in viable, albeit sick cells. Sumoylation has been implicated in a variety of diverse processes including transcription, DNA repair, recombination, mitosis and nucleo-cytoplasmic transport.

Modifications of *S. cerevisiae* cohesin complex

Subunit	Modification	Site	Enzyme	Function	Reference
Smc1	Sumoylation	?	Mms21	? rDNA maintenance	Takahashi et al., 2008
Smc3	Sumoylation	?	Mms21	? rDNA maintenance	Takahashi et al., 2008
	Acetylation	K112/K113	Eco1	Cohesin establishment during S-phase	Zhang et al., 2008a; Ben-Shahar et al., 2008; Unal et al., 2008; Rowland et al., 2009
Scc1	Phosphorylation	S175, S183, S194, S263, S273, S276, S325, S374, S389, S497	Polo	Enhanced cleavage of Scc1 by Separase	Alexandru et al., 2001
	Phosphorylation	S83	Chk1	Cohesion after DNA damage	Heidinger-Pauli et al., 2008
	Acetylation	K84, K210	Eco1	Cohesion after DSB	Ivanov et al., 2002; Heidinger-Pauli et al., 2009
	Phosphorylation	?	Rad53	After DNA damage	Sidorova and Breeden, 2003
	Sumoylation	?	?	?	Denison et al., 2005; Wohlschlegel et al., 2004
Scc3	Acetylation (*in vitro* only)	?	Eco1	?	Ivanov et al., 2002
Pds5	Sumoylation	?	Nif1/Siz2	Dissolution of cohesion	Stead et al., 2003
	Acetylation (*in vitro* only)	?	Eco1	?	Ivanov et al., 2002

Mammalian modifications					
Subunit	Modification	Site	Enzyme	Function	Reference
Smc1	Phosphorylation	957 + 966 (human)	Atm/Atr	DNA repair and checkpoint activation	Kim et al., 2002b; Yazdi et al., 2002; Kitagawa et al. 2004
Smc3	Phosphorylation	1076 + 1083 (human)	Atm CK2?	?	Hauf et al., 2005; Luo et al., 2008
Smc3	Acetylation	K105/K106 Human	ESCO1 (Eco1)	Cohesin establishment during S-phase	Zhang et al., 2008
Scc1	Phosphorylation	?	PLK1	Enhanced cleavage?	Hauf et al. 2005
Scc1	Sumoylation	?	Mms21?	?	Potts et al., 2006
SA2	Phosphorylation	?	PLK1	Prophase removal	Hauf et al. 2005; Gimenez-Abian et al. 2004
SA2	Sumoylation	?	Mms21?	?	Potts et al., 2006

Table 3: Modifications of the cohesin complex

1.9.1 The sumoylation cycle

Like ubiquitin, SUMO is covalently attached to lysines on target proteins. The sumoylation process is similar to ubiquitination and is depicted in Figure 6, and homologues of the different components of the pathway in mammals and yeast in Table 4. SUMO is produced as a precursor protein, which is C-terminally processed by a sumo-isopeptidase, Ulp1, to reveal a diglycine motif. It is then attached using the combined activities of an E1 activator (Aos1/Uba2), an E2 conjugator (Ubc9) and an E3 ligase. Three mitotic E3-SUMO ligases have been identified in *S. cerevisiae*: Siz1, Siz2 and Mms21, and one meiotic E3-ligase, Zip3. Targets can be mono-sumoylated, or SUMO

can form chains, catalysed again by the activities of E1, E2 and E3. Sumoylation is a reversible process, and can be removed from targets by the activities of the isopeptidases Ulp1 and Ulp2. Ulp2 also seems to be solely responsible for the disassembly of polysumo chains.

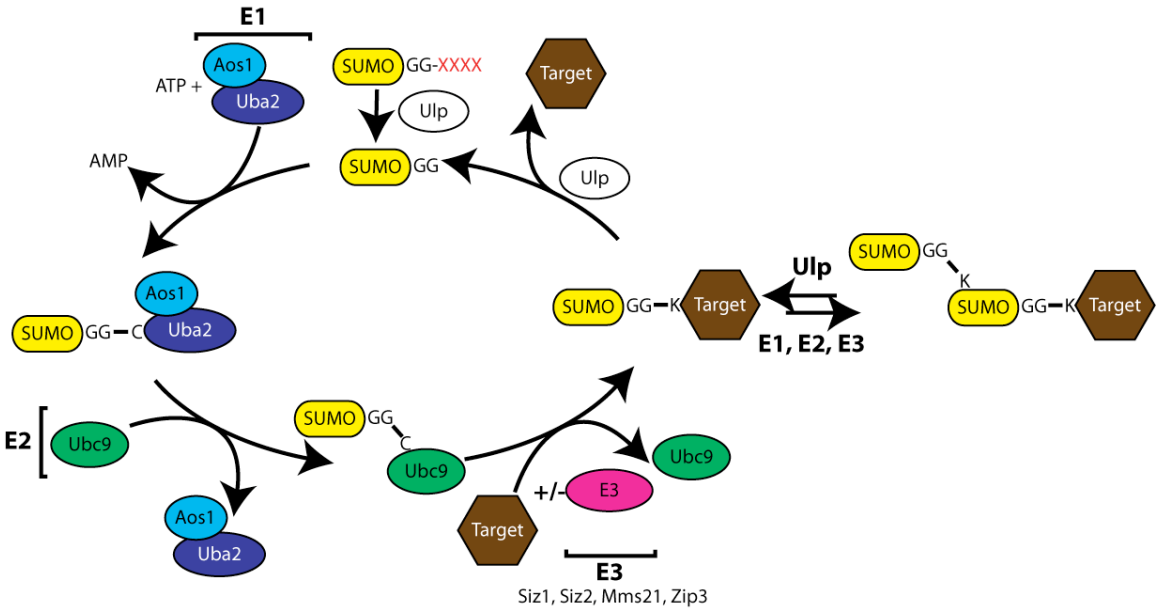


Figure 6: The SUMO cycle in *S. cerevisiae*

Sumoylation, unlike ubiquitination, is not absolutely E3-dependent, as Ubc9 (E2) has been shown to mediate site specific sumoylation of many physiological targets *in vitro*. However, it appears that *in vivo* the vast majority of sumoylation is E3-dependent, at least in yeast, where deletion of two E3 ligases, Siz1 and Siz2, results in a loss of over ninety percent of all SUMO conjugates. Interestingly, the other SUMO ligase present in yeast, Mms21, is part of the Smc5/6 complex, another SMC family member.

	Organism		
Component	Mammalian	*S. cerevisiae*	*S. pombe*
SUMO	SUMO-1 SUMO-2 SUMO-3 SUMO-4 (although this isoform does not seem to be conjugated *in vivo*)	Smt3	Pmt3
Activating enzyme (E1)	Aos1(SAE1) + Uba2(SAE2)	Aos1 + Uba2	Rad31 + Fub2(Uba2)
Conjugating enzyme (E2)	Ubc9	Ubc9	Ubc9
SUMO ligases (E3)			
SP-RING	PIAS1 PIAS3 PIASxα (ARIP3) PIASxβ (Miz1) PIASy	Siz1 Siz2 (Nfi1)	Hus5 Pli1
	Mms21	Mms21 Zip3	Nse2
	Zimp7 ? Zimp 10?		
other ligases	RanBP2 Pc2 HDAC4 RSUME?	*None known*	*None known*
Proteases	SENP1-3, SENP5-7	Ulp1 Ulp2 (Smt4)	Ulp1 Ulp2

Table 4: SUMO components in mammals and yeasts

1.9.2 The role of sumoylation

Several proteomic studies have been performed trying to identify the SUMO-modified proteome in *S. cerevisiae* (Denison et al., 2005; Hannich et al., 2005; Panse et al., 2004; Wohlschlegel et al., 2004; Zhou et al., 2004). These have identified more and more proteins as sumoylation targets. Sumoylation may control many cellular processes including: transcription; genome stability, DNA repair, recombination and the cell cycle; nucleo-cytoplasmic transport. The effect of modification by sumoylation seems to be

target specific. At a molecular level it acts by affecting the interaction of a target protein with other factors, as shown in Figure 7 (modified from Meulmeester and Melchior, 2008).

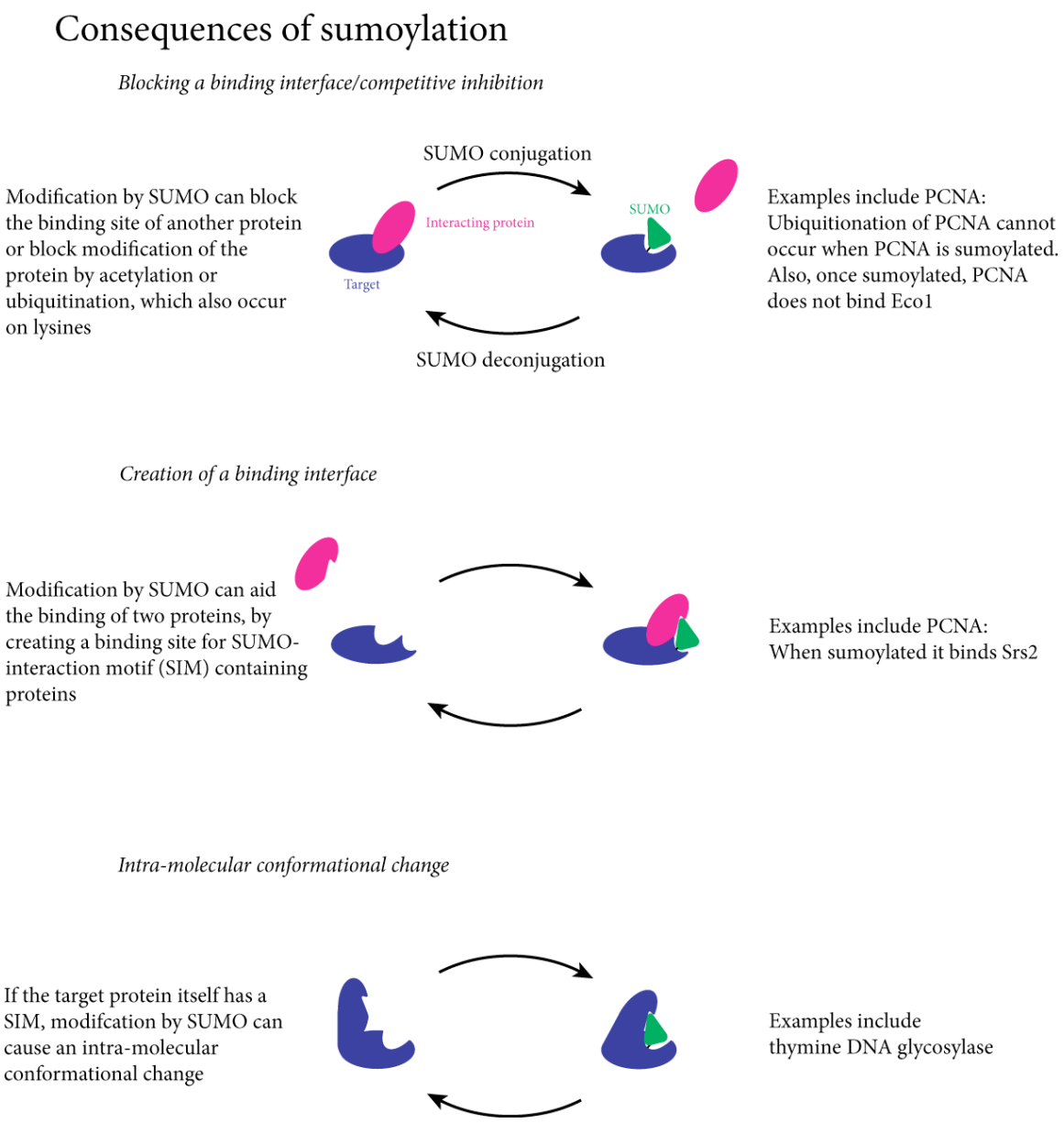


Figure 7: Consequences of modification by SUMO

Modified from Meulmeester and Melchior, 2008.

1.9.3 Cohesin is modified by SUMO in S. cerevisiae

Cohesin subunits have been identified in the screens for sumoylation targets in *S. cerevisiae* mentioned above (Denison et al., 2005; Hannich et al., 2005; Panse et al., 2004; Wohlschlegel et al., 2004; Zhou et al., 2004). The subunits identified are shown in Table 5.

			large scale study		
Cohesin subunit	Denison et al. 2005	Wohlschlegel et al. 2004	Panse et al. 2004	Zhou et al. 2004	Hannich et al. 2005
Smc1	5,5	3,4	*not identified*	*not identified*	*not identified*
Smc3	3,5	*not identified*	*not identified*	*not identified*	*not identified*
Scc1	1,1	9,14	*not identified*	*not identified*	*not identified*
Scc3	*not identified*	*not identified*	*not identified*	*not identified*	*not identified*
Pds5	*not identified*	*not identified*	*not identified*	*not identified*	*not identified*

Table 5: Cohesin subunits are sumoylated in *S. cerevisiae*

A summary of the results of several large scale proteomic studies in *S. cerevisiae* is shown. When a protein was identified, the number of unique peptides (first figure) and number of times the protein was identified (second figure) is shown.

Takahashi et al., (2008) verified that Smc1 and Smc3 are SUMO targets. Smc1 and Smc3 are polysumoylated or sumoylated at multiple sites dependent on the E3-ligase Mms21 in cells arrested in G2/M by nocodazole. Also, human Mms21 can stimulate the sumoylation of human Scc1 and SA2 (Scc3) but not Smc1 or Smc3 in HeLa cells (Potts et al., 2006). Pds5 has also been shown to be sumoylated in *S. cerevisiae* (Stead et al., 2003), although not detected by the proteomic studies. Interestingly, CTCF which, as already mentioned has a role in transcriptional regulation with cohesin, is also modified by SUMO in mammalian cells (MacPherson et al., 2009).

Takahashi et al. (2008) suggest that sumoylation of Smc1 and Smc3 plays a role in rDNA maintenance, and it has previously been shown that sumoylation of Pds5 promotes the dissolution of cohesion (Stead et al., 2003). However, it remains to be verified that Scc1 and Scc3 are sumoylation targets in yeast, and whether the sumoylation of the whole complex has other roles in cohesin function.

1.10 Objectives

- To verify that the cohesin complex is sumoylated in *S. cerevisiae*
- To investigate the function and importance of cohesin modification by SUMO

2 Results: Cohesin is sumoylated

2.1 Objectives

- To verify and characterize the sumoylation of cohesin

2.2 Approach

To verify that the cohesin subunits were *in vivo* targets of sumoylation I used an approach summarized in Figure 8. Strains were constructed in which Smt3 (*S. cerevisiae* homolog of SUMO) was tagged at its N-terminus with a His_6 and FLAG tag (HFSmt3), and cohesin subunit genes were tagged C-terminally with either HA or myc epitope tags. Cells were lysed under denaturing conditions and Smt3 conjugates purified using nickel agarose. Utilizing denaturing conditions during purification inhibits the activity of SUMO proteases, thereby preserving SUMO conjugates, and ensures that only proteins covalently attached to SUMO are purified. After SDS-PAGE and western blotting, it was examined whether HA or myc reactive species were present. This approach has been widely used to identify SUMO conjugates in yeast and mammalian cells.

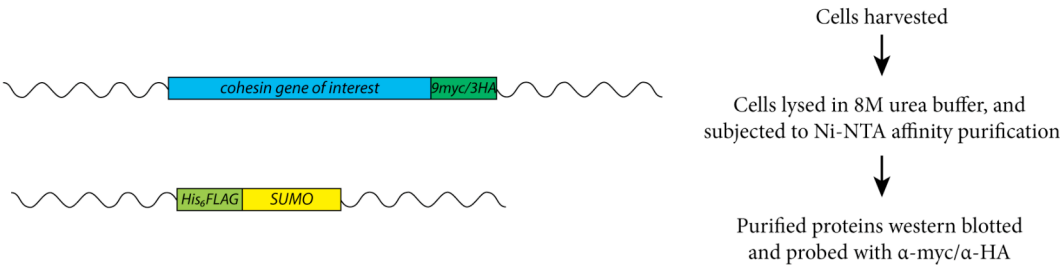


Figure 8: Approach

A subunit of the cohesin complex was tagged with myc or HA. SUMO was tagged N-terminally with His_6FLAG. After denaturing pull down of SUMO conjugates, the presence of the tagged subunit was determined.

Although Smt3 tagged strains have been used in previous studies, to ensure that the tagging of Smt3 did not affect any results obtained, I assayed the sensitivity of the

tagged strains to a variety of genotoxins, as I planned to investigate whether cohesin becomes sumoylated during its role in DNA repair. This has not been investigated and this strain is normally used in unchallenged growth conditions. I also assayed a strain which lacks the FLAG tag (HSmt3) as it has been previously reported that the FLAG tag affects conjugation to certain substrates. As can be seen in Figure 9, both the HFSmt3 and HSmt3 show no defect in growth under unchallenged conditions, nor any temperature sensitivity. They also show no difference to their untagged controls in sensitivity to the alkylating agent MMS, hydroxyurea (HU) or ultraviolet radiation (UV). However, they are more sensitive to bleomycin. The addition of the FLAG made little difference, as both HFSmt3 and HSmt3 display similar increased sensitivity. I decided to conduct further studies with the HFSmt3 strain as it would allow a second FLAG purification step if necessary.

Tagging of cohesin subunits is done routinely in conjunction with studies examining its properties in cohesion, DNA damage repair and transcription.

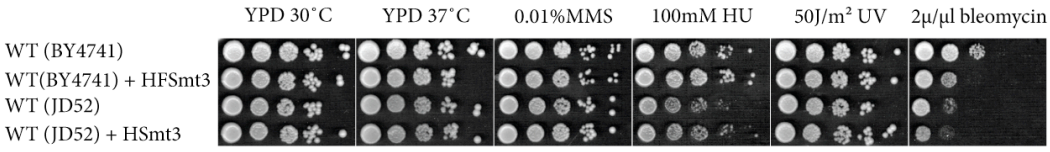


Figure 9: Tagging Smt3 N-terminally with His_6-FLAG or His_6 has no affect on viability, although it does cause sensitivity to bleomycin

10-fold dilutions, in PBS, of WT (BY background), HFSmt3 and WT (JD52) and HSmt3 were spotted onto YPD plates or plates containing the indicated genotoxins. To assay for UV sensitivity, cells were spotted onto YPD and then irradiated. Plates were incubated at 30°C, or 37° where indicated.

2.3 Cohesin is sumoylated in response to treatment with the alkylating agent MMS

I examined the cohesin sumoylation in asynchronous growing cells and after treatment with the alkylating agent MMS for 2 hours prior to harvesting (Figure 10). As has been outlined in the introduction, cohesin has a key role after DNA damage, and

sumoylation of other proteins involved in DNA repair, such as Rad52 and PCNA, is increased after treatment with 0.3% MMS (Hoege et al., 2002; Sacher et al., 2006).

All subunits of cohesin are sumoylated at a low level in asynchronous cells. Smc1 shows a single band that migrates higher than the unmodified band, Smc3 shows one very faint band, Scc1 a pair of bands and Scc3 a single band. Smc1, Smc3 and Scc1 have been identified as potential sumoylation targets by proteomic screens. Scc3 has not been identified as a sumoylation target previously. This may be because it has been excluded on the basis of its presence in the control purification. As can be seen, a large amount of unmodified Scc3 is also purified. However, the higher molecular weight bands are only enriched after purification in the HFSmt3 expressing strain.

After treatment with MMS, for all subunits the single, sometimes very faint, band is converted into a strong ladder of bands. The spacing of the bands suggest that after treatment with MMS cohesin subunits get modified with multiple Smt3 proteins- either as a ladder or mono-sumoylation of multiple sites.

Sumoylation of Scc1 is not affected by the tag attached to it, as both Scc1-3HA and Scc1-6HA are sumoylated (Figure 11). With the -3HA tag, the SUMO conjugate seen in asynchronously growing cells is well defined. Again, after treatment with MMS there is a ladder, although there are fewer bands present than with the -9myc, possibly due to reduced sensitivity due to fewer epitope tags, as with a -6HA tag more bands are seen.

Immunoprecipitation of Scc1-9myc also yields a ladder of higher molecular weight species (Figure 12).

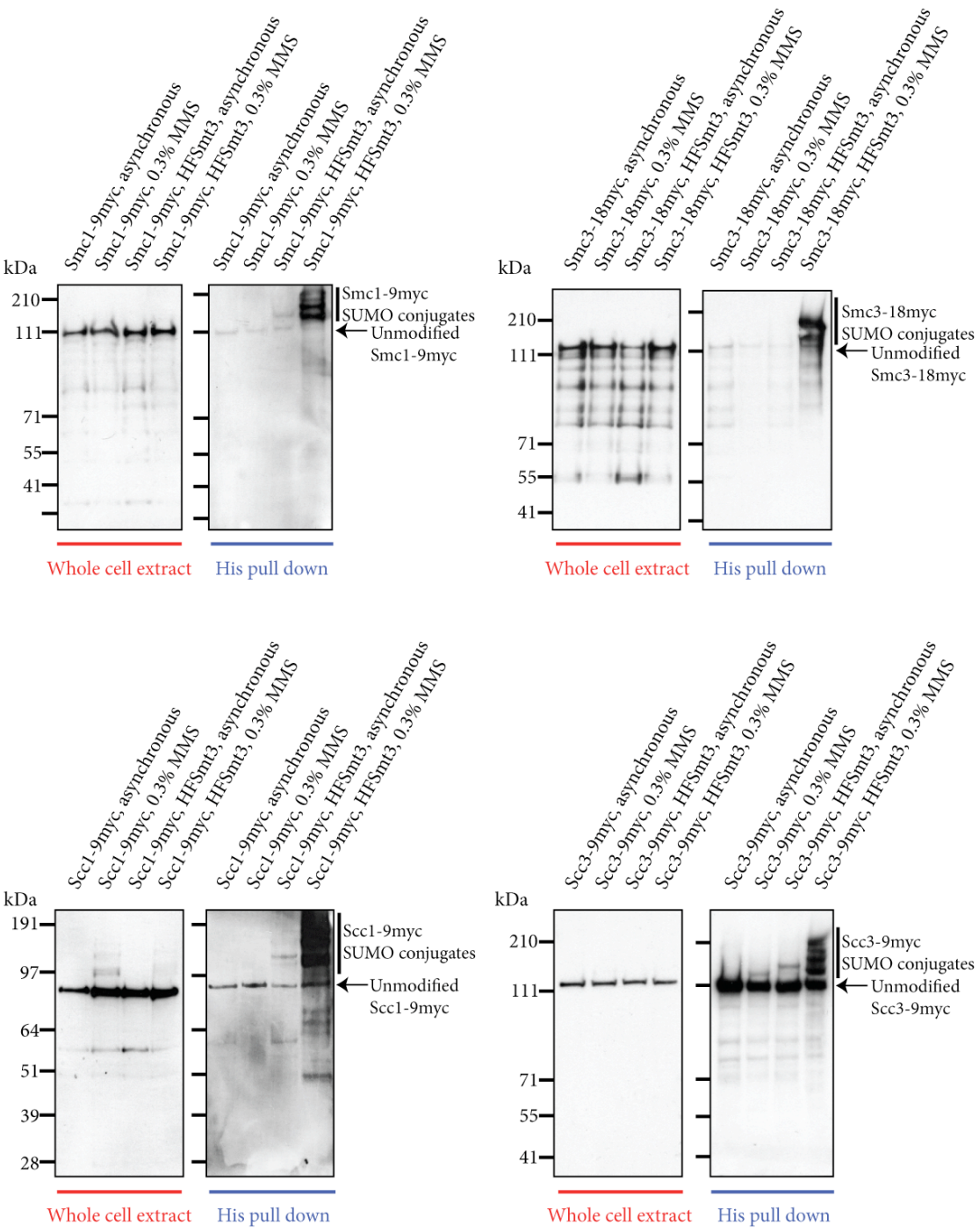


Figure 10: The cohesin complex is sumoylated and sumoylation is increased after incubation with 0.3% MMS

The indicated cohesin subunit was tagged with -9myc or -18myc at its endogenous locus in strains in which Smt3 was untagged and in strains in which Smt3 was tagged N-terminally with His_6-FLAG. Asynchronous cultures were split, half was harvested and half was incubated for 2 hours with 0.3% MMS, and then harvested. A His pull down using Ni-NTA beads was then performed to enrich for sumoylated proteins. The purified proteins and a whole cell extract sample were run on NuPAGE 4-12% Bis-Tris or 3-8% Tris-Acetate gels (Invitrogen), blotted onto PVDF membrane and then probed for myc reactive species.

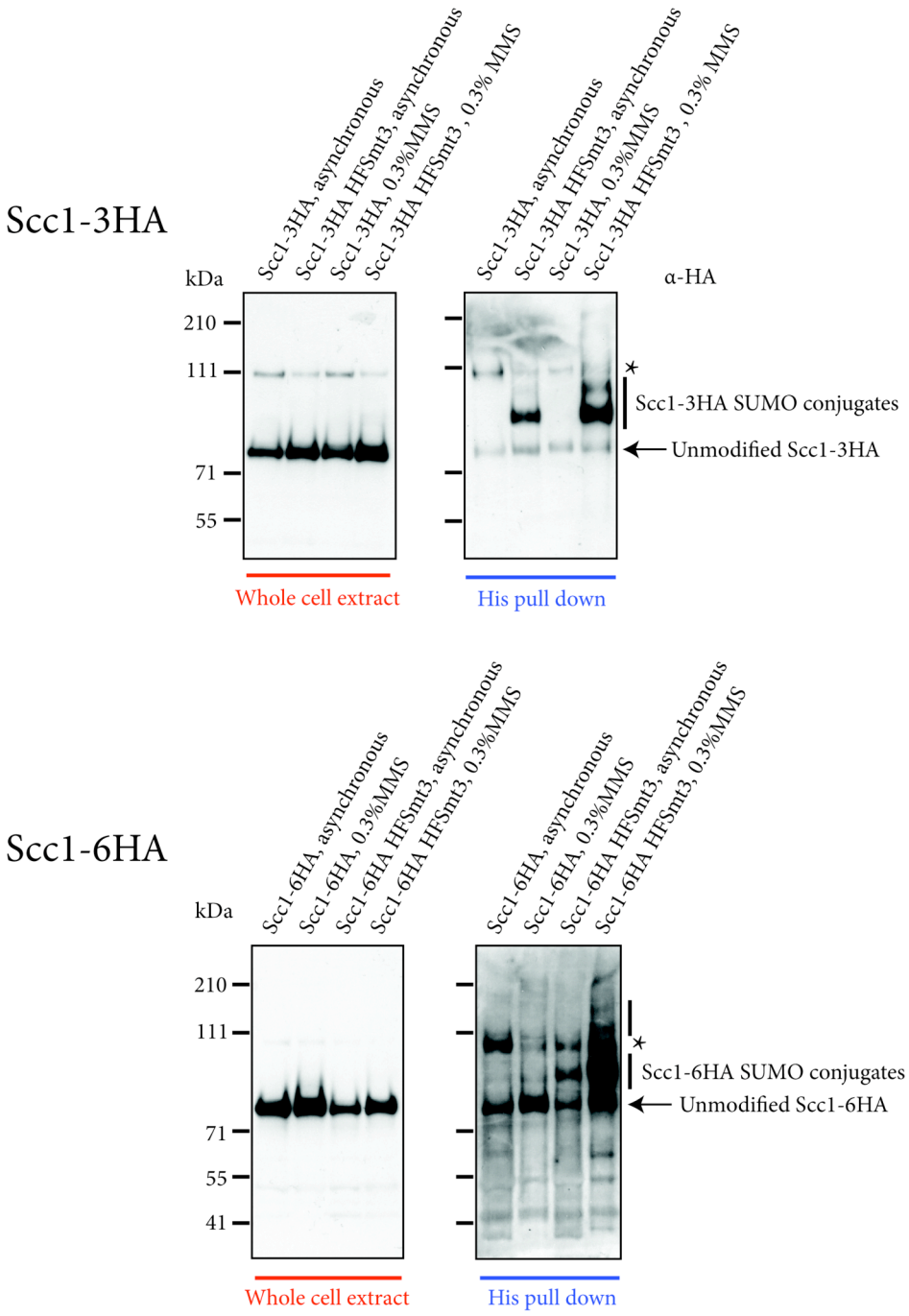


Figure 11: Scc1 tagged with HA is also sumoylated

Scc1 was tagged with -3HA or -6HA at its endogenous locus in strains in which Smt3 was untagged and in strains in which Smt3 was tagged N-terminally with His_6-FLAG. Asynchronous cultures were split, half was harvested and half was incubated for 2 hours with 0.3% MMS, and then harvested. A His pull down using Ni-NTA beads was then performed to enrich for sumoylated proteins. The purified proteins and a whole cell extract sample were run on NuPAGE 3-8% Tris-Acetate gels (Invitrogen), blotted onto PVDF membrane and then probed for HA reactive species.
A cross-reactive band is marked (*).

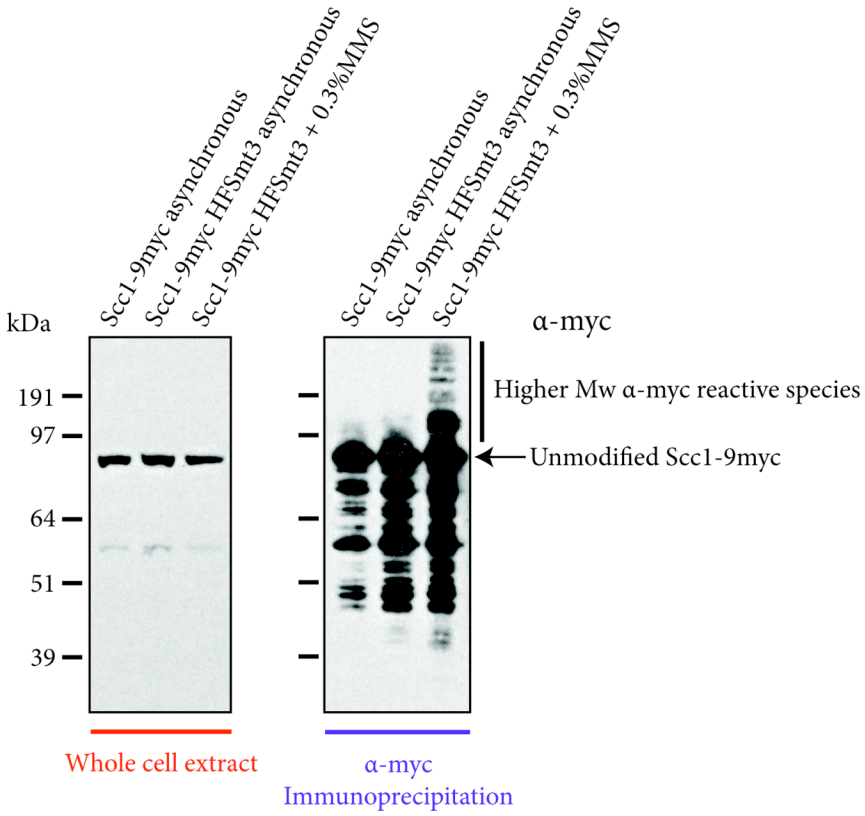


Figure 12: Higher molecular bands are present after Scc1 is immunoprecipitated after incubation with 0.3% MMS

Scc1 was tagged with -9myc at its endogenous locus. Asynchronous cultures were split, half was harvested and half was incubated for 2 hours with 0.3% MMS, and then harvested. Scc1 was immunopurified via its –myc tag. The purified proteins and a whole cell extract sample were run on NuPAGE 4-12% Bis-Tris gels (Invitrogen), blotted onto PVDF membrane and then probed for myc reactive species.

2.4 Scc1 is sumoylated in response to an unrepairable DSB

I decided to concentrate further studies on Scc1. Although all the subunits get sumoylated in response to MMS, the Scc1 response seems particularly strong. Although other subunits get modified in response to DNA damage, it is modification of Scc1 that appears to be key for cohesion generation after DNA damage, as outlined in the introduction.

To see if the SUMO ladder was MMS specific or could be stimulated by other types of DNA damage, I used a strain in which a site-specific endonuclease, under the

control of an inducible promoter (the GAL promoter), creates an unrepairable DSB. In the presence of raffinose, the promoter is in a neutral configuration and the endonuclease is not expressed. Addition of galactose, another sugar, induces the expression of the endonuclease and therefore the production of a DSB.

Scc1 was also tagged with -9myc at its endogenous locus and HFSmt3 was present on a centromeric vector, under the control of the GAL promoter. HFSmt3 would therefore be overexpressed concomitant with induction of the DSB.

To initially check that overexpression of HFSmt3 would give a similar result to that obtained with endogenously tagged Smt3 after treatment with MMS, the HFSmt3 plasmid was initially introduced into the strain that was used as a negative control for the previous assays (WT with Scc1 tagged with -9myc at its endogenous locus). Expression of HFSmt3 was induced either overnight or for 2 hours. Two hours was sufficient for robust expression of HFSmt3, and it is incorporated into Scc1 in asynchronously growing cells. In this case, the overexpression of HFSmt3 increased the abundance of Scc1 SUMO conjugates: more Scc1 appears to be sumoylated, and a weak ladder is obtained. Addition of 0.3% MMS again increased sumoylation significantly (Figure 13).

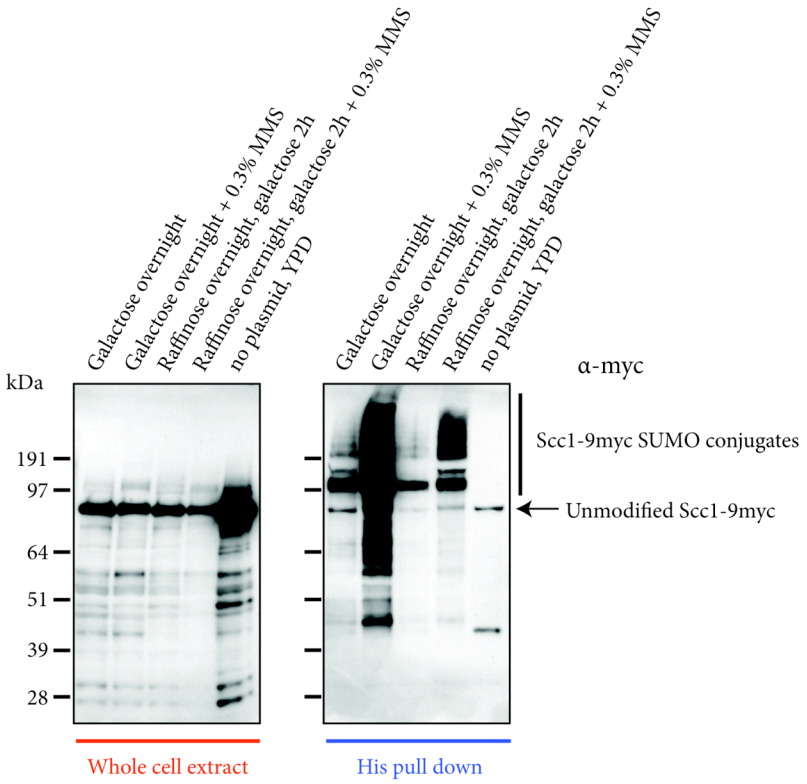


Figure 13: Overexpression of HFSmt3 increases levels of Scc1 SUMO conjugates

Scc1 was tagged with -9myc at its endogenous locus. A centromeric plasmid carrying HFSmt3 under the control of the GAL promoter was introduced into this strain. SUMO conjugates were purified by Ni-NTA affinity purification after expression of the plasmid had been induced overnight or for 2 hours, and the cells had been untreated or incubated for 2 hours with 0.3% MMS. The purified proteins and a whole cell extract sample were run on NuPAGE 4-12% Bis-Tris gels (Invitrogen), blotted onto PVDF membrane and then probed for myc reactive species.

Induction of a single unrepairable DSB and HFSmt3 induced an intense ladder of conjugates (Figure 14). No ladder was observed in the absence of HFSmt3. Therefore an unrepairable break produces a similar result to treatment with 0.3% MMS.

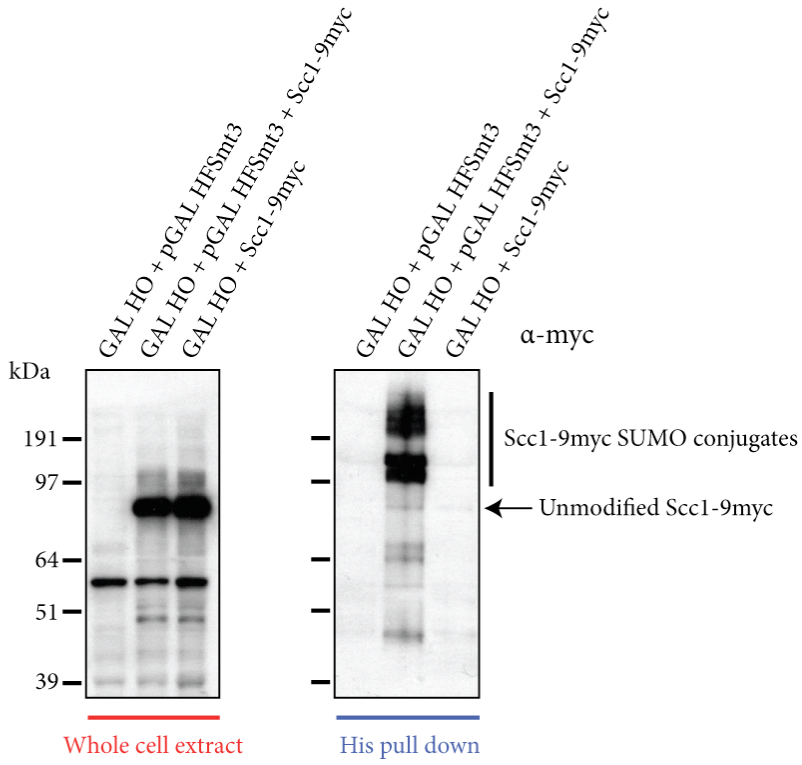


Figure 14: Induction of a single unrepairable DSB induces sumoylation of Scc1

The HO endonuclease, under the control of the GAL promoter, was used to induce a DSB at the MAT locus in a strain in which *HMR* and *HML* have been deleted. These deletions and the continual expression of the HO endonuclease cause this break to be unrepairable. In this strain, Scc1 was tagged with -9myc at its endogenous locus. Concomitant with expression of the DSB, expression of HFSmt3, present on a centromeric plasmid in the indicated strains, was also induced. After 2 hours of induction, cells were harvested, and SUMO conjugates purified by Ni-NTA chromatography. The purified proteins and a whole cell extract sample were run on NuPAGE 4-12% Bis-Tris gels (Invitrogen), blotted onto PVDF membrane and then probed for myc reactive species.

2.5 Scc1 is sumoylated in an Ubc9-dependent manner

All modification by SUMO depends on the E2 ligase Ubc9. Therefore, to confirm that the higher molecular weight bands observed were due to SUMO conjugation, an Ubc9 temperature sensitive allele, *ubc9-1,* was utilized. Strains were grown overnight at 30°C, the semipermissive temperature for *ubc9-1*, or overnight 25°C prior to a shift to 37°C for two hours. Cultures were then split, and MMS was added to half the culture for a further two hours (at the indicated temperature). As can be seen in Figure 15, sumoylation of Scc1 is abolished, even after treatment with MMS at the semi-permissive temperature (30°C). Therefore, the higher molecular weight bands seen are due to SUMO conjugation.

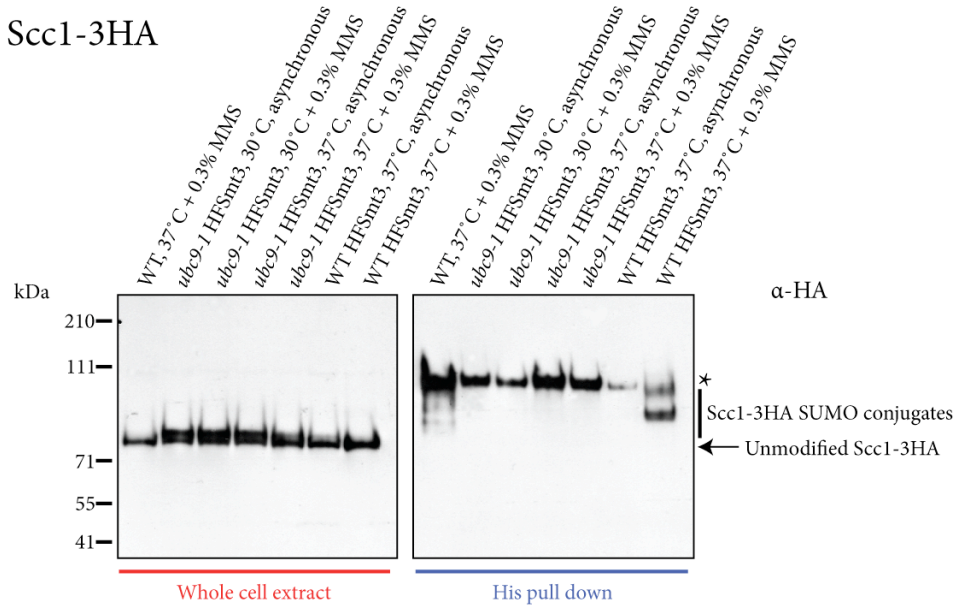


Figure 15: Sumoylation of Scc1 is dependent on the E2 ligase, Ubc9

Sumoylation of endogenously tagged Scc1 was analysed in WT and *ubc9-1* cells grown at 30°C (the semi-permissive) and 37°C, for 2h, (restrictive) temperature in cells with and without Smt3 tagged at its endogenous locus with HF and in asynchronously growing cells and after treatment with 0.3% MMS for 2 hours. Sumo conjugates were isolated by Ni-NTA chromatography, and the isolated proteins and whole cell extract samples run on NuPAGE 3-8% Tris-Acetate gels (Invitrogen) before transfer to PVDF and probing for α-HA reactive species.
A cross-reactive band is marked (*).

2.6 Determining the E3-ligase responsible for sumoylating Scc1

As mentioned in the introduction, it has been demonstrated that Mms21 is responsible for sumoylating Smc1 and Smc3 in nocodazole arrested cells (arrested at G2/M) (Takahashi et al., 2008), and that human Mms21 stimulates the sumoylation of human Scc1 in HeLa cells (Potts et al., 2006).

Mms21 is a subunit of the Smc5/6 complex, another SMC family complex. Smc5/6 has been implicated in a variety of processes including the segregation of repetitive regions, replication progression, and in DNA damage repair (De Piccoli et al., 2006; Torres-Rosell et al., 2007a; Torres-Rosell et al., 2005). There is evidence that cohesin and Smc5/6 may have overlapping or complementary functions. ChIP studies have shown that they have similar genomic localization (Lindroos et al., 2006). Mutants in

both complexes are DNA damage sensitive, and the Smc5/6 complex is required for cohesin loading at DSBs in mammalian cells (Potts et al., 2006). Mms21 may sumoylate cohesin after DNA damage and this might explain the requirement of the human Smc5/6 complex for cohesin loading at DSBs.

To determine the E3 ligase responsible for sumoylating Scc1, strains were constructed in which Smt3 was tagged N-terminally with His_6FLAG and Scc1 was tagged with 9myc, at their endogenous loci and the genes encoding the SUMO ligases were deleted, either alone or in combination. As sumoylation is greatly increased after DNA damage, the E3 ligase required after MMS treatment was determined (Figure 16).

siz1Δ, siz2Δ and *siz1Δsiz2Δ* are viable, with no obvious defects, despite a loss of ~90% of all sumoylation in *siz1Δsiz2Δ* mutants. Mms21, however, is essential. Mutations which abolish its E3 sumoylation activity are viable, albeit sensitive to DNA damage. A C-terminal deletion, *mms21CΔ,* lacks the RING domain, and acts as a catalytic dead mutant.

Sumoylation of Scc1 was maintained in *siz1Δ* and *siz2Δ* strains. The *mms21CΔ* mutation greatly reduced the abundance of SUMO conjugates (seen in several independent experiments). This is especially clear when examining low exposures (top panel), in which it appears that this mutation almost completely abolishes sumoylation. However, higher exposures (lower panel) reveal an almost full SUMO ladder. In contrast, the *siz1Δsiz2Δ* mutant has a truncated ladder. In conclusion, it seems that all three mitotic ligases have a role in sumoylating Scc1. Mms21 appears to be important for the level of sumoylation, but Siz1 and Siz2 seem to have a role in sumoylating particular sites.

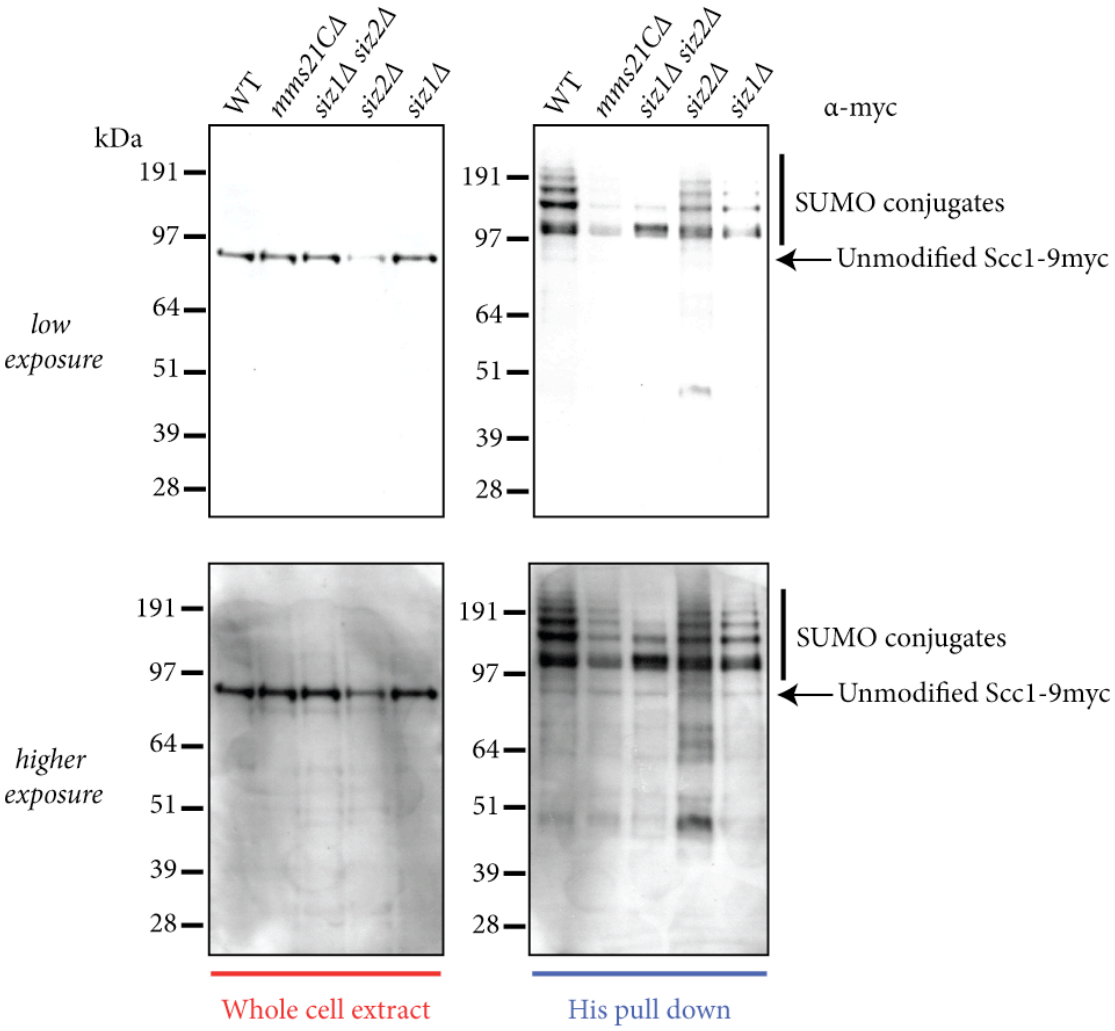


Figure 16: Determining the E3 ligase responsible for sumoylating Scc1

Sumoylation of endogenously tagged Scc1 was analysed in WT, *siz1Δ, siz2Δ, siz1Δsiz2Δ and mms21CΔ* strains in which Smt3 was tagged at its endogenous locus with HF after treatment with 0.3% MMS for 2 hours. SUMO conjugates were isolated by Ni-NTA chromatography, and the isolated proteins and whole cell extract samples run on NuPAGE 3-8% Tris-Acetate gels (Invitrogen) before transfer to PVDF and probing for α-myc reactive species.

2.7 Scc1 ladder is not due to polysumoylation

SUMO can form polysumo chains. There are three main sumoylation sites on Smt3: K11, K15 and K19 (Bylebyl et al., 2003). However, to determine whether the ladder seen is solely due to polysumoylation of a single lysine, a His_6-tagged mutated version of Smt3 in which all lysines were mutated to arginine (*K all R*) and hence definitely cannot form polysumo chains was created. Another construct was also created,

in which in addition to the *K all R* mutation the C-terminus was mutated to *RGG* (*I96R*). This construct was designed to be used for mass spectrometry analysis, as digestion by trypsin would produce a branched peptide with a GG dipeptide attached to the target lysine. This would potentially be easier to detect by mass spectrometry (discussed in the next chapter).

In *S. cerevisiae*, the ability to form SUMO chains is not essential for normal growth, but it is required for spore formation after meiotic cell division (Cheng et al., 2006). To confirm that the *K all R* and *K all R RGG* constructs allow normal growth, they were introduced, on centromeric plasmids, into *Smt3Δ* strains with or without Scc1 tagged. Smt3 *K all R* complemented for viability, and growth on MMS or HU. The Smt3 *K all R RGG* also complemented for viability, but was slightly sensitive to MMS and significantly more sensitive to HU (Figure 17).

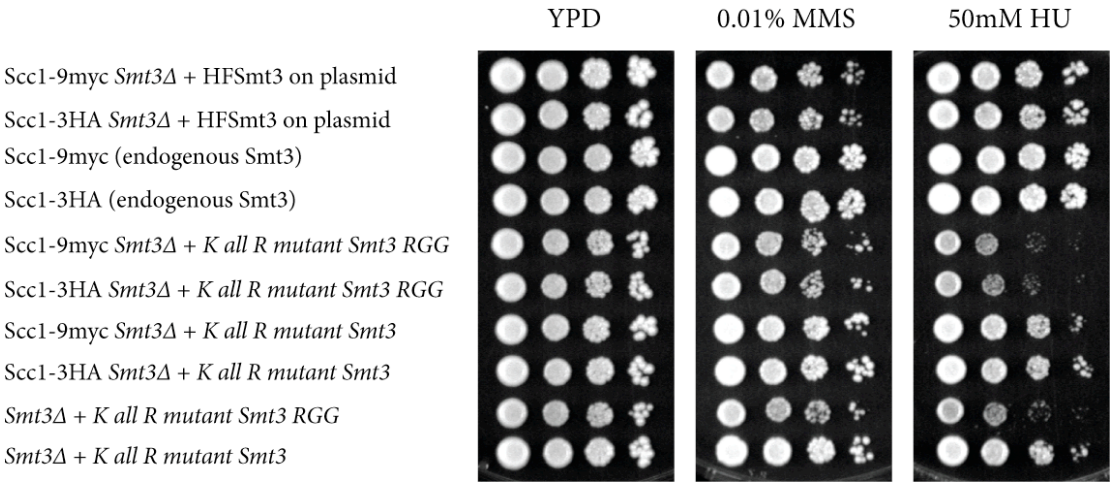


Figure 17: *Smt3 K all R* can complement for *smt3Δ*, but *I96R(RGG)* in combination with *Smt3 K all R* is sensitive to MMS and HU

10 fold dilutions, in PBS, were spotted onto YPD and YPD plates containing 0.01% MMS or 50mM HU. Plates were incubated at 30°C and then photographed.

After denaturing His purification of lysates from cells treated with MMS, a ladder of high molecular weight conjugates was observed in cells expressing both Smt3 *K all R* and Smt3 *K all R RGG*. The same result was observed when Scc1 was both -myc and –HA tagged (Figure 18). The ladder was very similar to that obtained by wild type

endogenously tagged HFSmt3, except each band was slightly lower, due to the difference in tag size (His_6 vs. His_6FLAG). Therefore the ladder obtained is not due to polysumoylation. The ladder could be due to mono-sumoylation of multiple lysines, or a combination of modifications. Scc1, as mentioned in the introduction, is subject to phosphorylation and acetylation. Acetylation of Scc1 has not been detected *in vivo*, and normally acetylation does not cause a change in mobility on SDS-PAGE gels. Phosphorylation does cause a change in mobility, so it may be that the ladder obtained is due to a combination of modification by phosphorylation and sumoylation. Alternatively Scc1 could be ubiquitinated, although ubiquitination of full length Scc1 has not been reported.

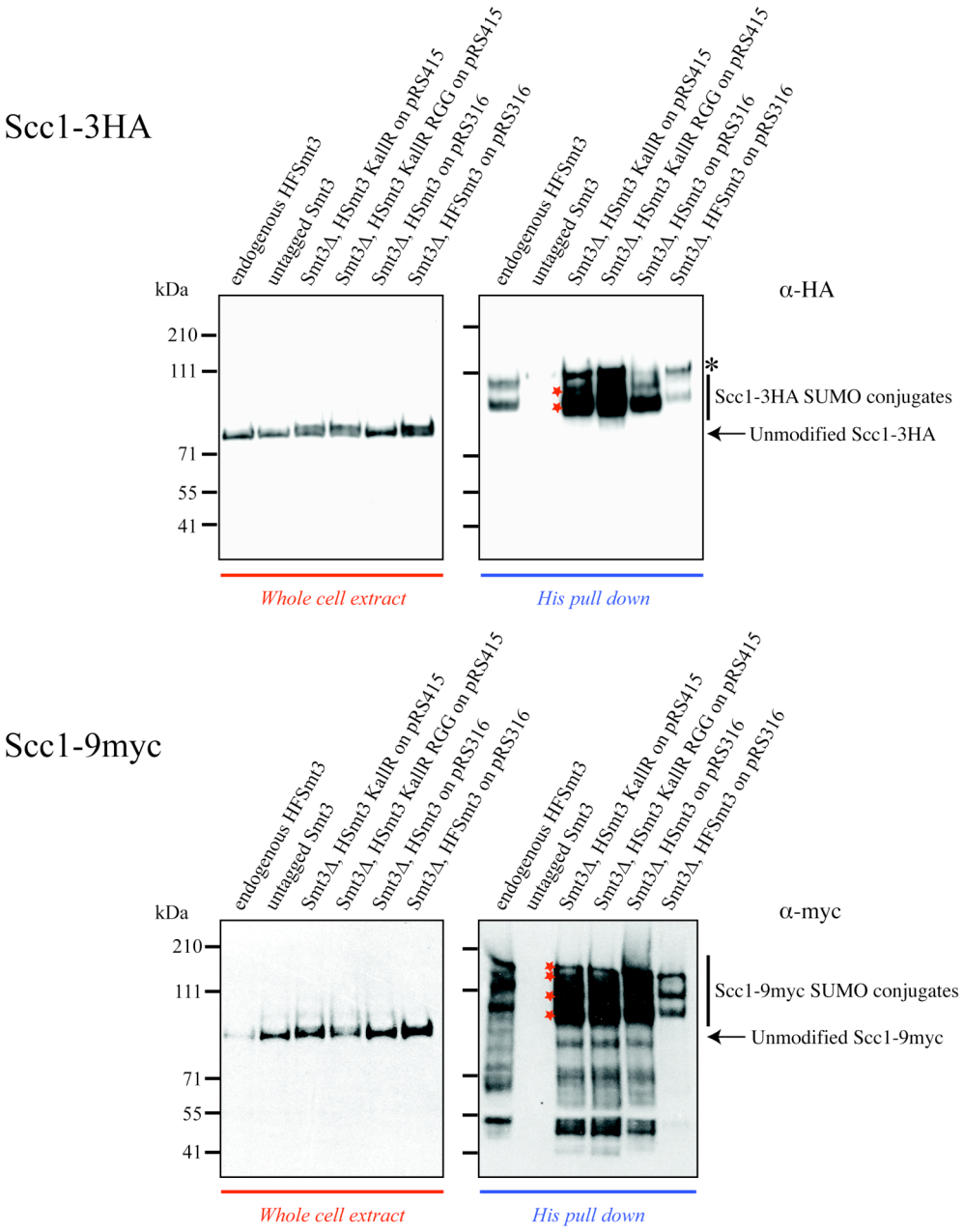


Figure 18: The Scc1 SUMO ladder after treatment with 0.3% MMS is not due to polysumoylation of a single site

Scc1 is tagged at its endogenous locus, with -3HA in the top panel and -9myc in the bottom panel. All cells were treated with 0.3% MMS before harvesting. SUMO conjugates, from strains expressing HFSmt3 encoded at its endogenous locus, HSmt3 *K all R* on a plasmid (in a *smt3Δ* strain), HSmt3 *K all R RGG* on a plasmid (in a *smt3Δ* strain), HSmt3 on a plasmid (in a *smt3Δ* strain), or HF Smt3 on a plasmid (in a *smt3Δ* strain), were purified via their His-tag. A strain expressing untagged Smt3 was used as a negative control. Conjugates were separated on NuPAGE 3-8% Tris-Acetate gels (Invitrogen), and Scc1 identified via its tag.
A cross-reactive band is marked (*). Scc1 SUMO conjugates are indicated (*).

2.8 *Scc1 sumoylation pattern varies in different cell cycle arrests*

Treatment with MMS can cause an S-phase arrest. Therefore, it is possible that the increase in sumoylation is due to an arrest in a particular cell cycle stage, rather than a response to DNA damage. It has previously been reported that Smc1 and Smc3 are sumoylated in G2/M (Takahashi et al., 2008).

To see if the sumoylation is increased in certain cell cycle stages, cells were arrested in S-phase (using HU) and G2/M (using nocodazole) and also treated with 0.3% MMS. A G1 arrest was not performed as Scc1 is not present during α-factor arrest (Guacci et al., 1997; Michaelis et al., 1997).

Higher molecular weight conjugates were obtained after His purification from both HU and nocodazole arrested cells (Figure 19). However, the pattern obtained is slightly different, although the significance of this is unclear, and may be due to differential phosphorylation. In both cell cycle arrests, the amount of higher molecular weight conjugates was significantly less than from cells treated with MMS.

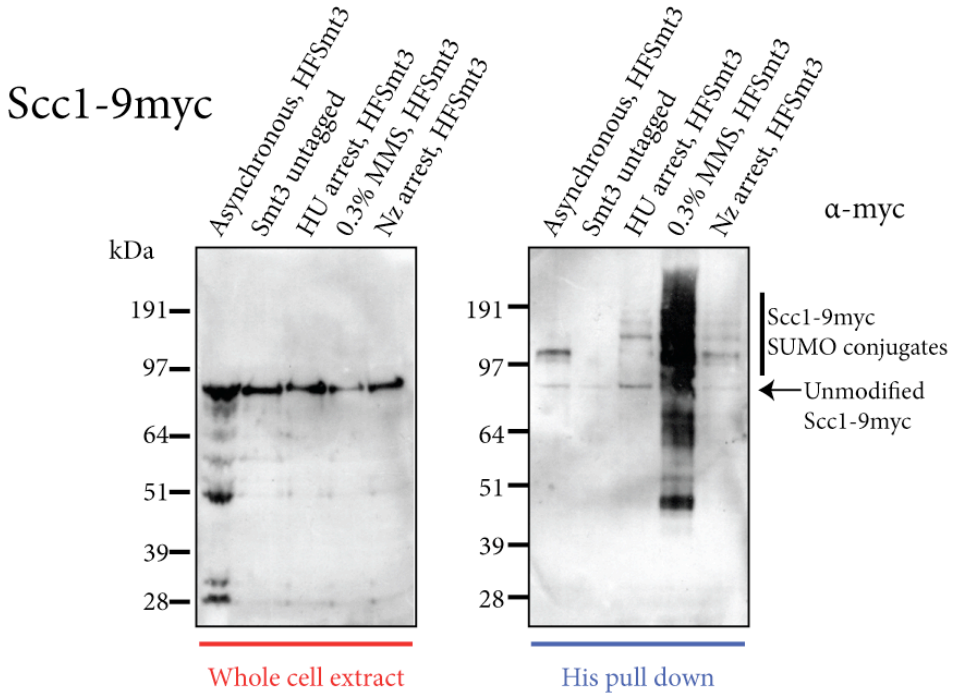


Figure 19: Scc1 sumoylation pattern in HU and Nocodazole arrest
SUMO conjugates were purified by Ni-NTA chromatography from asynchronously growing cells or cells arrested in S-phase (with 0.2M HU), in G2/M (with nocodazole (Nz)) or treated with 0.3% MMS. Scc1 was tagged at its endogenous locus, and Smt3 was tagged with HF (except in 'Smt3 untagged'). Conjugates were separated on NuPAGE 4-12% Bis-Tris gels (Invitrogen), and Scc1 identified via its tag.

2.9 Scc1 sumoylation does not depend on Chk1

Chk1 is implicated in the phosphorylation of Scc1 on S83A, which is vital for the subsequent establishment of cohesion after a DSB. It has been suggested that this phosphorylation is required for subsequent acetylation by Eco1. To see if Scc1 sumoylation also depends on phosphorylation by Chk1, SUMO conjugates were purified from wild-type and *chk1Δ* strains, with and without treatment with MMS (Figure 20). The ladder obtained from both after treatment with MMS is identical, suggesting that Chk1 is not required for Scc1 sumoylation, and that phosphorylation of Scc1 by Chk1 does not significantly affect the SUMO ladder obtained from WT cells.

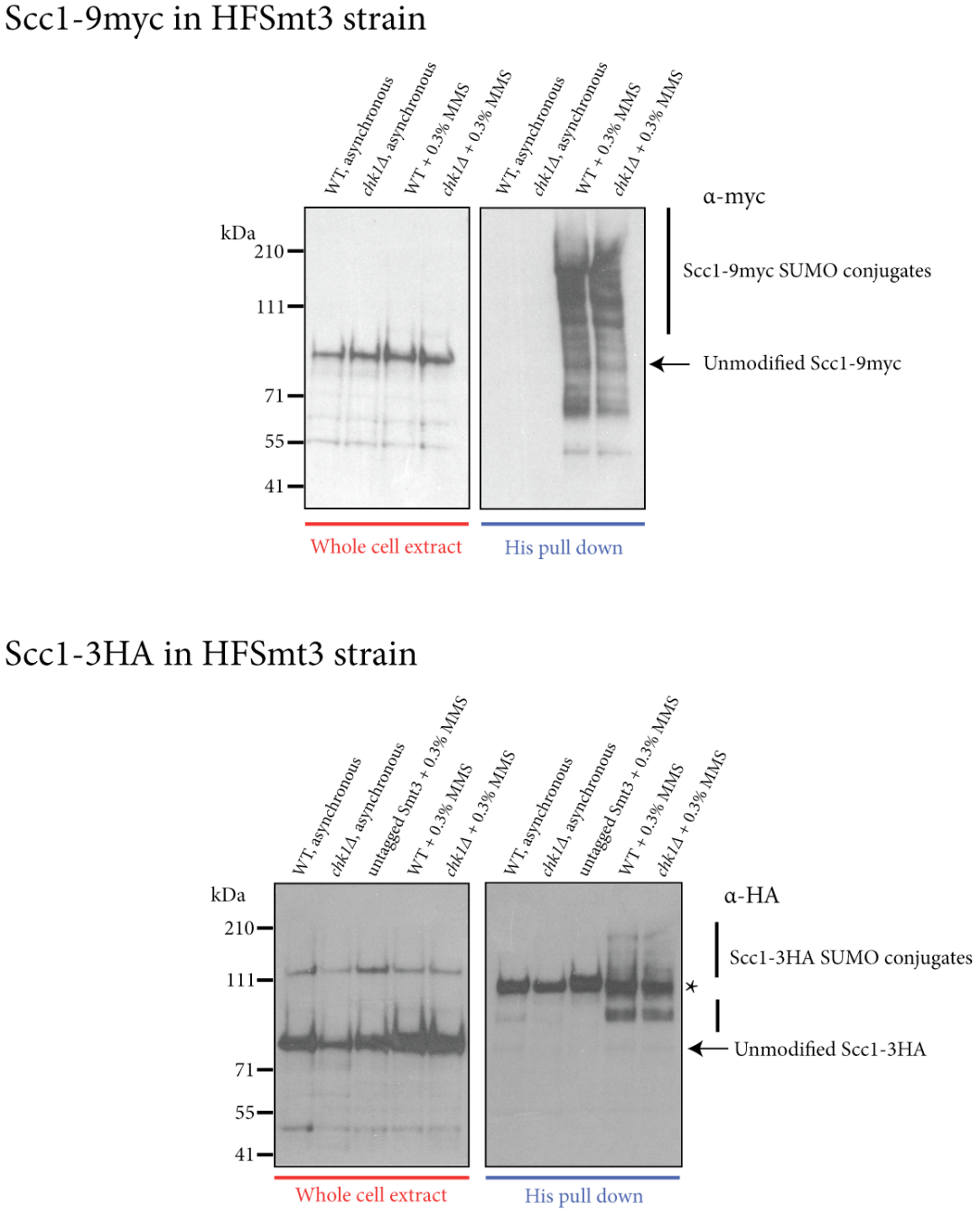


Figure 20: Sumoylation of Scc1 does not depend on Chk1

Scc1 is tagged at its endogenous locus, with -9myc in the top panel and -3HA in the bottom panel, in WT and *chk1Δ* cells. All strains, except the lane labelled 'untagged Smt3 + 0.3% MMS' had Smt3 N-terminally tagged at its endogenous locus with HF. WT and *chk1Δ* cells were grown up. Half the culture was harvested (asynchronous) and half was treated with 0.3% MMS for 2 hours, as labelled. SUMO conjugates were purified by His-pull down, separated on NuPAGE 3-8% Tris-Acetate gels (Invitrogen) and Scc1 was identified via its epitope tag.
A cross-reactive band is marked (*).

3 Results: Mapping Scc1 sumoylation sites by mass spectrometry

3.1 Objectives

- To develop approaches to allow the identification of sumoylation sites by mass spectrometry
- To identify the sites of Scc1 and other cohesin subunits sumoylation *in vivo* to allow the creation of mutants that cannot be sumoylated, to determine the function of modification of cohesin by SUMO

3.2 Approach

Unfortunately, sites of SUMO modification are difficult to predict. Sumoylation often occurs on consensus motif, ψ K X E/D (Bernier-Villamor et al., 2002; Johnson and Blobel, 1999), but this motif also occurs in many proteins that are not SUMO targets and many sumoylation events occur on lysines not in a consensus motif. Therefore, it seems logical to attempt to identify the sites of SUMO conjugation by mass spectrometry. There are two main problems in utilizing this approach: the quantity of sumoylated peptide is low compared to the unsumoylated form and the branched peptide produced after trypsinisation is difficult to identify. This section outlines the strategies I employed to try and overcome these difficulties, and the results of an initial purification.

3.3 Incorporation of SUMO with an internal HA tag

One of the major problems of utilizing mass spectrometry to map sumoylation sites is that the quantity of modified peptide in the mix is very low, and therefore it becomes very difficult to map sites. In order to enrich for SUMO conjugated peptides, an approach was devised in which Smt3 carries an internal HA tag. A two-step purification procedure would be performed, in which Scc1 would initially be purified via a –myc tag,

using myc conjugated agarose beads. Trypsinisation would release Scc1 peptides, and a second purification step would take place to enrich for sumoylated peptides, via the internal HA tag on Smt3. Figure 21 is a schematic of the proposed purification approach.

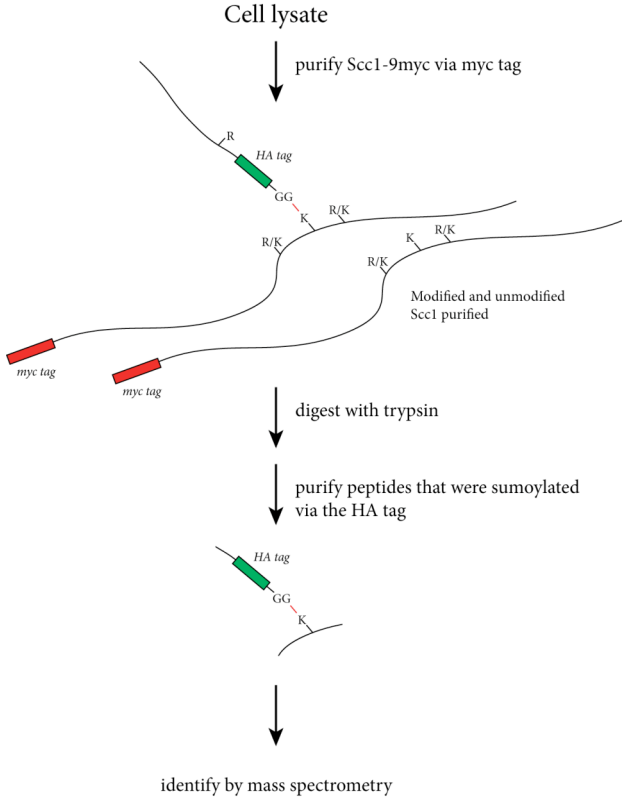


Figure 21: Purification of SUMO conjugates via an internal HA tag in Smt3

Four constructs were designed. Smt3 had either one or three copies of the HA epitope inserted at two different locations, either between R93 and the C-terminus or between R71 and the C-terminus, with a *R93Q* mutation, abolishing the trypsin site. Diagrams of the constructs are shown in Figure 22.

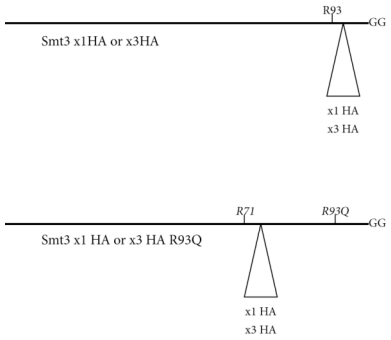


Figure 22: Schematic of SUMO constructs with an internal HA tag

To determine whether expression of these constructs can complement for *smt3Δ*, the constructs (on centromeric plasmids) were introduced into *smt3Δ* strains carrying HFSmt3 on a centromeric plasmid, to allow growth. The marker on the HFSmt3 plasmid was URA3, which can be selected against in yeast using 5-fluoroorotic acid (5-FOA). URA3 encodes oroline-5'-monophosphate (OMP) dicarboxylase, which is required for the biosynthesis of uracil. However, this enzyme converts 5-FOA to fluorodeoxyuridine, which is toxic to cells. Loss of URA3 allows growth in the presence of 5-FOA if media is supplemented with uracil. If the Smt3 constructs carrying an internal HA tag can complement for *smt3Δ* then they will allow the loss of the HFSmt3 plasmid and therefore growth on 5-FOA. As can be seen in Figure 23, although expression of the constructs had no affect when HFSmt3 was also expressed, they could not complement for *smt3Δ*.

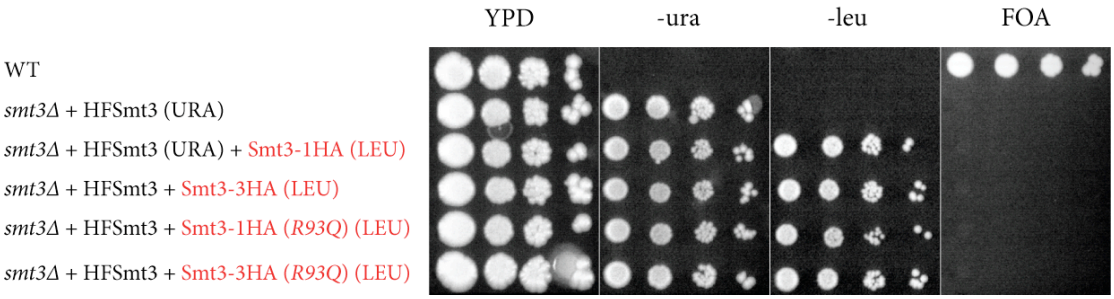


Figure 23: Smt3 constructs carrying an internal HA tag do not complement for *smt3Δ*

10-fold dilutions of WT and *smt3Δ* cells expressing HFSmt3 or HFSmt3 and one of the Smt3 constructs with an internal HA tag (labelled in red) expressed at endogenous levels were spotted onto YPD, -ura, -leu and 5-FOA plates. The marker for the HFSmt3 plasmid is URA3, and therefore strains carrying this plasmid can grow on –ura. Smt3 constructs containing an internal HA tag are on a plasmid that confers the ability to grow on –leu. Only *smt3Δ* strains that have the ability to survive without the HFSmt3 plasmid can grow on 5-FOA.

However, as the Smt3-HA constructs were viable in the presence of WT or N-terminally tagged Smt3, it was assayed whether the Smt3-HA constructs could be used to modify target proteins. Western analysis of whole cell extracts from strains expressing the constructs showed a ladder of –HA reactive species, suggesting that the constructs were indeed being incorporated (data not shown and whole cell extracts in Figures 24 and 25). However, it seemed that the different constructs were being incorporated to different extents. The constructs with the HA tag between R71 and the C-terminus and the *R93Q* mutation seemed to be incorporated to a greater extent (see whole cell extracts, Figure

24). It seems that the position of the HA tag in this construction allows for more efficient incorporation. Why this construction cannot complement for *smt3Δ,* when it seems to become incorporated, is unclear.

To see if the constructs were incorporated into Scc1, they were introduced into strains carrying Scc1 tagged with -9myc. Immunoprecipitation of Smt3 conjugates via the –HA tag after treatment with 0.3% MMS co-purified Scc1, and Smt3-1HA *R93Q* and Smt3-3HA *R93Q* had higher molecular weight myc reactive species as well, suggesting that these two constructs are incorporated into Scc1. To confirm this result, the reciprocal immunoprecipitation was performed. Scc1 was purified via its myc tag. This yielded a strong band corresponding to the unmodified Scc1, and some higher molecular weight forms more visible at higher exposures. α-HA reactive species corresponding to Smt3-1HA *R93Q* and Smt3-3HA *R93Q* conjugates co-purified with Scc1-9myc. Interestingly, these bands were not just above the molecular weight for unmodified Scc1, but corresponded to a whole range of sizes, suggesting that they correspond to either Scc1 degradation products or that immunoprecipitation of Scc1 co-purifies a range of other sumoylated proteins.

Unfortunately, although it appears that Smt3 with an internal HA tag does get incorporated into Scc1, using this approach to identify target sites relies on Smt3-HA being directly conjugated to the target protein, and not, for example, forming part of a chain. This is because the second stage of the purification occurs after trypsin digestion. If Smt3 HA is conjugated to WT Smt3, the only targets identified will be on Smt3. Although in the previous section it was demonstrated that the SUMO ladder obtained is not solely due to polysumoylation, this is no guarantee that no polysumoylation occurs. The internal HA mutant form could be incorporated specifically into SUMO chains, especially as for viability a WT copy of Smt3 (without the internal HA tag) also needs to be present.

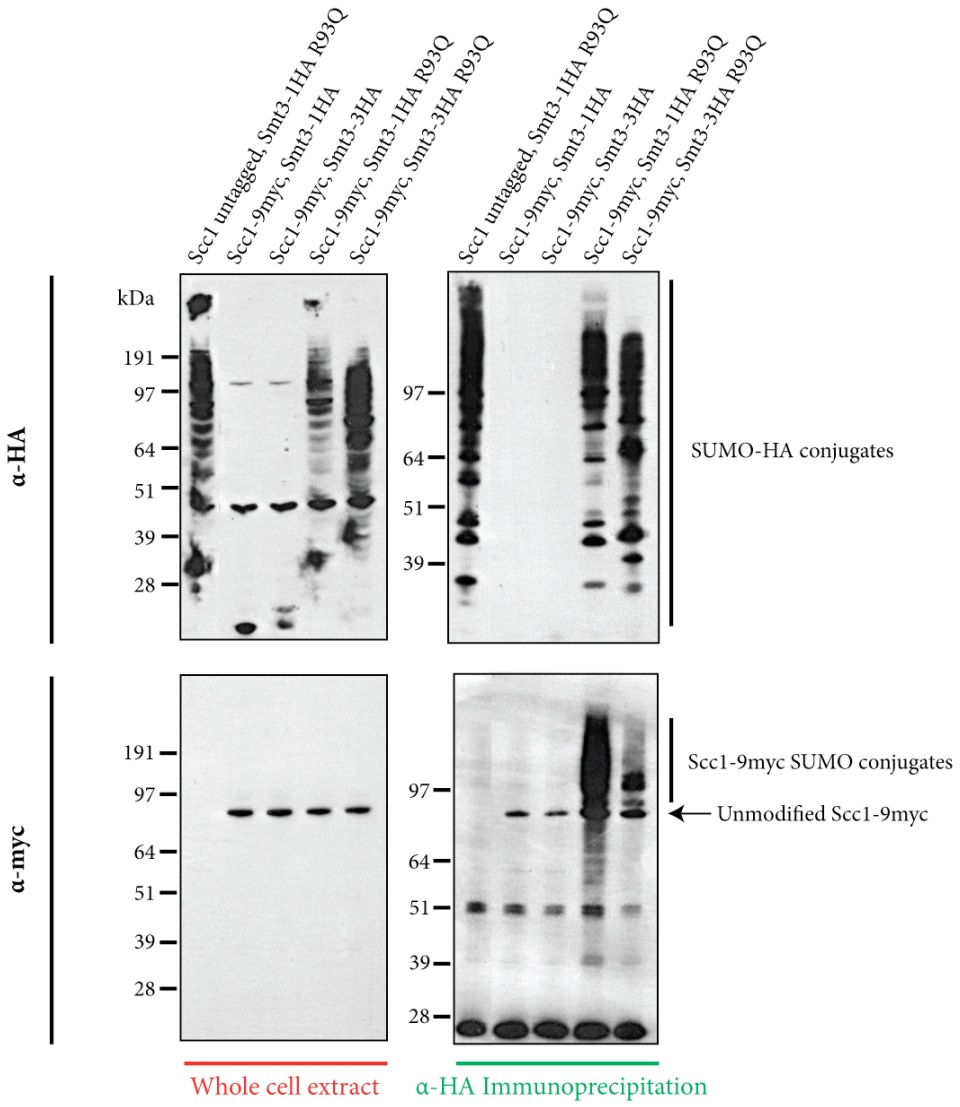


Figure 24: Smt3 constructs containing an internal HA tag and *R93Q* are used to modify Scc1 after treatment with 0.3% MMS

An anti-HA immunoprecipitation was performed to isolate proteins that were modified by the different Smt3 constructs containing an internal HA tag after treatment with 0.3% MMS. Scc1 was either untagged, as a negative control, or tagged with -9myc at its endogenous locus. α-myc and α-HA reactive species were detected after separation on NuPAGE 4-12% Bis-Tris gels (Invitrogen) and blotting onto PVDF.
NB WT, untagged Smt3 is also present in all of these strains.

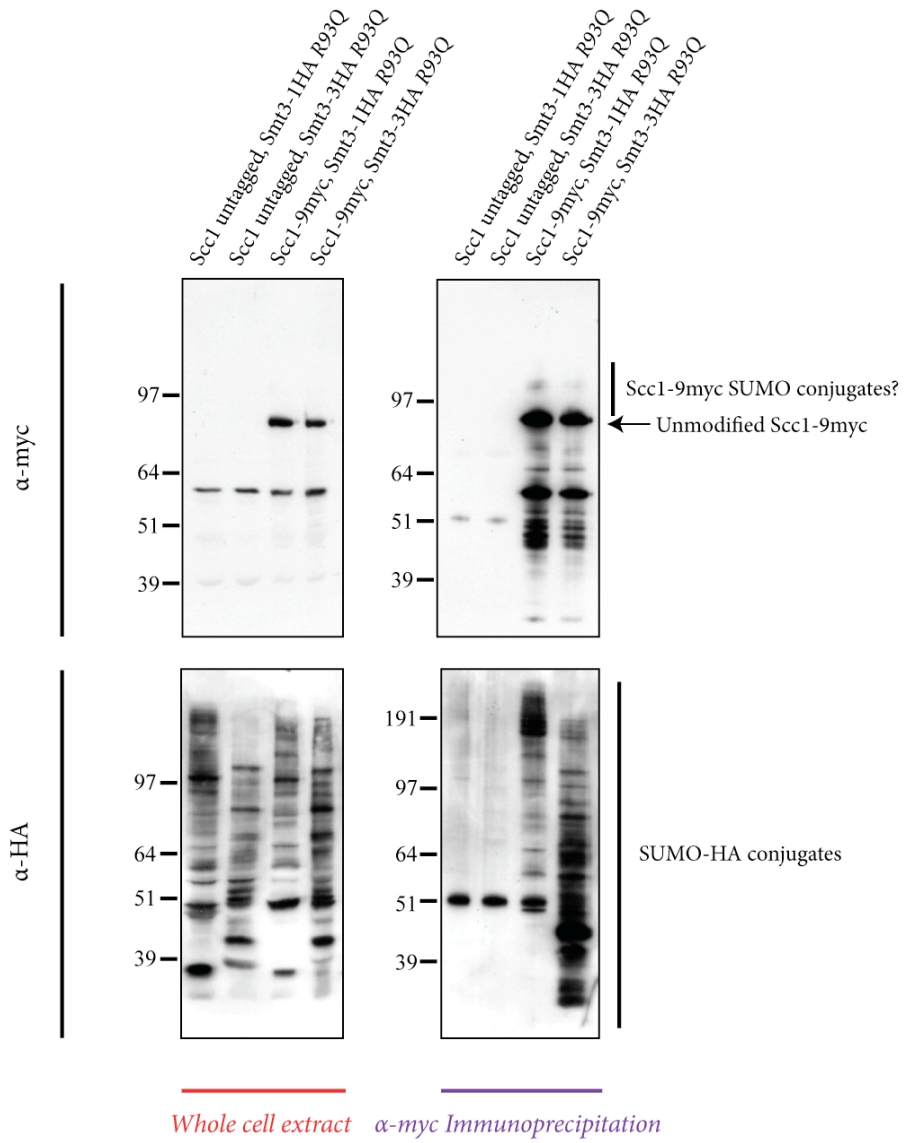


Figure 25: HA reactive species co-purify with Scc1-9myc after treatment with 0.3% MMS

An immunoprecipitation was performed to isolate Scc1-9myc after treatment with 0.3% MMS for 2 hours, from strains co-expressing Smt3 *R93Q* with 1 or 3 internal HA sequences. After immunoprecipitation α-myc and co-purifying α-HA reactive species were detected after separation on NuPAGE 4-12% Bis-Tris gels (Invitrogen) and blotting onto PVDF.
NB WT, untagged Smt3 is also present in all of these strains.

3.4 Incorporation of mutated SUMO producing a GG motif after trypsinisation

Digestion of SUMO-modified proteins by trypsin results in the five C-terminal amino acids from Smt3 (EQIGG) remaining covalently attached to the peptide containing the modified lysine residue in the substrate. As mentioned in the previous section, a version of Smt3 was constructed in which the C-terminal encoded RGG (*I96R*), and therefore an extra trypsin digestion site was inserted. Digestion with trypsin would therefore result in -GG remaining attached to the target lysine. This approach has already been utilized by Wohlschlegel et al. (2006). The EQIGG tag results in a mass shift of 484Da. Identification of Smt3 attachment sites using this mass shift is reported to be inefficient (Wohlschlegel et al., 2006), although possible (Denison et al., 2005; Zhou et al., 2004). Trypsin digestion of Smt3 *I96R* would result in a GG tag which would cause a mass shift of 114Da. This is identical to the mass shift after ubiquitination, sites of which can be readily identified by mass spectrometry. This motif was introduced into the *K all R* mutant, which cannot form polysumo chains.

As previously mentioned, this mutant can complement for *smt3Δ* and can be used to modify Scc1 (Figures 17 and 18).

3.5 Large scale purification of Scc1 after treatment with 0.3%MMS (in collaboration with Kirsty Pegram (Cell Cycle Group, CSC) and Richard Jones (NextGen Sciences Inc, Ann Arbor, USA))

A large scale purification of Scc1-3HA, via its –HA tag, from a strain co-expressing Smt3 *K all R RGG* was performed. To enrich for sumoylation, the cells were treated with 0.3% MMS before harvesting.

This purification was not done under denaturing conditions. Denaturing conditions favour the conservation of sumoylated species, and ensure that proteins identified are directly conjugated and not just interacting non-covalently. In this experiment, SUMO conjugates were preserved by the addition of NEM, and we exploited the fact that associated proteins would be purified, as this could allow the co-purification

of other cohesin subunits and therefore potentially allow the identification of the sumoylation sites of the whole complex.

Samples were taken throughout the process for western analysis (shown in Figure 26). Scc1 was present throughout, and became enriched. The purified sample was run on a SDS-PAGE gel and stained before being sent for analysis by mass spectrometry. Surprisingly, the most intense band ran at the molecular weight for Smc1 and Smc3, rather than Scc1. This result was confirmed in the mass spectrometry data: Smc1 was the most identified protein. Smc3 and Scc1 were also identified, although the other core subunit of the complex, Scc3 was not, nor were any of the cohesin associated proteins such as Pds5 or Wpl1. The number of unique peptides and the number of times subunits of the cohesin complex were identified are shown in Table 6 below.

Subunit	Number of unique peptides	Number of times identified
Smc1	130	845
Smc3	120	687
Scc1	31	488

Table 6: Cohesin subunits identified by mass spectrometry

As noted in the previous section, sumoylation and ubiquitination would produce the same mass shift after tryptic digestion. However, no –GG conjugates were detected. Acetylated Smc3 peptides were detected. Acetylation results in a molecular weight shift of +42Da. K113, previously identified as a site of acetylation by Eco1 was identified (2 peptides, 3 times). However, K112 was not. In addition, another acetylated residue in Smc3 was also identified, K280 (1 peptide, 1 time).

In conclusion, although Scc1, Smc1 and Smc3 were purified, the results of the mass spectrometry suggest that either SUMO conjugates were not maintained or were not enriched for enough.

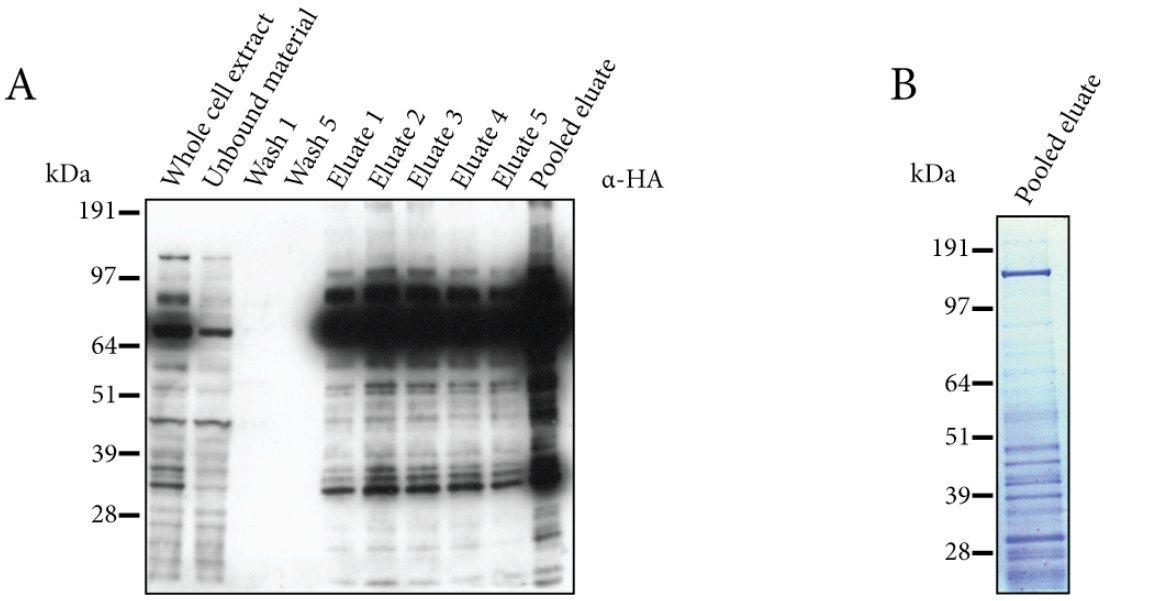


Figure 26: Purification of Scc1-3HA in a strain expressing His_6*Smt3 K all R RGG* for mass spectrometry

Cells were incubated with 0.3% MMS for 2h before harvesting. Scc1-3HA was immunopurified via its -3HA tag.
Panel A: Western analysis (α-HA) of the purification processes. Panel B: Gel analysis (stained with SimplyBlue Safestain) of the purification. The lane containing the pooled eluate was cut into 1mm strips and sent for mass spectrometry.

4 Results: Mapping Scc1 sumoylation sites by site-directed mutagenesis

4.1 Objectives

- To identify the sites of Scc1 sumoylation *in vivo* to allow the creation of mutants that cannot be sumoylated, to determine the function of modification of Scc1 by SUMO

4.2 Mutating individual lysines to arginine

Parallel to the mass spectrometry approach, identification of sites of Scc1 sumoylation was attempted by site directed mutagenesis. This approach has been successfully applied to identify sumoylation sites in a number of proteins.

A consensus sequence for sumoylation has been identified based on its interaction with Ubc9, ψ K X E/D, where ψ represents a bulky aliphatic residue, usually isoleucine, leucine or valine and X represents any amino acid (Bernier-Villamor et al., 2002; Johnson and Blobel, 1999). Sumoylation may also be promoted if this is followed by a phosphorylation site, creating a phospho-dependent SUMO motif, ψ K X E X X S P, or a series of negatively charged amino acids (Hietakangas et al., 2006; Yang et al., 2006). To be sumoylated, this sequence is often found in an unstructured region or loop, and Ubc9 does not recognize consensus sites in stable helical structures (Pichler et al., 2005). However, a number of proteins not subject to sumoylation contain this motif, and other physiological targets of sumoylation are not sumoylated on this consensus motif.

A number of sumoylation site prediction programs have been developed, based on the consensus motif and on previously identified sumoylation sites. These include SUMOsp and SUMOplot™ (Abcam). The Scc1 sequence was entered into these programmes, and the sites identified are shown in Table 7. Interestingly, each programme identified different lysines as sumoylation targets, and the new version of SUMOsp (Ren et al., 2009) predicts far fewer lysines than the original version (Xue et al., 2006). Sites

identified by SUMOplot™ or predicted with a high GPS score or matching MotifX by SUMOsp were chosen as primary sites to be mutated and tested. Scc1-9myc was cloned onto a plasmid, and potential Scc1 sites mutated (lysine to arginine, a similar amino acid which cannot be sumoylated) using a PCR based approach. These mutants were then integrated at the *URA3* locus in strains carrying HFSmt3, and in *scc1-73*, a temperature sensitive mutant.

Prediction programme	Lysine predicted	Comment
SUMOplotTM (Abcam)	K552	High probability
	K382	Low probability
	K252	Low probability
SUMOsp (Xue et al., 2006)	K345	Matches MotifX
	K521	Matches MotifX
Sites with a GPS Score of less than 1.5 are not shown	K52	GPS Score: 1.75
	K94	GPS Score: 5.67
	K99	GPS Score 4.33
	K105	GPS Score 2.00
	K165	GPS Score 9.00
	K324	GPS Score 8.00
	K345	GPS Score 3.38
	K391	GPS Score 4.33
	K460	GPS Score 4.00
	K494	GPS Score 1.69
	K500	GPS Score 5.00
	K509	GPS Score 2.28
	K521	GPS Score 2.35
	K552	GPS Score 2.15
	K556	GPS Score 4.00
SUMO sp version 2 (Ren et al., 2009)	K345	2.868 (Medium)
	K105	2.397 (Low)
	K252	2.574 (Low)
	K521	2.382 (Low)

Table 7: Lysines in Scc1 identified as potential sumoylation sites by prediction programmes

Whilst not expecting the ladder obtained after treatment with MMS to disappear after mutation of a single site, I hypothesized that removal of a major attachment site would significantly affect sumoylation, possibly by removing a band from the ladder or by reducing the levels of conjugates. However, removal of any single lysine did not

significantly affect the ladder obtained after treatment with 0.3% MMS. A representative figure is shown in Figure 27. K521 was identified by both SUMOsp (matches MotifX and a GPS score of 2.35) and SUMOsp version 2 (GPS score 2.382). K552 is a high probability site for SUMO modification according to SUMOplot™.

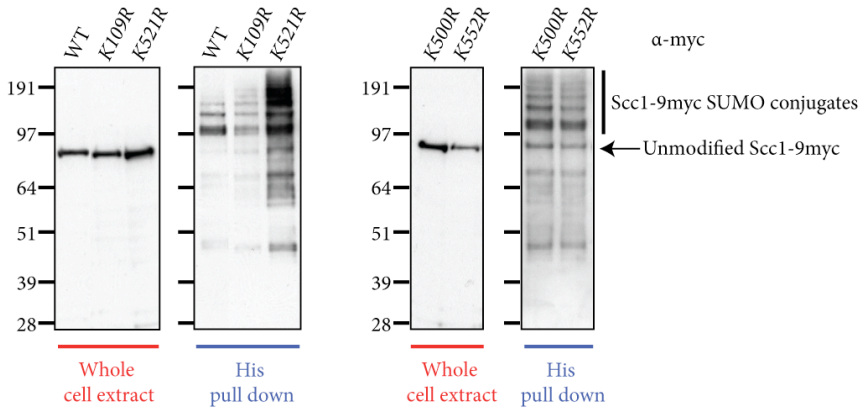


Figure 27: Mutation of single lysines in Scc1 has no effect on the SUMO ladder obtained after treatment with 0.3% MMS

Individual lysines in Scc1 were mutated to arginine, which cannot be modified by SUMO. These constructs were tagged with -9myc and integrated at the URA locus, where they were expressed under the control of Scc1's native promoter, in strains in which Smt3 was N-terminally tagged with HFSmt3. After treatment with 0.3% MMS for 2 hours, SUMO conjugates were purified by Ni-NTA chromatography, separated by SDS-PAGE on NuPAGE 4-12% Bis-Tris gels (Invitrogen), western blotted and probed with α-myc. Representative blots are shown. K521 is a high probability site for SUMO modification according to SUMOsp. K552 is a high probability site for SUMO modification according to SUMOplot™.

I went on mutating further individual lysines, plus, when lysines were consecutive, mutants in which all of them were mutated were created. Disappointingly, mutation of any of the 31 lysines in Scc1 individually to arginine, or the multiple mutants, produced no significant change in the ladder obtained (data not shown). The ability to complement for *scc1-73* temperature sensitivity and MMS sensitivity was also assayed. Again no significant changes were observed (data not shown).

4.3 Mutating all lysines to arginines

As mutating a single lysine to arginine failed to identify any sumoylation sites, I decided to take the opposite approach: to mutate all the lysines in Scc1 to arginine (*K all R*) and then identify sites that could be sumoylated by adding lysines back. To do this, the

K all R mutant, on a centromeric plasmid and under the control of the GAL promoter was synthesized by GeneCust (GeneCust Europe, Dudelange, Luxembourg). It was then determined whether the *K all R* mutant could complement for *scc1-73*.

The effect of expressing *K all R* was assayed by spotting 10-fold dilutions of cells onto media containing glucose, where *K all R* expression is repressed and galactose, where it is induced, and incubating plates at 25°C or 37°C, the non-permissive temperature for *scc1-73* (Figure 28). Expression of *Scc1 K all R* does not restore viability to *scc1-73* at 37°C. Interestingly, expression of this mutant was lethal at the permissive temperature. It was also lethal in the wild type. In these strains WT Scc1 is present, and therefore the mutated version is acting in a dominant manner.

Mutating all of the lysines to arginines will abolish all other modifications that occur on lysine residues, such as acetylation and ubiquitination. It has previously been described that after cleavage, the C-terminal region of Scc1 is subject to degradation via the N-rule pathway, and that the stabilization of this fragment is toxic (Rao et al., 2001). Therefore, it is likely that the *K all R* mutant is lethal because the C-terminal fragment cannot be ubiquitinated.

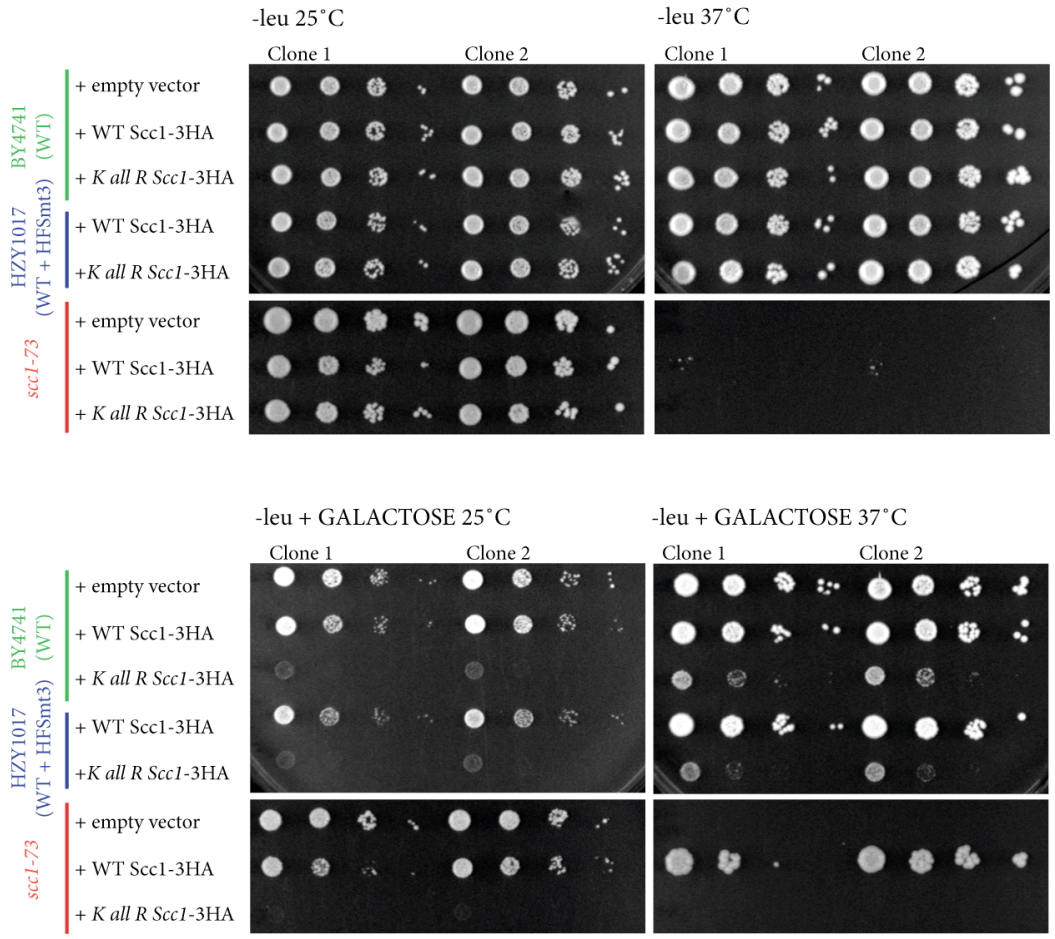


Figure 28: Expression of *Scc1 K all R* is lethal, even in WT cells

10-fold dilutions of WT, WT cells with HFSmt3 and *scc1-73* cells transformed with empty vector, or vectors carrying GAL WT Scc1-3HA or GAL *K all R Scc1-3HA* were spotted onto –leu (to maintain the plasmid) containing glucose, repressing expression of the constructs, or galactose, inducing expression. Plates were incubated at 25°C (the permissive temperature for *scc1-73*) or 37°C (the restrictive temperature).

To confirm that the *K all R* mutant cannot be sumoylated, a denaturing pull down of SUMO conjugates was performed from cells expressing either WT or *K all R* Scc1-3HA. The ladder of higher molecular weight bands normally seen is abolished in the *K all R* mutant (Figure 29).

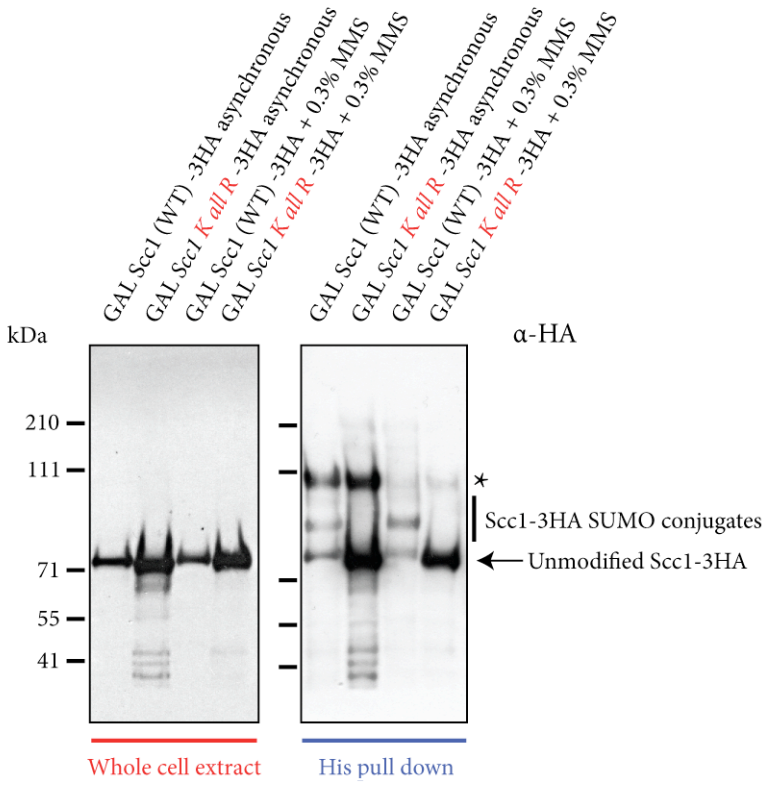


Figure 29: *Scc1 K all R* is not sumoylated

Expression of GAL WT Scc1-3HA or GAL *Scc1 K all R* -3HA (labelled in red) was induced in cells carrying HFSmt3 for 2 hours by the addition of galactose to the media, following which the culture was split and half was harvested immediately and half incubated with 0.3% MMS for a further 2 hours before harvesting. SUMO conjugates were purified via the His tag on Smt3, separated on NuPAGE 3-8% Tris-Acetate gels (Invitrogen) and western blotted. Scc1 SUMO conjugates were identified by probing with α-HA.
A cross-reactive band is marked (*).

4.4 ABC constructs

Before adding back individual lysines to the *K all R* mutant, I wanted to try to narrow down the sites in which sumoylation could occur. To do this naturally occurring restriction enzyme sites present in Scc1 were utilized to allow the formation of constructs with mutated and WT portions, as depicted in Figure 30. This approach relies on Scc1 folding correctly when portions of it are mutated, and, if sumoylation relies on association with other cohesin subunits or loading onto DNA, it being capable of this too.

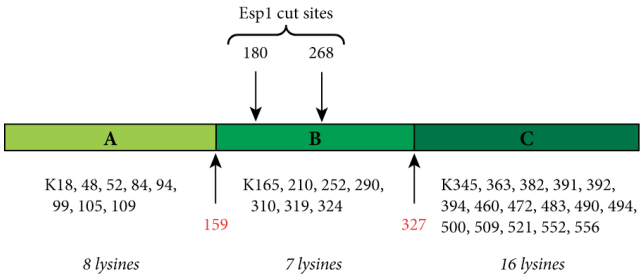


Figure 30: SCC1

Scc1 can be divided into three sections, designated A, B, and C. A and B can be divided by *EcoRI* which cleaves after base 476, separating the protein after amino acid 159. B and C can be separated by *ClaI*, which cleaves after 981, separating the protein after amino acid 327. The number of lysines in each section, and the position of the Esp1 cleavage sites is also shown.

In the subsequent descriptions, WT portions of the protein will be represented by a capital letter in black, whilst mutant portions of the protein in which lysines have been mutated to arginine will be represented by lower case italic letters in red.

Again, the effect of expression of the constructs, and the ability of these constructs to complement for *scc1-73* was determined by growth on galactose (Figure 31). Expression of all constructs, except A*bc* had no effect on the WT strain and complemented for *scc1-73* at 37°C. A*bc* is lacking a lysine residue C-terminal to the Esp1 cleavage site, and therefore stabilization of the C-terminal region may explain its toxicity, as above for the *K all R* mutant.

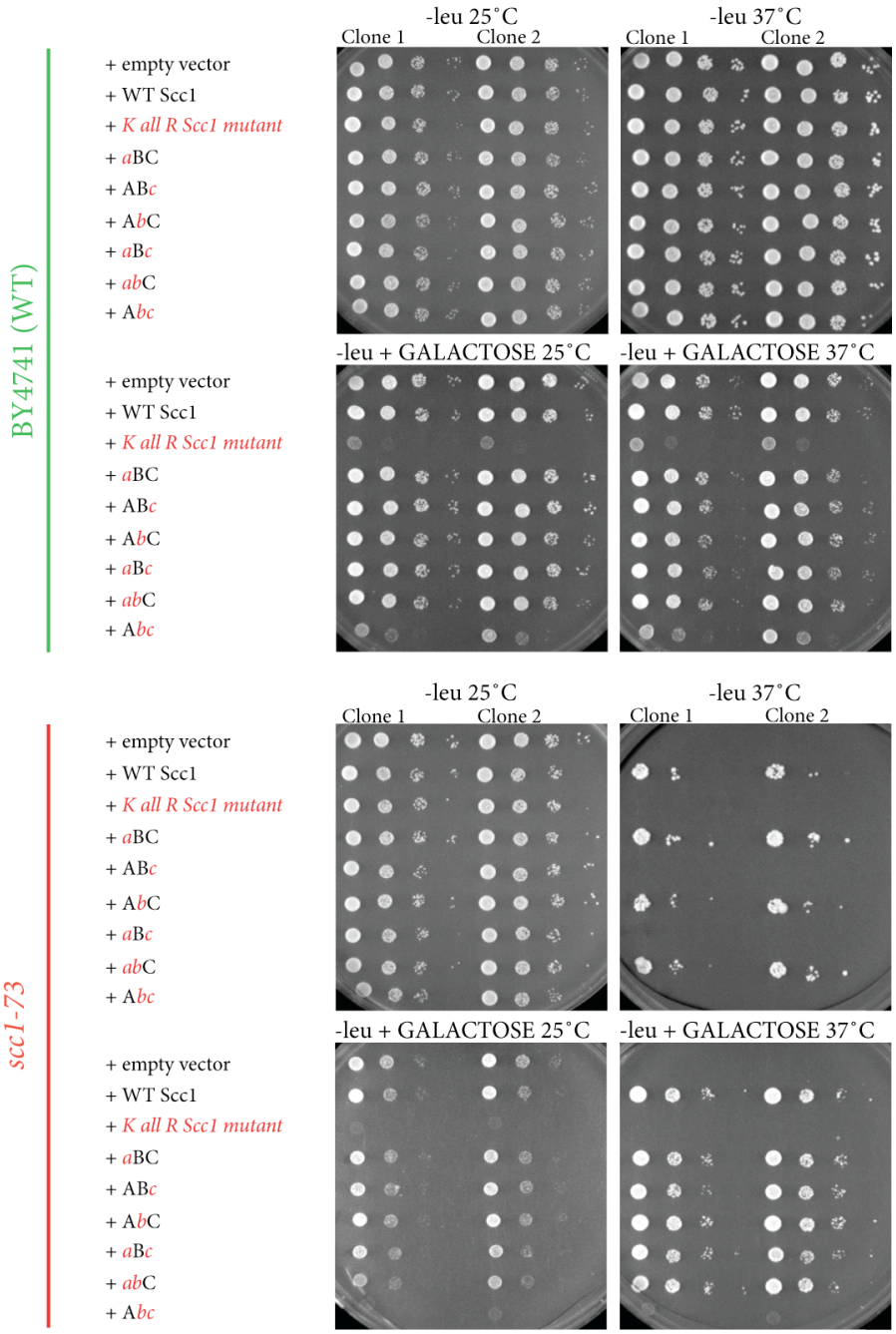


Figure 31: Analyzing the effect of expression of the ABC constructs on the viability of WT and *scc1-73* cells

10-fold dilutions of WT and *scc1-73* cells transformed with empty vector, or vectors carrying GAL WT Scc1-3HA, *K all R Scc1* and ABC mutants were spotted onto –leu (to maintain the plasmid) containing glucose, repressing expression of the constructs, or galactose, inducing expression. Plates were incubated at 25°C (the permissive temperature for *scc1-73*) or 37°C (the restrictive temperature).

The ability of all the constructs to be sumoylated after treatment with 0.3% MMS was then assayed (Figure 32). *a*BC is robustly sumoylated, and the pattern and intensity of the bands looks identical to the WT, suggesting that the bulk of sumoylation occurs in these two regions. This is corroborated by the finding that A*bc* is not sumoylated. AB*c* and A*b*C also show sumo bands, but interestingly *a*B*c* and *ab*C, with only one WT region seem to be sumoylated to a greater extent than when WT region 'A' is present. Also, although not quantified, it seems that the ratio of modified to unmodified forms is higher when region 'C' is WT.

These findings suggest that sumoylation sites occur in both regions 'B' and 'C'.

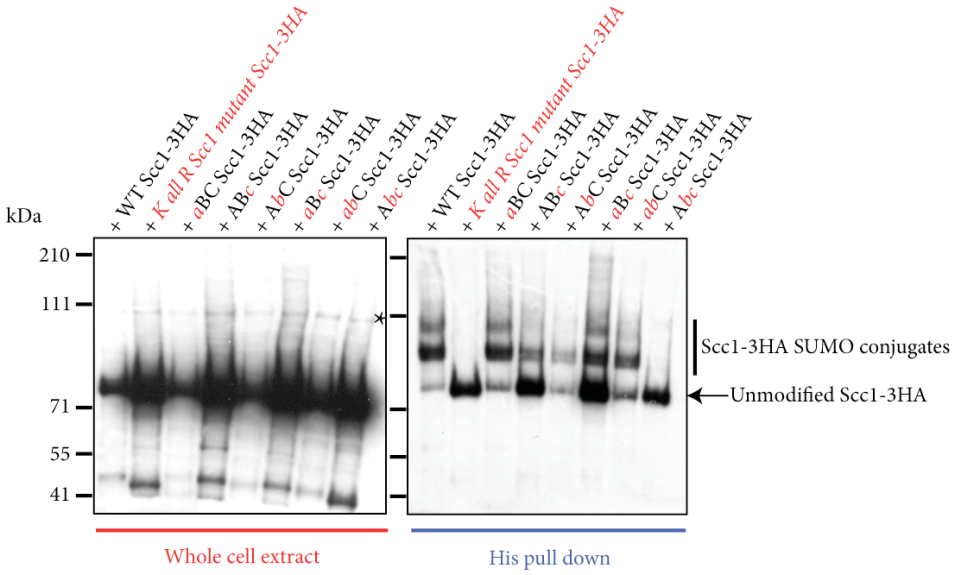


Figure 32: Analyzing sumoylation of ABC constructs after treatment with 0.3% MMS

Expression of GAL WT Scc1-3HA, *Scc1 K all R* -3HA or the ABC constructs (also tagged with -3HA) was induced in cells carrying HFSmt3 for 2 hours by the addition of galactose to the media. Subsequently the culture was split and half was harvested immediately and half incubated with 0.3% MMS for a further 2 hours before harvesting. SUMO conjugates were purified via the His tag on Smt3, separated on NuPAGE 3-8% Tris-Acetate gels (Invitrogen) and western blotted. Scc1 SUMO conjugates were identified by probing with α-HA.
A cross-reactive band is marked (*).

4.5 Adding back individual lysines

4.5.1 Addition of a single lysine C-terminal to the cleavage site is sufficient for viability, although it does not appear to be sumoylated.

The finding that the *K all R* mutant is lethal, and that this is probably due to stabilization of the C-terminal fragment after cleavage with Esp1, suggested the possibility that the addition of a single lysine could complement for viability. A consensus motif has not been identified for ubiquitination. I chose K319 to add back: it is C-terminal to the major (and most C-terminal) Esp1 cleavage site, and it is present in section B, so is therefore a possible sumoylation target.

R319K can complement for *scc1-73* at 37°C (Figure 33). This means that *Scc1 K all R* R319K can form a cohesin complex that fulfils cohesin's essential functions. It also suggests that the *K all R* mutation does not perturb Scc1's folding. Denaturing pull down of SUMO conjugates shows that sumoylation of this mutant cannot be detected. This suggests that K319 is a target for ubiquitination and is required for degradation of Scc1 (although other lysines may also be able to fulfil this function). However, it could be subject to another modification. It also leads to the conclusion that sumoylation of Scc1 is not essential for viability. However, *Scc1 K all R* R319K is sensitive to MMS. Sumoylation could be essential for this response, but alternatively it could be due to acetylation. As mentioned in the introduction, acetylation of K210 and K84 has been recently implicated in the establishment of cohesion after a DSB (Heidinger-Pauli et al., 2009), and obviously *Scc1 K all R* R319K lacks these two sites. However, the authors did not show the sensitivity of non-acetylatable mutants to MMS, so it is difficult to compare the sensitivities to MMS. In addition, individual *K210R* or *K84R* mutants did not display any MMS sensitivity in my assay, although they may both need to be mutated simultaneously to see an effect.

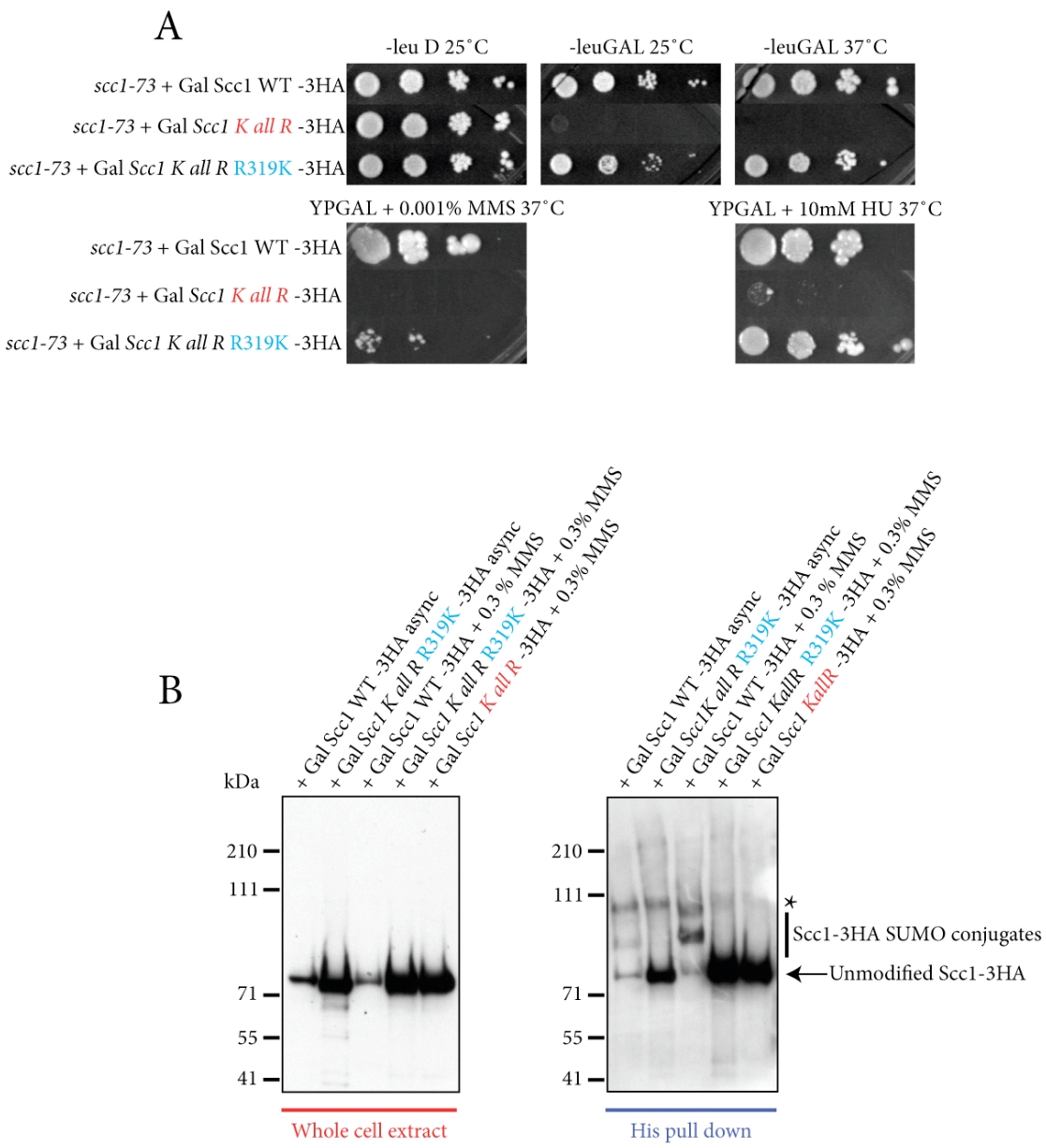


Figure 33: Mutation of residue 319 in *Scc1 K all R* back to lysine restores viability but not sumoylation

Panel A: 10-fold dilutions of *scc1-73* cells transformed with vectors carrying GAL WT Scc1-3HA, *K all R Scc1 (labelled in red)* and *K all R* R319K *Scc1* (labelled in blue) constructs were spotted onto –leu (to maintain the plasmid) containing glucose, repressing expression of the constructs, or galactose, inducing expression. Plates were incubated at 25°C (permissive temperature for *scc1-73*) or 37°C (restrictive temperature). To assay DNA damage sensitivity, cells were also spotted onto YPGAL + 0.001% MMS and YPGAL +10mM HU and incubated at 37°C. Panel B: GAL WT Scc1-3HA, *K all R Scc1* and *K all R* R319K *Scc1* constructs were transformed into strains expressing HFSmt3. Expression of the constructs was induced for 2 hours by the addition of galactose. The culture was then split and half was harvested immediately and half incubated with 0.3% MMS for 2h. SUMO conjugates were purified via the His tag on Smt3, separated on NuPAGE 3-8% Tris-Acetate gels (Invitrogen) and western blotted. Scc1 SUMO conjugates were identified by probing with α-HA. A cross-reactive band is marked (*).

4.5.2 Adding back lysines individually

To continue to investigate whether sumoylation of Scc1 is required in response to DNA damage, the mapping of potential sumoylation sites was continued. Lysines were added back to *scc1 K all R* individually by site-directed mutagenesis. Sumoylation after treatment with MMS was determined (Figure 34). The effect of expression on *scc1-73* at permissive and restrictive temperature, as well as in the presence of MMS was also determined (Figure 35).

Scc1 can be sumoylated on multiple lysines. The level of sumoylation varied. K165, K290, K500 and K460 were all strongly sumoylated. K252, K345, K382, K392 K394, K472, K509, K521, K552, K556 were also sumoylated. Sometimes, multiple bands were obtained. In particular, K165 and K460 had ladders that look very similar to ladders obtained with WT Scc1. This suggests that in these cases Scc1 is either subject to a combination of modifications, or is polysumoylated in this case on a single lysine. The experiment will have to be repeated with *Smt3 K all R* to distinguish between these possibilities.

As predicted from the requirement of a lysine C-terminal to the Esp1 cleavage site at 268 for ubiquitination, expression of *K all R* R165K, R210K, and R252K was lethal, even at the permissive temperature (Figure 34. Expression is also lethal in the WT, data not shown). However, expression of *K all R* R472K, R483K, R460K, R490K, R494K, R500K, R509K, R521K, R552K or R556K, which all have a lysine after the cleavage sites, is also toxic. All of these mutants affect cohesin's function in a dominant manner. *K all R* R310K, R324K R345K, R382K, R392K, R394K can complement, if only partially in the cases of R345K and R382K, but do not allow growth on MMS. *K all R* R290K, R363K and R391K complement and also allow some growth on MMS. The ability of a mutant to complement for *scc1-73* and/or to allow growth on MMS does not seem to correlate with the ability of the mutant to be modified by SUMO. R290K can complement for *scc1-73*, allows growth on MMS, and is sumoylated. On the other hand, R460K and R500K, are potential sumoylation sites, but expression of these mutants is lethal. It may be that some lysines can act as both sumoylation and ubiquitination sites, whereas others only act as sumoylation sites, or that the folding of some mutants is defective, somehow affecting

cohesin's function in a dominant manner. It is interesting to note that no mutants were created which had no effect on growth, i.e. did not complement but allowed growth at the permissive temperature. Instead, all of the mutants had a positive or dominantly negative effect.

It is also interesting to note that *K all R* R290K, R363K, and R391K allow growth on MMS, whereas some of the other mutations which complement do not. This suggests a role for these lysines after DNA damage. This resistance to MMS is also surprising given the importance of K84 and K210 for cohesion after a DSB (Heidinger-Pauli et al., 2009), as these are both mutated in all of these constructs.

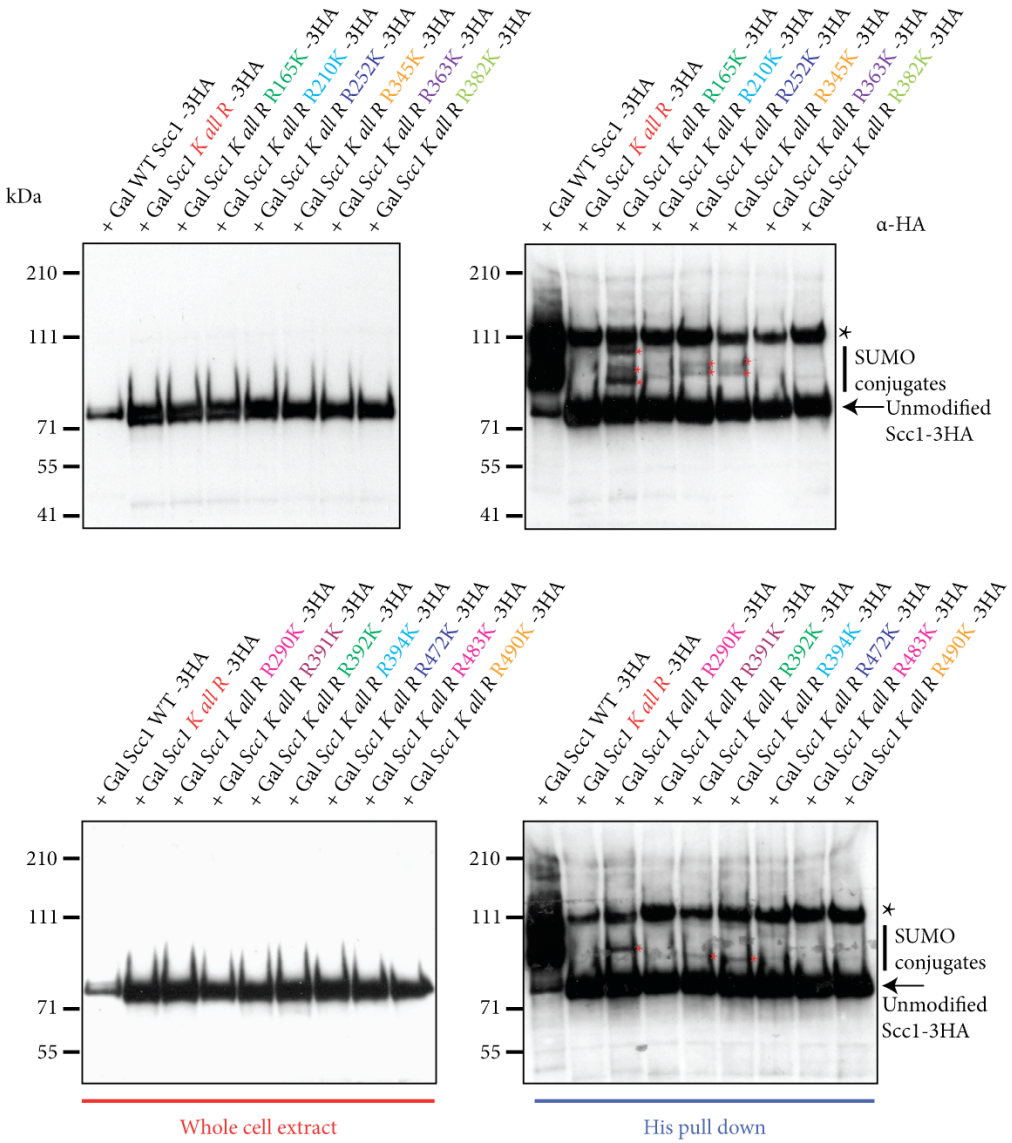


Figure 34: Identifying potential sumoylation sites (1)

GAL WT Scc1-3HA, *K all R Scc1* and *K all R + indicated lysine added back Scc1 (labelled in colour)* constructs were transformed into strains expressing HFSmt3. Expression of the constructs was induced for 2 hours by the addition of galactose to the media. MMS to a final concentration of 0.3% was added for a further 2 hours. SUMO conjugates were purified via the His tag on Smt3, separated on NuPAGE 3-8% Tris-Acetate gels (Invitrogen) and western blotted. Scc1 SUMO conjugates were identified by probing with α-HA. A cross-reactive band is marked (*). Scc1 SUMO conjugates are indicated (*).

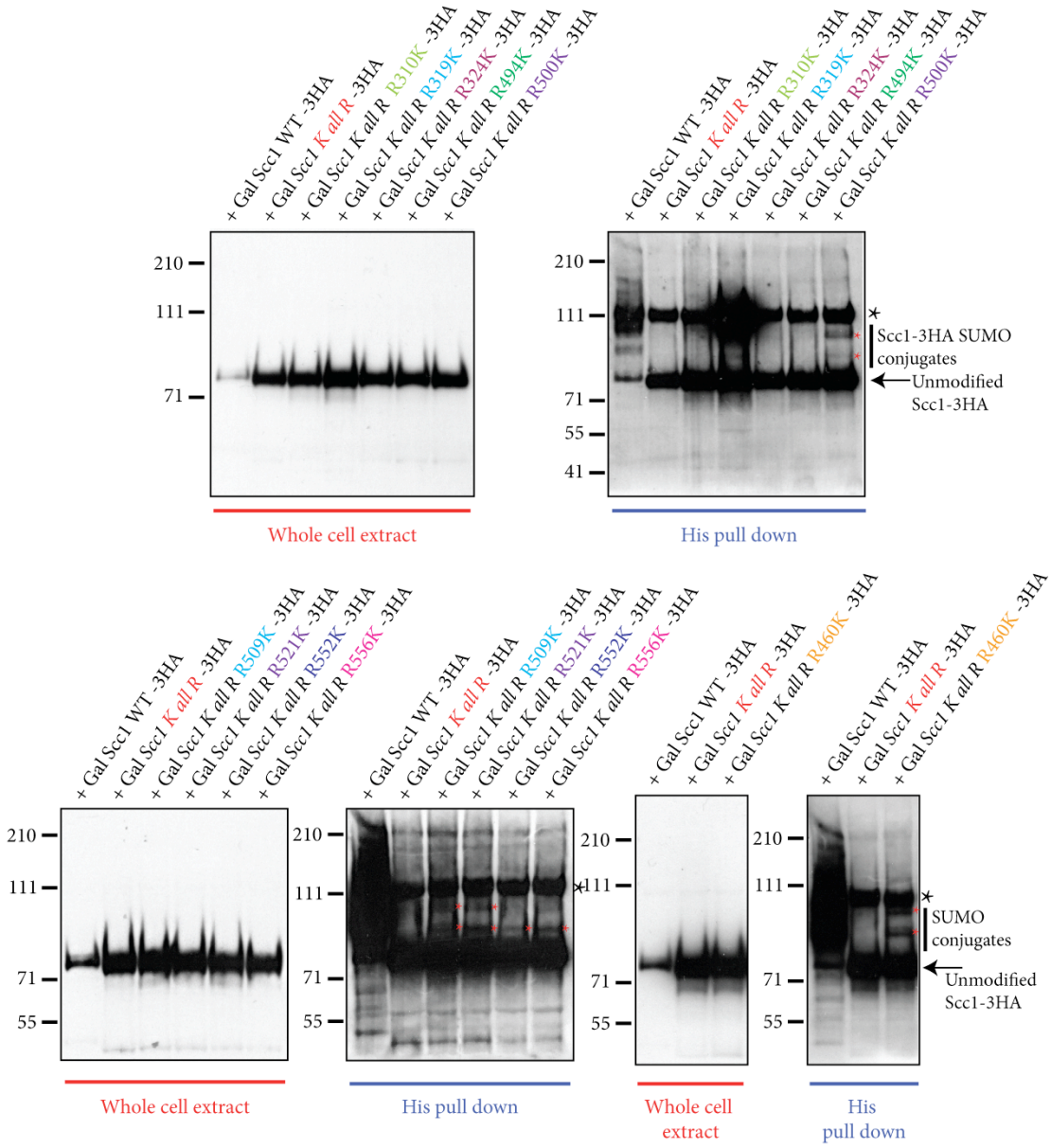


Figure 34 continued: Identifying potential sumoylation sites (2)

GAL WT Scc1-3HA, *K all R Scc1* and *K all R + indicated lysine added back Scc1 (labelled in colour)* constructs were transformed into strains expressing HFSmt3. Expression of the constructs was induced for 2 hours by the addition of galactose to the media. MMS to a final concentration of 0.3% was added for a further 2 hours. SUMO conjugates were purified via the His tag on Smt3, separated on NuPAGE 3-8% Tris-Acetate gels (Invitrogen) and western blotted. Scc1 SUMO conjugates were identified by probing with α-HA. A cross-reactive band is marked (*). Scc1 SUMO conjugates are indicated (*)

Figure 35: Effect of expressing *K all R Scc1 + indicated lysine* mutants
10-fold dilutions of *scc1-73* cells transformed with vectors carrying GAL WT Scc1-3HA, *K all R Scc1* and *K all R Scc1 + indicated lysine* mutants were spotted onto –leu (to maintain the plasmid) containing glucose, repressing expression of the constructs, or galactose, inducing expression. MMS sensitivity was assayed by spotting onto YPGAL +0.001% MMS. Plates were incubated at 25°C (the permissive temperature for *scc1-73*) or 37°C (the restrictive temperature).

4.6 GeneCust constructs

Three different constructs were made by GeneCust using the above results. In these constructs, combinations of lysines in WT Scc1 were mutated to arginine. In the first construct, K165 and K290, which both have prominent sumoylation bands, were mutated to arginine. In the second construct (*K2ndR*) K165, K290, K252, K 345 K391, K392, K394, K500, K509 and K521 were mutated, and in the third construct (*K3rdR*) K165, K290, K252, K345, K391, K392, K394, K500, K509, K521, K319, K324, K382, K472, K494, K552, K556 were mutated. Unfortunately, the K460 site was identified after these constructs had been made, and therefore was not mutated. Ongoing work is being undertaken to mutate this site.

The constructs were integrated into the genome at Scc1's endogenous locus, and expression was under the endogenous promoter (this is different to previous experiments, in which constructs were present on centromeric plasmids and under the control of the GAL promoter). All of the mutants were viable, and were not temperature sensitive (Figure 36). However, both *K2ndR* and *K3rdR* were slightly sensitive to both HU and MMS. However, the sumoylation pattern after treatment with MMS was no different in the mutants compared to the WT (Figure 37). Although K460 is present, in the *K3rdR* construct 17 of 31 lysines in Scc1 are mutated, and *K3rdR* has a very similar SUMO ladder to the WT, with no loss of bands or reduction in intensity. Although K460 was identified as a major site for SUMO attachment, the ratio of sumoylated to unmodified after purification was low (Figure 34), suggesting that K460 cannot be solely responsible for the sumoylation observed. Instead, perhaps different lysines can be modified in a more WT protein, possibly including those in section 'A'.

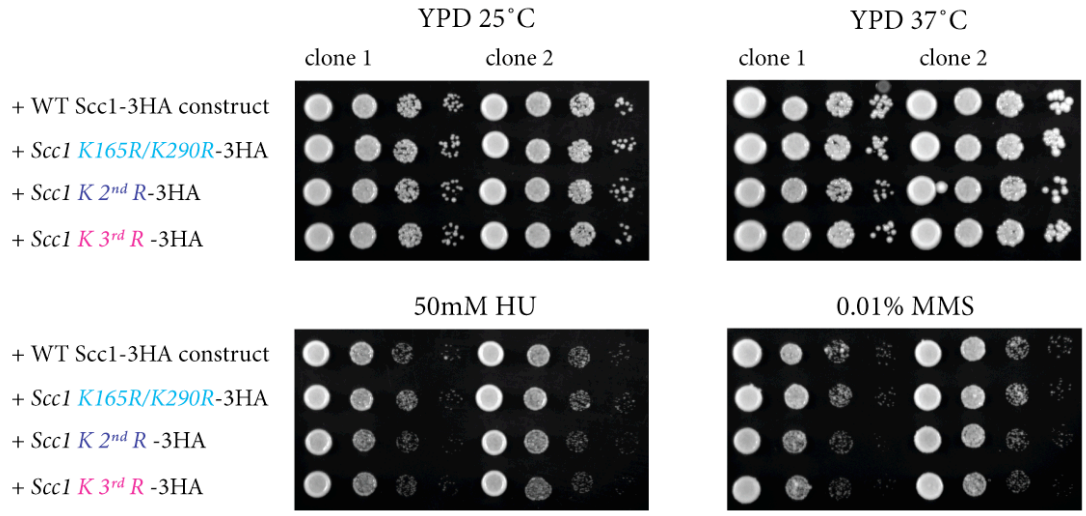


Figure 36: All constructs, including *K3rdR*, are viable

10-fold dilutions of WT (BY4741) cells with the indicated construct integrated at Scc1's endogenous locus were spotted onto YPD or YPD containing 50mM HU or 0.01% MMS. Plates were incubated at 30°C unless otherwise indicated.

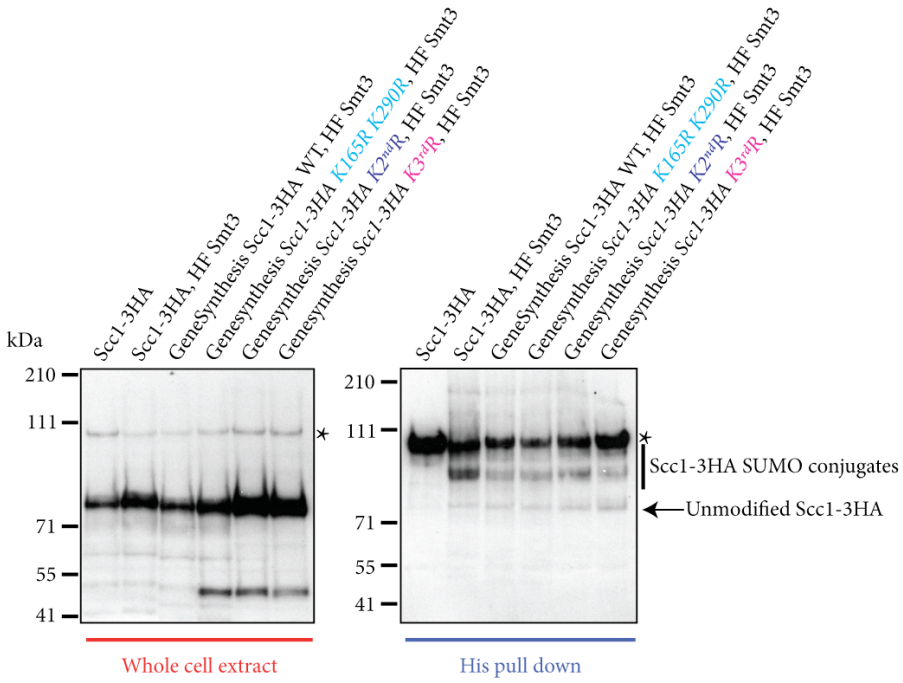


Figure 37: All constructs are sumoylated, even *K3rdR*, which lacks 17 lysines

Scc1-3HA, *Scc1 K165/290R*, *Scc1 K2ndR* and *Scc1 K3rdR* were integrated at Scc1 (to replace endogenous Scc1) in strains expressing HFSmt3. These strains, and strains expressing Scc1-3HA with and without HFSmt3, as positive and negative controls respectively, were grown in YPD, and then treated with 0.3% MMS before harvesting. SUMO conjugates were purified via the His tag on Smt3, separated on NuPAGE 3-8% Tris-Acetate gels (Invitrogen) and western blotted. Scc1 SUMO conjugates were identified by probing with α-HA. A cross-reactive band is marked (*).

5 Results: Functional analysis of sumoylation of Scc1 using Ubc9 and Ulp1 fusions

5.1 Objectives

- To characterize the function of sumoylation of cohesin

5.2 Approach

As the removal of identified sumoylation sites on Scc1 did not prevent sumoylation, a different approach was utilized to try and characterize the function of sumoylation. Ubc9 fusion-directed sumoylation (UFDS), in which Ubc9 (E2) is fused to the C-terminus of a target protein, is a technique developed in mammalian cells to increase the amount of a specific sumoylation target (Jakobs et al., 2007). It has the advantage that sumoylation can be studied *in vivo*, and sumoylation has been shown to occur at previously identified sumoylation sites, at least for p53 and STAT-1. Using this approach, Jakobs et al. (2007) discovered that sumoylation of STAT-1 inhibits its phosphorylation at Y701.

We used this technique, which has not been used so far in yeast, to analyze the sumoylation of Scc1. Fusing Ubc9 to the C-terminus of Scc1 has other advantages as it might allow the characterization of the function of sumoylation of cohesin rather than just Scc1. As reported in chapter 2, all cohesin subunits are sumoylated in response to MMS. It may be that any function of Scc1 sumoylation can be compensated by sumoylation of other cohesin subunits.

This approach could also be used to enrich for sumoylated proteins to aid in site identification by mass spectrometry.

5.3 *Scc1-Ubc9 fusion is sumoylated*

A construct was generated encoding Scc1-Ubc9-9myc under the control of the GAL promoter, carried on a centromeric plasmid. This fusion can complement for the temperature sensitivity of *scc1-73* but not *ubc9-1* (Figure 38). This suggests that fusing Scc1 with Ubc9 does not affect cohesion. However, Ubc9 fused to Scc1 cannot fulfil its normal role, possibly because of defective localization. The Scc1-Ubc9 fusion is sumoylated (Figure 39).

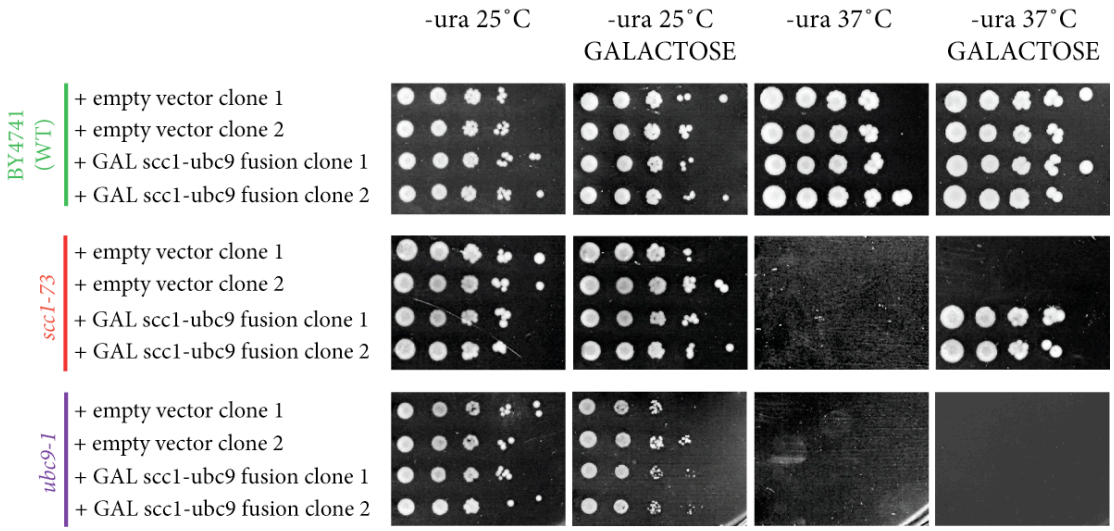


Figure 38: Scc1-Ubc9-9myc fusion complements for *scc1-73* but not *ubc9-1*

10-fold dilutions of WT(BY4741) *scc1-73* and *ubc9-1* cells transformed with centromeric vectors carrying GAL Scc1-Ubc9-9myc were spotted onto –ura (to maintain the plasmid) containing glucose, repressing expression of the constructs, or galactose, inducing expression. Plates were incubated at 25°C (the permissive temperature for *scc1-73* and *ubc9-1*) or 37°C (the restrictive temperature).

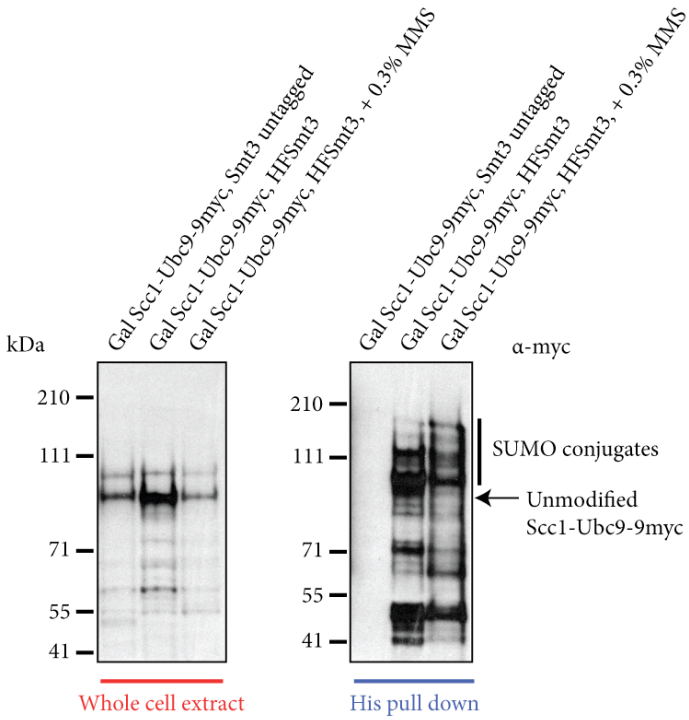


Figure 39: Scc1-Ubc9-9myc is sumoylated

WT strains and strains expressing HFSmt3 were transformed with the centromeric vector carrying GAL Scc1-Ubc9-9myc. Expression of Scc1-Ubc-9myc was induced for 2 hours by the addition of galactose to the media. The culture was then split and half was harvested immediately and half incubated with 0.3% MMS for a further 2 hours. SUMO conjugates were purified via the His tag on Smt3, separated on NuPAGE 3-8% Tris-Acetate gels (Invitrogen) and western blotted. Scc1-Ubc9 SUMO conjugates were identified by probing with α-myc.

5.4 Ulp1 fusion directed de-sumoylation

In addition to fusing Ubc9 to Scc1, I also obtained a construct encoding Scc1 fused the catalytic domain of Ulp1, a SUMO-isopeptidase (Scc1-Ulp1-3HA). This is a novel idea, which attempts to use the same approach to reduce sumoylation and hence mimic a non-sumoylatable state (from Jordi Torres-Rosell). For consistency, an Scc1-Ubc9-3HA construct obtained from Jordi Torres-Rosell was also used, to keep the tag and method of construction consistent for all future experiments. Again all constructs were under the control of the inducible GAL promoter. Scc1-Ulp1-3HA and Scc1-Ubc9-3HA co-immunoprecipitate with Smc3, suggesting that these constructs can form cohesin complexes. However, although Scc1-Ubc9-3HA can complement for *scc1-73*, Scc1-Ulp1-

3HA cannot (Torres-Rosell, unpublished data). The fusion of Ulp1 to Scc1 affects the essential function of cohesion, either through affecting folding or formation of the complex, or, through inhibition of sumoylation of the complex.

5.5 Scc1-Ubc9 and Scc1-Ulp1 are both recruited to a DSB

As mentioned in the introduction, upon induction of a DSB, cohesin is enriched in the vicinity of the break (Strom et al., 2004; Unal et al., 2004). Given that treatment with MMS or induction of an unrepairable DSB induces the sumoylation of cohesin, and that some of the mutational analysis suggests that mutants that cannot be sumoylated are sensitive to MMS, it is pertinent to investigate whether recruitment of the Scc1 to a DSB is perturbed in the fusions.

The fusions, and Scc1-3HA as a control, on centromeric plasmids, were introduced into a strain that contains the HO endonuclease, which cuts at the *MAT* locus on chromosome III, under the control of the GAL promoter, and in which the *HMR* and *HML* loci have been deleted, and therefore repair cannot occur. Thus, in galactose a single unrepairable break is formed. The fusions are also under the control of the GAL promoter. Therefore expression of the fusions and induction of the DSB occur simultaneously.

Strains were grown overnight in media containing lactate, which maintains the GAL promoter in a neutral position, and then both a DSB and expression of the fusions were induced by the addition of galactose. The strains also contain Ddc2 (binding partner of Mec1 and involved in the DNA damage response) tagged with GFP. This allows induction of the break to be followed microscopically, as a DSB will cause the appearance of a GFP focus.

Before induction, and 2h, 4h and 6h after induction of the break, cells were taken to monitor induction of the DSB, expression of the fusions and to assay recruitment to the break by ChIP (Figure 40). Although expression of the fusions peaked quickly, reaching maximal levels after 2h, it took longer for the DSB to be formed, with 80% of cells containing a DSB (as monitored by Ddc2-GFP focus formation) after 6h (Panel A). Interestingly, a ladder of higher molecular weight bands can be seen above Scc1-Ubc9-3HA, particularly in higher exposures. As these are whole cell extracts, and SUMO

conjugates have not been purified, this suggests that Scc1 is being sumoylated to a high level (Panel B).

Real-time PCR analysis of the ChIPs was performed with 10 primer pairs spanning the DSB (described in Shroff et al., 2004), and a primer pair corresponding to a region on chromosome VI, which allows DSB-independent effects, as well as experiment-to-experiment variations in efficiency of crosslinking and immunoprecipitation to be controlled for. The RT-PCR results are shown in Figure 40C. All data is normalized to ChrVI.

ChIP analysis shows that Scc1-3HA, Scc1-Ubc9-3HA and Scc1-Ulp1-3HA are all recruited to the DSB. This recruitment increases with time, and is maximal 4-6 hours after induction, concomitant with the peak in the percentage of cells with a Ddc2 focus. Enrichment of all three constructs is similar: peaking close to the break and decreasing with distance on both sides.

Although 4h after induction recruitment of all three constructs is similar, surprisingly 6h after induction recruitment of Scc1-Ulp1-3HA is actually increased relative to Scc1-Ubc9-3HA and Scc1-3HA. This is contrary to what was expected, as the sumoylation of Scc1 in response to DNA damage had led to the hypothesis that this sumoylation would be important for cohesin's recruitment. It is interesting that fusion of Scc1 to Ulp1 and Ubc9 did not produce opposite results, as both fusions are recruited, and the Ubc9 fusion is enriched to the same extent as Scc1-3HA. Instead it appears that Scc1-Ulp1 continues to be loaded. It will be important to fully characterize the effect of the Ubc9 and Ulp1 fusions before any concrete conclusions can be drawn.

Also, it is possible that sumoylation of cohesin may play a role in cohesion after a DSB rather than affecting the recruitment to the break, as already suggested for the phosphorylation of S83 and acetylation of K84 and K210 in Scc1 (Heidinger-Pauli et al., 2008; Heidinger-Pauli et al., 2009). This cannot be assayed by ChIP. Instead, an experimental set up developed by the Koshland lab will be used. In this approach, cohesion is generated during S-phase using a temperature-sensitive allele of Scc1. Cells are arrested in G2/M by nocodazole, and the formation of two DSBs, which cause the excision of a small region, is induced. This region contains a lacO array, and the cells express LacI-GFP. At the same time, induction of Scc1-3HA or either of the two fusions is induced. The cells

are then shifted to the non-permissive temperature, to inactivate S-phase generated cohesion. If Scc1-3HA or the fusions are competent for cohesion after a DSB, then a single GFP focus will be present. However a loss of cohesion will generate two GFP foci. Using this approach it will also be possible to determine whether the fusions are competent for genome-wide cohesion after a DSB, by moving the lacO array to a different chromosome, which does not contain HO endonuclease cleavage sites. These experiments are ongoing.

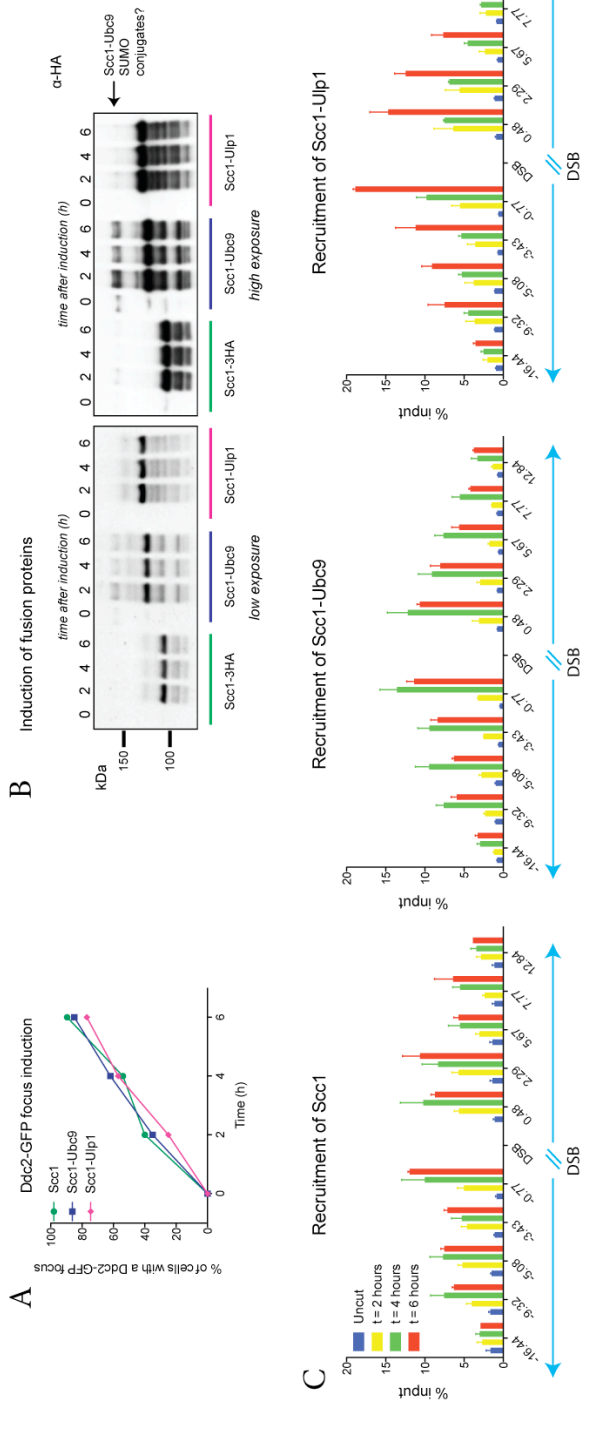


Figure 40: Scc1-3HA, Scc1-Ubc9-3HA and Scc1-Ulp1-3HA are all recruited to a DSB
The HO endonuclease, under the control of the GAL promoter, was used to induce a DSB at the *MAT* locus in a strain in which *HMR* and *HML* have been deleted. These deletions and the continual expression of the HO endonuclease cause this break to be unrepairable. This strain was transformed with the centromeric plasmids encoding GAL Scc1-3HA, GAL Scc1-Ubc9-3HA and GAL Scc1-Ulp1-3HA. Cells were grown up in YPlactate, and expression of the HO endonuclease and the Scc1 constructs induced by the addition of galactose. Samples were taken before and 2, 4 and 6 hours after induction. Panel A: The accumulation of Ddc2 foci. Panel B: Expression of the Scc1 constructs, analyzed by western blot. Panel C: Distribution of the constructs at the break. Input DNA and DNA coimmunoprecipitated with α-HA were amplified using primer pairs corresponding to sequences around the *MAT* locus. Values shown are % input chromatin, normalized to Chr VI. Distance from the break of each primer pair is indicated.

6 Results: Scc1 does not get cleaved in response to MMS in *S. cerevisiae*

Intriguingly, in *S. pombe* it has been reported that Separase-mediated cleavage is required for DNA repair (Nagao et al., 2004). The authors found that mutations in Securin, (Cut2) essential for Separase stability, and Separase (Cut1) are sensitive to UV, X-ray and γ-irradiation. The temperature sensitivity of the Securin mutant, $Cut2^{EA2}$, could be rescued by overexpression of Separase (Cut1), and levels of Cut1 were decreased in $Cut2^{EA2}$, suggesting that the Securin phenotype is due to a reduction Cut1 levels. The authors also found that expression of a non-cleavable Scc1 mutant ($Rad21^{RREE}$) negatively affected repair. After irradiation, Scc1 was retarded on SDS-PAGE gels, corresponding to modification by phosphorylation. Cleavage products were also hyper-shifted.

However, it was unclear from this study whether DNA damage directly induced cleavage of Scc1. The above experiments were performed in asynchronous cells, and the cleavage products obtained could have been due to passage through mitosis. Does cleavage actually occur before the metaphase to anaphase transition? Also, it was unclear whether there was any accumulation of cleavage products, or if they just became retarded on SDS-PAGE gels.

To address this question, I made use of the *Scc1 K all R* mutant, which has the advantage of having a stabilized C-terminal fragment. This allows the easy detection of the C-terminal cleavage product. Both WT and *K all R* Scc1-3HA were placed under the control of the GAL promoter on centromeric plasmids. These plasmids were introduced into WT and *esp1-1* (Separase) temperature sensitive cells. The cells were synchronized using alpha factor. Once arrested, the temperature was shifted to 37°C to inactivate *esp1-1* and galactose added to induce expression of the Scc1 constructs. The cells were then released into a synchronous cell cycle. 30 minutes after release (when the cells were in mid to late S-phase) 0.3% MMS was added to half of the culture. Samples were taken every 15 minutes for FACS and western analysis (Figure 41).

In WT (W303) cells expressing WT Scc1-3HA, cleavage product is seen in α-factor arrested cells (as Esp1/separase is known to be active in G1). Overexpression via the

GAL promoter allows Scc1 to accumulate. After passage through mitosis (90 minutes and 210 minutes) the same amount of cleavage product can be observed. When WT (W303) cells express *K all R Scc1-3HA* a similar result is observed, except there is much more cleavage product. *esp1-1* cells progress more slowly through the cell cycle, and a G2/M delay was observed. When the cells do undergo division, it results in some cells with a DNA content of less than 1n. Despite this division, Scc1 is never cleaved, as no cleavage products are observed, either when WT or *K all R* Scc1-3HA is expressed.

Treatment with 0.3% MMS arrests the cell cycle. WT (W303) cells arrested in late S phase, and *esp1-1* cells arrested in mid S-phase, due to slight differences in the kinetics of the release from α-factor. Again, WT cells expressing WT Scc1-3HA had a band corresponding to the cleavage fragment. 30 minutes after release, immediately prior to the addition of MMS, no cleavage product is detected. After 1h and 2h with MMS, no cleavage product is detected either. When WT (W303) cells express *K all R Scc1-3HA* a cleavage product is again observed during α-factor arrest. This band is still present 30 minutes after release, although at reduced levels. After treatment with MMS, the band corresponding to Scc1's C-terminal cleavage fragment further decreases in intensity. In the *esp1-1* controls, no cleavage is seen.

These results strongly suggest that contrary to the situation in S. *pombe* after irradiation, treatment with MMS during S-phase does not induce the cleavage of Scc1.

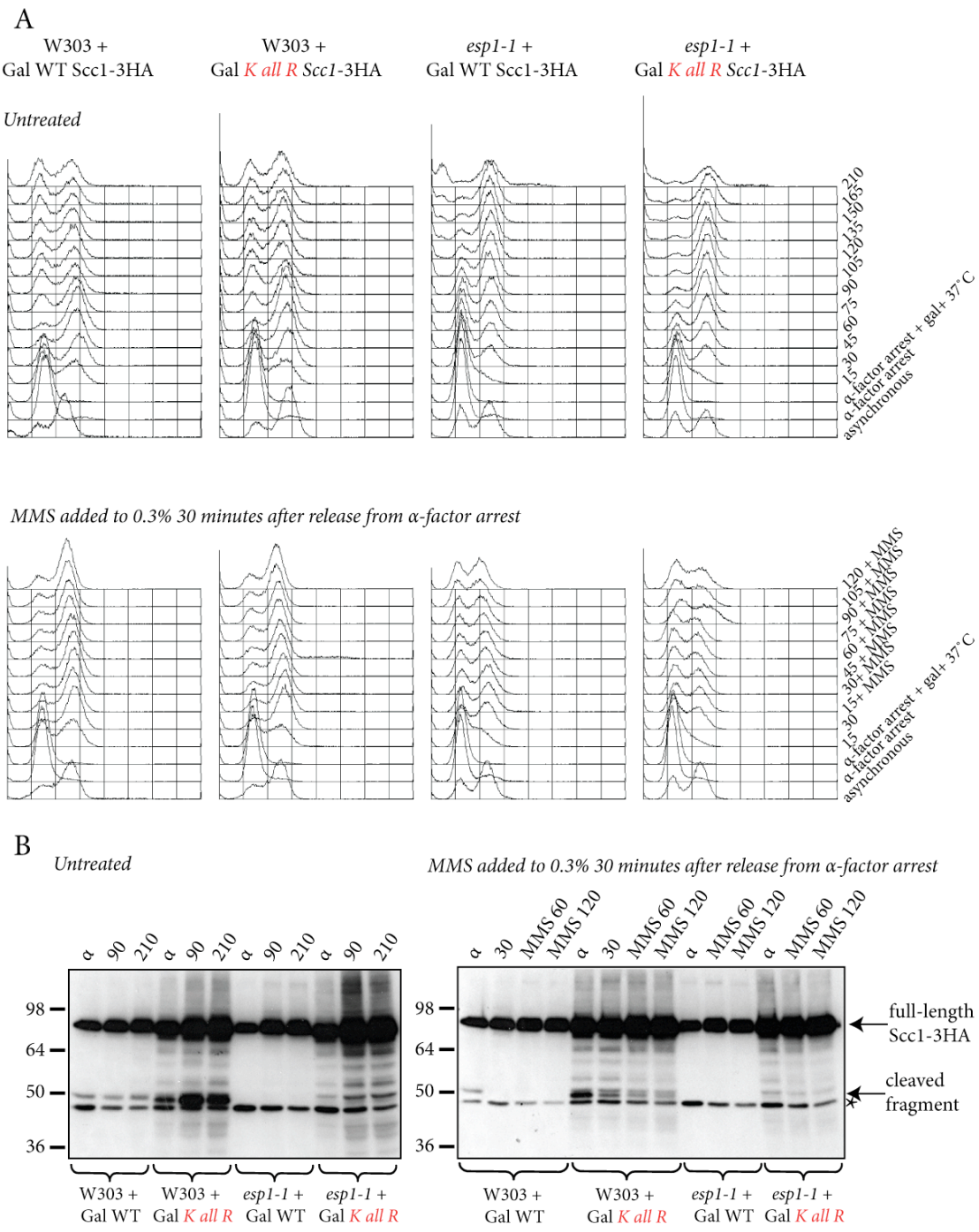


Figure 41: Scc1 is not cleaved after treatment with 0.3% MMS in S-phase

WT (W303) and *esp1-1* cells were transformed with plasmids encoding Gal WT Scc1-3HA or Gal *K all R Scc1*-3HA. Strains were arrested in G1 by α-factor, after which expression of the constructs was induced by the addition of galactose, and the temperature shifted to 37°C, to inactivate *esp1-1*. Cells were released into the cell cycle and left untreated, or 0.3% MMS was added 30 minutes after release. Samples were taken every 15 minutes. Panel A: FACS analysis showing cell cycle progression in an unperturbed cell cycle (top) and after treatment with MMS (bottom). Panel B: Western blot analysis. The left hand blot shows samples from an unperturbed cell cycle. The right hand blot shows samples from MMS treated cells.

7 Discussion

7.1 Summary of results

7.1.1 Cohesin is sumoylated

All cohesin subunits are sumoylated, and this sumoylation is increased after DNA damage, either caused by treatment with MMS or the induction of an unrepairable DSB.

Sumoylation of several other proteins, including PCNA and Rad52, is also increased after treatment with MMS (Hoege et al., 2002; Sacher et al., 2006). However, sumoylation of other targets, for example septins, is not induced after treatment with MMS (Johnson and Blobel, 1999; Reindle et al., 2006). Interestingly, the sumoylation of PCNA and Septins are both dependent on Siz1, and yet they occur in different cell cycle phases. PCNA is modified during S-phase and Septins are modified during mitosis (Hoege et al., 2002; Johnson and Blobel, 1999). Therefore, it is not a general mechanism that all sumoylation targets are hyper-modified after treatment with MMS, even if sumoylation is induced by the same E3 ligase.

Rad52 is sumoylated in response to a range of genotoxins, including MMS, 4-nitroquinoline 1-oxide (4-NQO), X-rays and Zeocin, as well as during meiosis, following the creation of programmed DSBs by Spo11. Accumulation of DSBs, and their recognition by the MRX complex, triggers RAD52 sumoylation during mitosis and meiosis (Sacher et al., 2006). It remains to be investigated whether sumoylation of cohesin is dependent on Mec1, Tel1, or the MRX complex in an analogous fashion to Rad52. I have already determined that the sumoylation of Scc1 is not Chk1 dependent. Chk1, which is downstream of both Mec1 and Tel1, has previously been shown to be important for cohesion establishment in G2/M after DNA damage, and it is thought that phosphorylation of S83 in Scc1 by Chk1 allows it to be acetylated by Eco1 (Heidinger-Pauli et al., 2008; Heidinger-Pauli et al., 2009), reviewed in the introduction.

7.1.2 Siz1, Siz2 and Mms21 all have a role in sumoylating Scc1

In *S. cerevisiae*, Smc1 and Smc3 are sumoylated by Mms21 in cells arrested in G2/M by nocodazole (Takahashi et al., 2008). Human Mms21 can stimulate the sumoylation of human Scc1 and SA2 (Scc3), although not Smc1 or Smc3 in HeLa cells (Potts et al., 2006). However, it is less clear that Mms21 is responsible for Scc1 sumoylation in *S. cerevisiae*. Instead it seems that all three SUMO E3 ligases contribute to the sumoylation of Scc1 after treatment with MMS. Truncating Mms21, so that it no longer has ligase activity reduced the abundance of SUMO conjugates. Although deletion of Siz1 or Siz2 individually had little effect on the SUMO ladder produced, in the *siz1Δsiz2Δ* double mutant the ladder was less extensive. Therefore it appears that although Mms21 is responsible for the bulk of the sumoylation, either Siz1 or Siz2 is responsible for the sumoylation of certain sites or the formation of polysumo chains. The sumoylation of cohesin mediated by Siz1 and Siz2 cannot be required for the DNA damage response, as *siz1Δsiz2Δ* double mutants are not damage sensitive, despite a decrease in ~90% of SUMO conjugates (Johnson and Gupta, 2001). On the other hand, the *mms21CΔ* allele does render cells sensitive to genotoxins (Andrews et al., 2005; Zhao and Blobel, 2005).

It has previously been reported that sumoylation of many substrates can be stimulated, *in vivo,* by either Siz1 or Siz2. Many Siz substrates are still sumoylated, although at dramatically reduced levels, in *siz1Δsiz2Δ* double mutants (Reindle et al., 2006). The same study also showed that Siz1, Siz2 and Mms21 can all promote the sumoylation of certain shared substrates, for example Net1 (Reindle et al., 2006).

As the sumoylation of Smc1 and Smc3 was found to be dependent on Mms21 in nocodazole arrested cells, it would be interesting to repeat this experiment, to determine E3 ligase specificity, under these conditions.

7.1.3 Scc1 ladder not solely due to polysumoylation.

The ladder of Scc1 conjugates seen after Ni-NTA purification of conjugates from MMS treated cells is not solely due to polysumoylation. To perform this experiment, we used a version of SUMO in which all the lysines were mutated to arginine (*Smt3 K all R*).

In *S. cerevisiae*, it has been found that the ability to form SUMO chains is not essential for normal growth, as mutants with *Smt3 K11R, K15R, K19R* have no obvious defect (Bylebyl et al., 2003) but is required for spore formation after meiotic cell division (Cheng et al., 2006). *Smt3 K all R* strains have been reported to grow slowly and be sensitive to MMS (Bylebyl et al., 2003; Mullen and Brill, unpublished result). However, I found that my *Smt3 K all R,* expressed under its own promoter from a centromeric vector, fully complemented for viability, and growth on MMS and HU in *Smt3Δ* strains.

I found that the *Smt3 K all R* did not affect the ladder obtained. There are several interpretations of this result. Scc1 could be monosumoylated at a number of sites. Alternatively, Scc1 could be modified by a combination of modifications, all of which cause a shift on SDS-PAGE gels.

This experiment also does not rule out the possibility that when target lysines are available on Smt3, that polysumo chains form.

7.1.4 Scc1 is sumoylated in both S-phase and G2/M arrested cells.

It was found that Scc1 was sumoylated in both a S-phase (HU arrest) and a G2/M arrest (nocodazole arrest), although the pattern of bands was slightly different in the two arrests. As previously mentioned, Smc1 and Smc3 are sumoylated during G2/M (Takahashi et al., 2008). It has not been investigated thoroughly whether these subunits are sumoylated in other cell cycle phases. It would be informative to release from α-factor into a synchronous cell cycle and to monitor sumoylation of all of the subunits, to investigate the timing of sumoylation. This would also ensure that the modifications are not due to activation of either the DNA damage or Spindle checkpoints.

7.1.5 Development of approaches to identify Scc1 sumoylation sites by mass spectrometry.

Two approaches have been developed to aid in Scc1 sumoylation site identification. These have aimed to overcome the problems normally associated with site identification by increasing the ratio of sumoylated peptide by immunoprecipitating

sumoylated tryptic fragments and by creating a Smt3 mutant which, when digested with trypsin, leaves a –GG motif, that because of its smaller mass is more easily identified.

7.1.6 Smt3 with an internal HA

Smt3 with an internal HA was designed so that after digestion of purified Scc1 with trypsin, SUMO modified peptides could be enriched for by an α-HA immunoprecipitation (see Figure 21). An HA tag was inserted into Smt3 in two locations: very near its C-terminus and further into the protein, with an additional *R93Q* mutation to abolish a trypsin digestion site. It was the latter construct that was used to modify proteins *in vivo*, including Scc1, both when one or three copies of HA were present. Unfortunately, these constructs were not viable in the absence of WT Smt3. This strategy relies on Smt3 with an internal HA being directly conjugated to the target. If it only forms polysumo chains on WT Smt3 the only sites for modification identified will be in Smt3. To examine whether Smt3 is polysumoylated I used a *Smt3 K all R* mutant. The HA tag and the *R93Q* mutation would have to be inserted into this construct to ensure that Smt3 with an internal HA is being directly conjugated to Scc1.

7.1.7 K all R RGG (I96R) results in a diglycine motif after trypsin digestion.

An *Smt3 K all R RGG* construct was developed for mass spectrometry. After trypsin digestion, a diglycine motif would remain attached to a target lysine, instead of the larger EQIGG motif left after digestion of WT Smt3. This would be easier to identify. This construct complemented for *smt3Δ* viability, but was slightly sensitive to MMS and significantly sensitive to HU. *Smt3 I96R* has previously been used to aid site identification (Wohlschlegel et al., 2006).

7.1.8 Mass spectrometry of an immunopurification of Scc1 identifies Smc1 and Smc3, but no sumoylated peptides

Scc1-3HA was immunopurified from cells treated with 0.3% MMS expressing *Smt3 K all R RGG.* Purified material was sent for mass spectrometry. Although Smc1, Smc3 and Scc1 were identified, puzzlingly it was not Scc1 that was the most abundant protein, but Smc1. Smc1 and Smc3 were the two most identified species. This either indicates that it is easier to identify Smc1 and Smc3 than Scc1, or a problem in the purification. The latter is more probable as from the coomassie staining it was obvious that Scc1 was not the most abundant species, but a protein of higher molecular weight. Disappointingly, in addition, no –GG conjugated peptides were identified, i.e. no peptides that had been modified by either *Smt3 K all R RGG* or ubiquitin. However, a previously described acetylation site (K112) on Smc3 was identified.

7.1.9 Future directions for mass spectrometry

There are other modifications that could be made to try and identify sumoylation sites by mass spectrometry. The quantity of sumoylated target could be increased either by overexpression of Smt3 or by decreasing desumoylation by introducing the *ulp1-1* allele. Overexpression of HFSmt3 increased the amount of SUMO modified Scc1 even without MMS treatment (see Figure 13). *ulp1-1* is a temperature sensitive allele of the SUMO protease Ulp1.

Alternatively, it has been demonstrated that the physiological SUMO targets Pol30, Cdc11 and Cdc3 can be sumoylated at identified sites *in vitro*, even in the absence of an E3 ligase (Wohlschlegel et al., 2006). Sumoylating Scc1 *in vitro* would allow the production of large amounts of modified protein, but any sites identified would have to be verified as the sites of modification *in vivo*.

However, an *in vitro* system requires the cloning, expression and purification of each component required for sumoylation. Another approach, also used by Wohlschlegel et al., (2006) (based on (Baba et al., 2005; Uchimura et al., 2004)) uses the pDUET bacterial expression system (Novagen) which facilitates the coexpression of up to eight

different proteins allowing the simultaneous expression of Uba2p/Aos1p, Ubc9, Smt3p, and a GST or MBP tagged substrate of interest in *E. coli*. A significant amount of Smt3 is conjugated to the substrate of interest. In addition, the expression of additional components such as SUMO E3 ligases can be easily incorporated into the system.

If the large scale purification were to be repeated, denaturing conditions would be used, as the addition of NEM is obviously not sufficient to preserve Scc1 SUMO conjugates. Recently a protocol has been published to identify sumoylation sites which could easily be performed in our laboratory. A yeast strain is generated which expresses His_8-Smt3 and the protein of interest fused to an *in vivo* biotinylation peptide. SUMO conjugates are purified by Ni-NTA chromatography under denaturing conditions, and the target then purified using immobilized streptavidin (Wohlschlegel, 2009).

7.1.10 Conjugation site identification by site directed mutagenesis.

Site directed mutagenesis has been used successfully to identify sumoylation sites. For example, this technique was used to identify the sumoylation sites on Rad52 (Sacher et al., 2006). Initially I mutated single lysines to arginine, starting with those identified using prediction software. SUMO modification can occur on the consensus motif, ψ K X E/D, which is the Ubc9 interaction site. However, this motif also occurs in many proteins that are not SUMO targets and many sumoylation events occur on lysines not in a consensus motif. In addition, sumoylation can only occur on this motif when it occurs in an unstructured loop, and sumoylation does not occur if the site is present in an α helix. Addition of this constraint would increase the accuracy of the programmes. Also, it seems logical to predict that only a surface exposed lysine can be sumoylated. Unfortunately there is no full crystal structure of *S. cerevisiae* Scc1. Crystal structures of bacterial homologues have been solved, so homology modelling could be performed to identify surface lysines. However, some lysines may be hidden by interaction with other subunits of the complex. In any case, mutation of any single lysine in Scc1 had no effect on the ladder of conjugates obtained, and did not affect viability or MMS sensitivity. It has already been observed that even in cases where a primary acceptor lysine can be identified,

mutation of this site may result in modification of other lysines that are not normally used for conjugation (Ulrich, 2009).

7.1.11 Expression of Scc1 K all R is lethal- the importance of ubiquitination

Since mutating single lysines had not identified the sumoylation site, I decided to mutate all the lysines to arginine (*K all R*). This would determine if any modification that occurs on lysines residues is important. Expression of *Scc1 K all R* is lethal, even when WT Scc1 is also present.

It has previously been described that after cleavage, the C-terminal region of Scc1 is subject to degradation via the N-rule pathway, and that the stabilization of this fragment is toxic (Rao et al., 2001). It is unclear why the C-terminal fragment is toxic. There is evidence that the C-terminal fragment can bind to the Smc1/3 heterodimers *in vivo*, leading to the possibility that it may compete with full length Scc1 and therefore prevent the formation of the tripartite ring (Arumugam et al., 2003). It has also been shown that the fragment has the potential to disrupt the interaction between Smc1 and Smc3 and dissolve cohesion, even in the presence of full length Scc1 (Weitzer et al., 2003). This may be a mechanism for preventing the re-establishment of cohesion during the metaphase to anaphase transition. It is likely that the *K all R* mutant is lethal because the C-terminal fragment cannot be ubiquitinated and degraded. However, expression of *Scc1 K all R* could be lethal for other reasons. It was not determined whether *Scc1 K all R* can associate with other cohesin subunits, or whether it is competent for cohesion. However, *Scc1 K all R* can be cleaved by Esp1/Separase (Figure 41).

To explore whether adding back a single lysine C-terminal to the cleavage site could restore viability, I added back a single lysine at position 319. Amazingly this mutant was able to complement for the temperature sensitivity of *scc1-73*, although it was MMS sensitive. Incredibly, Scc1 can function when it contains just one lysine, making it unlikely that the *Scc1 K all R* mutant is misfolded.

Both *Scc1 K all R* and *Scc1 K all R* R319K were not sumoylated. Therefore this suggests that K319 is subject to a different modification. Acetylation of Scc1 has not been

demonstrated *in vivo*. It is likely that this residue is ubiquitinated after cleavage, and is rapidly degraded, however this was not explored further.

7.1.12 Identifying potential sumoylation targets

As site directed mutagenesis of lysines to arginines had been unsuccessful, I tried the opposite approach of adding lysines back to the *Scc1 K all R* mutant. Initially, sections of WT sequence were added back to the mutant. All of the mutants produced were viable except A*bc*, expression of which was lethal in a dominant fashion. In this mutant, all the lysines added back are N-terminal to the Esp1 cleavage site, and therefore this mutant may be lethal for the same reason as the *K all R* mutant: the C-terminal fragment is stabilized and cannot be degraded. This mutant was also not sumoylated after treatment with MMS. All the other mutants were, leading to the conclusion that the sumoylation sites were in the regions designated B and C. However, A*bc* might not be sumoylated because it is incorrectly folded, and there are normally sumoylation sites in A.

Lysines from regions B and C were added back individually to the *Scc1 K all R* mutant. This allowed the identification of several lysines that could be modified by SUMO. When several of these lysines were added back individually a ladder of conjugates was observed. It is possible therefore that polysumoylation of cohesin is important after treatment with lethal doses of MMS. However, expression of most of the mutants, including some of those which could be modified by SUMO, and therefore probably have a surface exposed lysine, was lethal. Either the lysine added back was not subject to ubiquitination, or the mutant was misfolded. Other mutants were observed such as *Scc1 K all R* R363K and R391K which are not sumoylated, can complement for *scc1-73* temperature sensitivity and are only slightly sensitivity to MMS. This would suggest that sumoylation of Scc1 is not essential for normal growth. However, removal of Scc1 sumoylation may be compensated for by sumoylation of other cohesin subunits, hence the lack of phenotype.

Scc1 K all R R290K, R363K and R391K only have one lysine residue, and yet are only slightly more sensitive than the WT to MMS. These are not the potential acetylation sites implicated in cohesion establishment after damage (Heidinger-Pauli et al., 2009).

The potential sumoylation sites were then mutated together. If this mutant was not sumoylated, it could be used for further studies to investigate thoroughly the role of Scc1 sumoylation. Unfortunately, when this mutant was made the potential sumoylation site at K460 had not yet been identified. However, in the *K3rdR* mutant, even when 17/31 lysines were mutated, the resulting protein was functional and was sumoylated. This result could be because other sites are normally modified, but were not identified because of protein misfolding, or it could indicate that modification of Scc1 is extremely important and will occur even when all of the preferred binding sites are mutated.

7.1.13 Ulp1 and Ubc9 fusions

It has been observed that often many subunits of a multisubunit complex are modified by SUMO (Denison et al., 2005; Hannich et al., 2005; Panse et al., 2004; Sterner et al., 2006; Wohlschlegel et al., 2004). Cohesin is similar: Smc1, Smc3, Scc1 and Scc3 as well as the cohesin associated protein Pds5 are all sumoylated. Since sumoylation of a particular lysine in a single subunit, Scc1, does not seem to be important, it follows that SUMO attachment to any of these subunits may be functionally redundant.

I therefore used UFDS and a related technique developed by J. Torres-Rosell in which Ulp1 is fused to the target to analyze the function of cohesin sumoylation, as it may allow or prevent the sumoylation of the whole cohesin complex (the range of the fusions is still being investigated). However, it may also affect other nearby targets. The Scc1-Ubc9 fusion complements for the temperature sensitivity of *scc1-73*, however the Scc1-Ulp1 fusion does not (this study and Torres-Rosell et al., unpublished data).

As sumoylation is induced after DNA damage, the recruitment of these fusions to a DSB was investigated by ChIP. Given the result that all cohesin subunits are sumoylated in response to MMS, and Scc1 is sumoylated in response to a DSB, coupled with the ability of Scc1-Ubc9 to complement for *scc1-73*, it was hypothesized that Scc1-Ubc9 would be hyper recruited and Scc1-Ulp1 would not. Unexpectedly, both fusions were recruited. WT Scc1, Scc1-Ubc9 and Scc1-Ulp1 were all recruited to similar levels 2 and 4 hours after break induction, but whereas WT Scc1 and Scc1-Ubc9 levels did not increase further, Scc1-Ulp1 levels did. Therefore, fusion of Ulp1 to Scc1 does not block the

recruitment of cohesin, but actually promotes it. Although all of the fusions are recruited to the break, this experiment does not indicate whether the cohesin recruited is competent for cohesion. It has been published that Scc1 S83A mutants (which cannot be phosphorylated) and K84R K210R mutants are both recruited to a break, but cohesion does not occur (Heidinger-Pauli et al., 2008; Heidinger-Pauli et al., 2009). Therefore it may be that the –Ulp1 fusion cannot be made cohesive. This possibility is being investigated using an approach developed by the Koshland lab (described in Section 5.5). Surprisingly, preliminary data from our laboratory suggests that the –Ubc9 fusion is not competent for cohesion (discussed below).

Another approach to determine the function of sumoylation, similar to UFDS, is the covalent attachment of SUMO by a linear gene fusion (either N or C) to the target of interest. This can mimic the activities of the SUMO conjugate (Huang et al., 2003; Long et al., 2004; Ross et al., 2002; Yurchenko et al., 2006). This may be useful in determining whether constitutive sumoylation of individual subunits has an effect.

7.1.14 Scc1 is not cleaved after DNA damage in S/G2 phase in S. cerevisiae

In *S. pombe* mutations in both Cut2 (Securin) and Cut1 (Separase), and uncleavable Rad21 (Scc1) all cause DNA damage sensitivity (Nagao et al., 2004). The authors of this study conclude that Cut1 cleavage of Scc1 is required after DNA damage. However, using the *Scc1 K all R* mutant, which has a stabilized C-terminal fragment which can be easily detected by SDS-PAGE and western blot, I found no evidence that Scc1 is cleaved after DNA damage in S/G2 phase in *S. cerevisiae*.

There are several possible reasons for this. I used a lethal dose of MMS to induce DNA damage in a synchronous cell cycle, prior to mitosis. In the Nagao et al., (2004) study an asynchronous culture is UV irradiated. Therefore, the result may reflect differences in the type of DNA damage and the amount of damage. Also, the authors did not observe an enrichment of the cleaved product, only a retardation on SDS-PAGE gels. As an asynchronous culture was used, the cleavage observed could simply be due to passage through the cell cycle if the damage induced was not sufficient to cause a cell cycle arrest. It was not investigated whether irradiation could cause cleavage in S-phase or G2

arrested cells. My study relies on *Scc1 K all R* being incorporated into the cohesin complex. This has not been shown and although it can be seen that the mutant is cleaved during an unperturbed cell cycle in an Esp1 dependent manner, it may be that cleavage of the *Scc1 K all R* mutant does not occur after DNA damage.

The possibility of Scc1 cleavage after DNA damage could be investigated more thoroughly by arresting cells in G2/M and inducing a DSB using the HO endonuclease. This type of damage does not require passage of the replication fork for it to be detected. A N-terminally tagged Scc1 with a mutation that would allow stabilization of the N-terminal fragment could be used. This would have the advantage of not being lethal, as overexpression of the N-terminal fragment of Scc1 has no affect on viability (Rao et al., 2001).

7.2 What is the function of cohesin sumoylation?

Stresses, including osmotic, heat shock, oxidative, hypoxic and genotoxic stress can either induce changes in global sumoylation, or changes in the sumoylation state of individual targets (Tempe et al., 2008). Oxidative and ethanol stresses increase global sumoylation in *S. cerevisiae* (Zhou et al., 2004). Genotoxins seem to affect the sumoylation of specific targets, rather than inducing global changes. How sumoylation affects the activity of the target appears to be substrate specific.

Cohesin is sumoylated in asynchronously growing cells, and sumoylation is upregulated after DNA damage. However, sumoylation of Scc1 does not seem to be essential or required for growth on MMS, as several single lysine mutants, which did not appear to be sumoylated, complemented for the temperature sensitivity of *scc1-73* and allowed growth on MMS containing plates. However, where feasible it does occur. Even in the *Scc1 K3rdR* mutant, in which 17/31 lysines are mutated, including all of the potential sumoylation sites identified by site directed mutagenesis (except K460), Scc1 is sumoylated to a similar level to the WT. This raises the possibility that sumoylation of other subunits of the cohesin complex may complement for the lack of Scc1 sumoylation in the single lysine Scc1 mutants, but wherever possible Scc1 is sumoylated. This possibility was addressed using the –Ubc9 and –Ulp1 fusions.

What is the role of cohesin sumoylation after DNA damage? I initially hypothesized that sumoylation would be required to recruit cohesin to a DSB. However, both the –Ubc9 and –Ulp1 fusions are recruited, and at 6h recruitment of the –Ulp1 fusion is greater. This lead to the hypothesis that cohesin sumoylation was instead required for cohesion, in an analogous manner to phosphorylation of S83 or acetylation of K84 and K210 in Scc1 (Heidinger-Pauli et al., 2008; Heidinger-Pauli et al., 2009). However, preliminary data from our laboratory surprisingly suggests that it is the –Ubc9 fusion that is not cohesive after a DSB. This experiment was performed using an approach devised by the Koshland laboratory: cohesion is generated during S-phase using a temperature-sensitive allele of Scc1, cells are arrested in G2/M, and the formation of two DSBs, which cause the excision of a small region, is induced. This region contains a lacO array, and the cells express LacI-GFP. At the same time, induction of Scc1 or either of the two fusions is induced. The cells are then shifted to the non-permissive temperature, to inactivate S-phase generated cohesion. Expression of Scc1 or Scc1-Ulp1 resulted in a single GFP focus, demonstrating that they are competent for cohesion after a DSB. Cells expressing Scc1-Ubc9 had a high proportion of cells with two GFP foci, meaning that the sisters were not remaining cohesed, and that therefore Scc1-Ubc9 is not competent for cohesion.

This finding is especially unexpected as the Scc1-Ubc9 fusion can complement for the temperature sensitivity of *scc1-73*, and therefore must be capable of generating and maintaining cohesion during an unperturbed cell cycle. Scc1-Ubc9 must be deficient in a DNA damage specific function. It may be that the Scc1-Ubc9 fusion cannot maintain cohesion for long periods: it would be interesting to see whether cohesion can be maintained during a prolonged G2/M arrest (using nocodazole). This finding also suggests that strains carrying Scc1-Ubc9 as their sole copy of Scc1 should be DNA damage sensitive. Surprisingly, it seems that the Scc1-Ulp1 fusion is recruited to a DSB and can maintain cohesion, yet it cannot complement for *scc1-73*.

If sumoylation is not promoting the recruitment or loading of cohesin at a DSB, and may be detrimental for either establishment or maintenance of cohesion, what is its function and why is it upregulated after DNA damage? Sumoylation could dissolve cohesion, by promoting removal through a cleavage dependent or independent 'prophase-like' mechanism. Alternatively sumoylation of cohesin could be important for

relocalization of the break to the nuclear periphery, which could in turn stimulate cohesin removal. A schematic of the events that might be occurring in WT, Scc1-Ubc9 and Scc1-Ulp1 cells with and without DNA damage is presented in Figure 42.

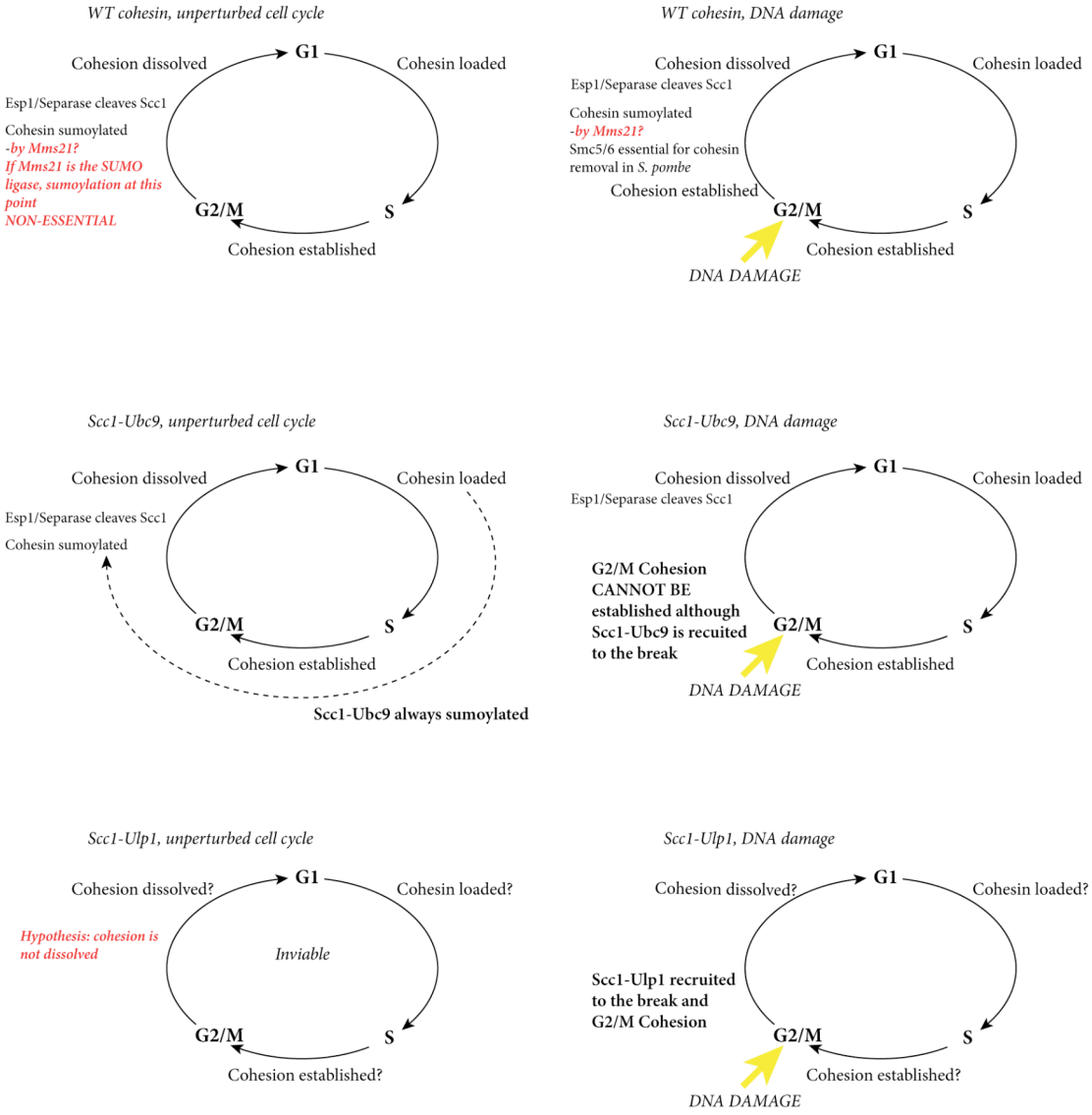


Figure 42: Is cohesin sumoylation required for cohesion dissolution?

Potential models of what may occur during the cell cycle and after DNA damage in cells expressing WT cohesin, Scc1-Ubc9 and Scc1-Ulp1 based on my results.

7.2.1 Sumoylation promoting dissolution of cohesion

After DNA damage, cohesion is maintained but at the same time repair needs to occur. Cohesin may need to be rapidly cycled to maintain cohesion but to also allow recombination proteins to access the site and for strand invasion and other processes involved in repair to take place.

This cycling could occur through a 'prophase-like' removal, in which no subunits are cleaved and cohesin is somehow removed. Sumoylation could act as a marker, just as SA2 (Scc3) phosphorylation acts as a marker for prophase removal in mammalian cells. Alternatively, the complex could be cleaved or degraded, through Esp1 or via another pathway, possibly SUMO-dependent ubiquitination. Although Cut2 (Esp1) has been shown to be required for DNA damage in *S. pombe* (Nagao et al., 2004), my results suggest that if cleavage does occur, it is not Esp1 dependent, as a decrease in cleavage products was observed after synchronous cells were treated with MMS in S/G2 phase.

If sumoylation does cause the dissolution of cohesion, how are Scc1-Ubc9 strains viable under normal conditions? Perhaps there is something different about cohesin after DNA damage. As mentioned in the introduction, cohesin is subject to a number of post-translational modifications after DNA damage, including phosphorylation and acetylation. It may be that a combination of modifications is required.

If this mechanism is in operation, then perhaps it operates in a cell-cycle specific manner as well. For example, Smc1 and Smc3 are sumoylated in G2/M. Perhaps this modification, in combination with the Plk1-dependent phosphorylation of Scc1, promotes cohesin removal at the metaphase to anaphase transition.

This would also potentially provide an explanation for the inviability of the Scc1-Ulp1 fusion despite the fact that it is both recruited to a break and is competent for cohesion. Perhaps it has a defect instead in the dissolution of cohesion. This could be investigated by analyzing a synchronous cell cycle to determine whether sister chromatids separate with correct kinetics, and whether Scc1 is cleaved. This model would also predict that expression of Scc1-Ulp1 is lethal even when WT Scc1 is present, which can easily be determined. Alternatively, sumoylation could be required for correct localization of cohesin to CARs. It has previously been reported that cohesion defects can occur if

cohesin does not localize correctly to CARs. Smc1 hinge and loop1 mutations have been identified which prevent cohesin localization to CARs but not the stable binding of cohesin to chromatin, which occurs with proper cell cycle timing and requires ATP and Scc1. These mutants are unable to establish cohesion (Milutinovich et al., 2007). The distribution of Scc1-Ulp1 on undamaged chromosomes could be determined by ChIP. However, Scc1-Ulp1 is obviously competent for localization to a DSB, although the mechanism of recruitment is different.

If sumoylation is required for unloading, this could be examined using a shut-off experiment. After induction of a break and of the fusions, expression of the fusions could be 'shut-off', by the addition of galactose, which inhibits transcription from the GAL promoter. The kinetics of disappearance of the fusions from the break could then be monitored by ChIP.

7.2.2 Sumoylation promoting relocalization of a DSB to the nuclear periphery, and/or SUMO dependent-ubiquitination of cohesin.

Unrepairable DSBs, such as those formed by overexpression of the HO endonuclease when *HMR* and *HML* are deleted, relocalize to the nuclear envelope (Kalocsay et al., 2009; Nagai et al., 2008). Kalocsay et al., (2009) suggest that this relocalization may prevent erroneous recombination which may result in chromosome translocations, loss of heterozygosity or deletion of repetitive sequences. Alternatively it may be required to sequester the damaged DNA until homology is provided, or to maintain the activity of genes close to a DSB. This relocalization requires the DNA damage checkpoint, Rad51 and the SUMO modification of H2A.Z (Kalocsay et al., 2009).

Alternatively, relocalization could allow the SUMO-dependent proteolysis of a target present at a DSB. The Slx5/8 complex is a SUMO dependent ubiquitin ligase. Slx5/8 form a complex present at nuclear pores and in foci throughout the nucleus. Both subunits have been found at DSBs (Cook et al., 2009; Nagai et al., 2008). Although Slx5 and Slx8 are not required for viability, *slx5Δ* and *slx8Δ* strains have elevated rates of Gross Chromosomal Rearrangement (GCR) and an increase in spontaneous Ddc2-YFP foci. Rad53 is also activated in asynchronous cultures (Zhang et al., 2006). *slx5Δ* and *slx8Δ*

mutants are also sensitive to HU (Mullen et al., 2001). In addition, the proteasome has also been found to be recruited to DSBs (Krogan et al., 2004).

Polysumo chains are the preferred substrate of Slx5/8, and ubiquitin is preferentially attached to SUMO (Mullen and Brill, 2008). As mentioned previously, a ladder is sometimes seen when just one lysine is present in Scc1, suggesting that polysumoylation could be taking place. So far the transcriptional regulator Mot1 is the only *in vivo* target of SUMO-dependent ubiquitin-mediated proteolysis identified in *S. cerevisiae* (Wang and Prelich, 2009). However, relocalization of DSB to the nuclear pore and the localization of Slx5 and Slx8 at the break suggest that the SUMO-directed ubiquitination and proteolysis of a target at the break is important, possibly for repair, as suggested by Nagai et al. (2008). It may be that cohesin is this target. Relocalization of a DSB to nuclear pores/the nuclear periphery in strains expressing Scc1-Ulp1 or Scc1-Ubc9 fusions is being investigated, as is the recruitment of Slx5/8 and proteasome recruitment to the DSB.

7.2.3 Is the Smc5/6 complex required for cohesin sumoylation and therefore removal?

A recent publication has suggested that Smc5/6 complex mutants have defects in removing cohesin after damage in *S. pombe* (Outwin et al., 2009). Could Smc5/6 promote the removal of cohesin by catalyzing its sumoylation? As mentioned previously, human Mms21 sumoylates Scc1 and SA2 (Scc3) in HeLa cells (Potts et al., 2006), and Mms21 is responsible for Smc1 and Smc3 sumoylation in *S. cerevisiae* (Takahashi et al., 2008), and has been shown to have a role in Scc1 sumoylation after DNA damage (this study). The Smc5/6 and cohesin complex have very similar genomic localization, and are both recruited to a DSB (De Piccoli et al., 2006; Lindroos et al., 2006; Potts et al., 2006). The Smc5/6 complex has also been found to be required for relocalization of damage. Mms21 is required for the relocalization of damage in the rDNA to an extranucleolar site, to prevent hyper-recombination and the excision of extrachromosomal circles (Torres-Rosell et al., 2007b). Also, the Smc5/6 complex is required for both relocalization and homologous recombination during alternative lengthening of telomeres in human cells

(Potts and Yu, 2007). It would also fit with the model in which the Smc5/6 promotes partioning. Mutations in budding yeast Smc5 and Smc6 affect the segregation and stability of the ribosomal array on chromosome XII (Torres-Rosell et al., 2005). It might also provide an explanation for the DNA damage sensitivity of Mms21 ligase-dead mutants (Andrews et al., 2005; Zhao and Blobel, 2005).

However, it has previously been reported that the human Smc5/6 complex recruits human cohesin to DSBs (Potts et al., 2006), and the authors speculate that Smc5/6 performs this function via sumoylation of cohesin by Mms21. This would not fit with a model in which Mms21 mediated sumoylation is required for cohesin removal that in turn is required for DNA repair. However, in *S. cerevisiae*, the Smc5/6 complex has not been shown to be required for the recruitment of cohesin, and in addition it appears that cohesin directs Smc5/6 localization, as the Smc6 binding pattern is altered in Scc1 mutants (without damage) (Lindroos et al., 2006).

7.3 Alternative explanations

7.3.1 Could the fusions be affecting the sumoylation of another factor?

The Scc1-Ubc9 fusion is able to sumoylate Smc1 (Torres-Rosell, unpublished data). It is possible that the fusion is able to sumoylate other factors, including those which also localize to a DSB after DNA damage. Scc1-Ubc9 cannot be competent to sumoylate all targets however, as the fusion cannot complement for the temperature sensitivity of *ubc9-1*. By the same mechanism perhaps the Ulp1 fusion is capable of desumoylating proteins other than the cohesin complex.

A number of proteins involved in DNA repair are SUMO modified, including PCNA (Hoege et al., 2002), Rad52 (Sacher et al., 2006), Ku70 (Zhao and Blobel, 2005), Sgs1 (Branzei et al., 2006), TopI (Jacquiau et al., 2005), Top II (Bachant et al., 2002) and H2A.Z (Kalocsay et al., 2009) in *S. cerevisiae*. It could be that it is the modulation of the sumoylation of another factor is responsible for my observations.

Alternatively, it could be sumoylation of another cohesin protein that is responsible for the phenotypes observed, for example Pds5. Pds5 is required to maintain

cohesion, and Pds5 sumoylation plays a role in cohesin dissolution (Stead et al., 2003). For this to be the explanation, the sumoylation of Pds5 would have to be such that cohesion maintenance during a normal cell cycle is unperturbed, but maintenance during a prolonged arrest is affected.

To investigate these possibilities it will be necessary to test if other proteins are hypermodified in the Scc1-Ubc9 fusion.

7.3.2 Could the fusions be affecting transcription?

A number of proteins involved in transcriptional control are sumoylated in response to stress. This is likely to control gene expression, allowing an appropriate cellular response to be induced. As mentioned in the introduction, cohesin also has a role in the regulation of transcription. Perhaps sumoylation of Scc1/cohesin affects this function of cohesin.

7.4 Summary

Cohesin is sumoylated during unperturbed growth, but cohesin becomes hyper-modified after DNA damage. Although potential sites of modification on Scc1 were identified, when these were mutated there was little effect on either viability or the sumoylation pattern obtained. Several Scc1 mutants containing a single lysine were obtained which were viable, insensitive to MMS and were not modified by SUMO. This suggests that either sumoylation of Scc1 is not essential and not required after DNA damage, or that sumoylation of the rest of the cohesin complex can compensate.

Using Ubc9 fusion directed sumoylation (UFDS) and Ulp1 fusion directed desumoylation, which potentially allow the modulation of sumoylation of the whole complex, the function of cohesin sumoylation is being investigated. Intriguing results using these fusions have already been obtained. Both fusions are recruited to a DSB. However, strains expressing Scc1-Ubc9 are unable to maintain cohesion after damage. This result suggests that sumoylation may be required for the dissolution of cohesion, and that this is potentially necessary after DNA damage, possibly for repair to occur.

Future work will continue to investigate this. In addition, it will be examined whether cohesin sumoylation could play a similar role during a normal cell cycle. It would also be interesting to determine whether Rec8 containing cohesin is sumoylated after Spo11 induced breaks during meiosis, and the effect of expressing Rec8-Ubc9 or Rec8-Ulp1 fusions.

8 Materials and methods

8.1 Chemicals

Unless otherwise stated, all chemicals and reagents were obtained from: Invitrogen, GE Healthcare, Bio Rad, Kodak, New England Biolabs, Promega, Roche, Sigma, Fluka, BDH Laboratory Supplies, BIO 101 Systems, Fisher Scientific, Operon and Qiagen.

8.2 S. cerevisiae strains, growth conditions and general methods

8.2.1 S. cerevisiae strains

A list of strains used in this study is provided in Table 8 below.

Strain description	**Strain name**	**Genotype**	**Reference**
WT (BY)	BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
strains isogenic to BY4741:			
HFSmt3	HZY1017	*6HisFLAG-smt3::kanMX6*	Zhou et al., 2004
Scc1-9myc	CCG5441	*Scc1-9myc NAT*	This study
Scc1-9myc, HFSmt3	CCG4944	*Scc1-9myc NAT, 6HisFLAG-smt3::kanMX6*	This study
Smc1-9myc	CCG5448	*Smc1-9myc NAT*	This study
Smc1-9myc, HFSmt3	CCG5348	*Smc1-9myc NAT, 6HisFLAG-smt3::kanMX6*	This study
Smc3-18myc	CCG5455	*Smc3-18myc HIS*	This study
Smc3-18myc, HFSmt3	CCG4948	*Smc3-18myc HIS 6HisFLAG-smt3::kanMX6*	This study
Scc3-9myc	CCG5885	*Scc3-9myc NAT*	This study
Scc3-9myc, HFSmt3	CCG5879	*Scc3-9myc NAT, 6HisFLAG-smt3::kanMX6*	This study
Scc1-3HA	CCG7038	*Scc1-3HA HYG*	This study
Scc1-3HA, HFSmt3	CCG7041	*Scc1-3HA HYG, 6HisFLAG-smt3::kanMX6*	This study
Scc1-6HA	CCG7044	*Scc1-6HA NAT*	This study
Scc1-6HA, HFSmt3	CCG7047	*Scc1-6HA NAT, 6HisFLAG-smt3::kanMX6*	This study

ubc9-1, HFSmt3, Scc1-3HA	CCG 7945	*trp1ΔNAT, ubc9-1myc TRP*	This study
Scc1-9myc, HFSmt3, *siz1Δ*	CCG 5339	*6HisFLAG-smt3::kanMX6 Scc1-9MYC NAT siz1ΔHYG*	This study
Scc1-9myc, HFSmt3, *siz2Δ*	CCG5342	*6HisFLAG-smt3::kanMX6 Scc1-9MYC NAT siz2ΔHYG*	This study
Scc1-9myc, HFSmt3, *siz1Δ siz2Δ*	CCG5706	*6HisFLAG-smt3::kanMX6 Scc1-9MYC NAT siz1ΔHYG siz2ΔHIS*	This study
Scc1-9myc, HFSmt3, *mms21CΔ*	CCG5345	*Scc1-9MYC (NAT) mms21CΔHYG*	This study
Scc1-9myc, HFSmt3, *chk1Δ*	CCG7943	*Scc1-9myc NAT, 6HisFLAG-smt3::kanMX6 chk1ΔHYG*	This study
Scc1-3HA, HFSmt3, *chk1Δ*	CCG7941	*Scc1-3HA HYG, 6HisFLAG-smt3::kanMX6 chk1ΔNAT*	This study

Strain description	**Strain name**	**Genotype**	**Reference**
WT (JD52)	JD52	*Mata, trp1-Δ1, ura3-52, his3-Δ200, leu2-3,112, lys2-801*	(Dohmen et al., 1995)
Isogenic to JD52: HSmt3	EJ362	*His6Smt3::TRP*	(Nathan et al., 2006)

Strain description	**Strain name**	**Genotype**	**Reference**
BBY48 (WT)	BBY48 (WT)	*Matalpha, trp1-1, ura3-52, his2Δ200, leu2-3, 112, lys2-801*	(Johnson et al., 1997)
Isogenic to BBY48: *Smt3Δ* + p316HFSMT3 (SMT3 promoter) in BBY48		*Smt3ΔHIS + p316-HF-SMT3* (SMT3 promoter)	from E. Johnson
Scc1-3HA, His-Smt3 *K all R RGG*	CCG7673	*Smt3ΔHIS Scc1-3HA HYG + pRS415 His-Smt3 K all R RGG LEU*	This study

Strain description	**Strain name**	**Genotype**	**Reference**
W303 (WT)	W303	*Mata can1-100, leu2-3, his3-11, trp1-1, ura3-1, ade2-1*	
Isogenic to W303: *esp1-1*	RJD 2629	*tetO112 ::URA3x3, 3tetR-GFP::HIS3, esp1-1*	(Azzam et al., 2004)
scc1-73	*scc1-73*	*scc1-73*	From K. Nasmyth

Strain description	Strain name	Genotype	Reference
Gal inducible unrepairable DSB + DDC2-GFP	CCG2781 (based on JKM179)	*MATalpha ade1-100 leu2,3-112 lys5 ura3-52 trp::hisG hoD hml::ADE1 hmr::ADE1 ade3::GAL-HO DDC2-GFP KanMX4*	De Piccoli et al., 2006
ubc9-1	CCG6133	*Mata leu2 ura3 his3 trp1 ade2 lys2 bar1 pep4:HIS3 ubc9-1:TRP1*	This study

Table 8: Strains used in this study

In addition, many of these strains were transformed with the plasmids described below. For example, WT (BY4741), HFSmt3 and *scc1-73* were all transformed with Scc1 mutant constructs to assay their sumoylation state and their effect on viability, as described in the text.

8.2.2 S. cerevisiae plasmids

A list of plasmids used in this study is provided in Table 9. Custom mutants were synthesized by GeneCust (GeneCust Europe, 30b rue Dominique Land, L-3505, Dudelange, Luxembourg).

Description	Name	Backbone	Reference
GAL HFSmt3	pSmt3	pRS315	(Johnson and Blobel, 1997)
His *Smt3 K all R* under the control of the Smt3 promoter on a centromeric vector	*Smt3 K all R*	pRS415	This study, constructed by GeneCust
His *Smt3 K all R RGG (I96R)* under the control of the Smt3 promoter on a centromeric vector	*Smt3 K all R RGG*	pRS415	This study, constructed by GeneCust
Smt3 with x1 internal HA tag under the control of the Smt3 promoter on a centromeric vector	Smt3 x1HA	pRS415	This study, constructed by GeneCust
Smt3 with x3 internal HA tags under the control of the Smt3 promoter on a centromeric vector	Smt3 x3HA	pRS415	This study, constructed by GeneCust
Smt3 with x1 internal HA tag and the *R93Q* mutation under the control of the Smt3 promoter on a centromeric vector	Smt3 x1HA *R93Q*	pRS415	This study, constructed by GeneCust

Smt3 with x3 internal HA tags and the *R93Q* mutation under the control of the Smt3 promoter on a centromeric vector	Smt3 x3HA *R93Q*	pRS415	This study, constructed by GeneCust
GAL Scc1 on an integrating vector	pFU67	YIp204	Uhlmann and Nasmyth 1998
A -9myc tag was added to Scc1 in pFU67. pFU67 was then used to construct the single lysine to arginine Scc1 mutants (named K18R to K556R)			This study
Scc1-3HA under the control of the GAL promoter on a centromeric vector	GAL WT Scc1-3HA	pRS415	This study, constructed by GeneCust
Scc1 K all R-3HA under the control of the GAL promoter on a centromeric vector	GAL *Scc1 K all R*	pRS415	This study, constructed by GeneCust
GAL Scc1 K all R was used to construct the single lysine Scc1 mutants in sections B and C (named K all R R165K to R556K)			This study
Scc1 A*bc* -3HA mutant: WT 'A' and K all R '*b*' and '*c*' under the control of the GAL promoter on a centromeric vector	GAL A*bc* Scc1-3HA	pRS415	This study
Scc1 a*B*c -3HA mutant under the control of the GAL promoter on a centromeric vector	GAL *a*B*c* Scc1-3HA	pRS415	This study
Scc1 *ab*C -3HA mutant under the control of the GAL promoter on a centromeric vector	GAL *ab*C Scc1-3HA	pRS415	This study
Scc1 AB*c* -3HA mutant under the control of the GAL promoter on a centromeric vector	GAL AB*c* Scc1-3HA	pRS415	This study
Scc1 aBC -3HA mutant under the control of the GAL promoter on a centromeric vector	GAL *a*BC Scc1-3HA	pRS415	This study
Scc1 A*b*C -3HA mutant under the control of the GAL promoter on a centromeric vector	GAL A*b*C Scc1-3HA	pRS415	This study
WT Scc1-3HA with a selectable marker (LEU) upstream of the promoter. Construct flanked each side by regions of homology to allow integration at the *SCC1* locus.	LEU-Scc1-3HA	pUC57	This study, constructed by GeneCust
Scc1-3HA with *K156R* and *K290R*. A selectable marker (LEU) upstream of the promoter. Construct flanked each side by regions of homology to allow integration at the *SCC1* locus.	Scc1 *K165R K290R* -3HA	pUC57	This study, constructed by GeneCust

Scc1-3HA with *K156R, K290R, K252R, K345R, K391R, K392R, K394R, K500R, K509R, K521R*. A selectable marker (LEU) upstream of the promoter. Construct flanked each side by regions of homology to allow integration at the *SCC1* locus.	Scc1 *K2ndR* -3HA	pUC57	This study, constructed by GeneCust
Scc1-3HA with *K156R, K290R, K252R, K345R, K391R, K392R, K394R, K500R, K509R, K521R, K319R, K324R, K382R, K472R, K494R, K552R, K556R*. A selectable marker (LEU) upstream of the promoter. Construct flanked each side by regions of homology to allow integration at the *SCC1* locus.	Scc1 *K3rdR* -3HA	pUC57	This study, constructed by GeneCust
Scc1-Ubc9-9myc fusion under the control of the GAL promoter on a centromeric vector	GAL Scc1-Ubc9-9myc	pRS416	This study
GAL Scc1-3HA	pSM957	YCplac111	Torres-Rosell, unpublished
GAL Scc1-3HA-Ubc9	pTR918	YCplac111	Torres-Rosell, unpublished
GAL Scc1-3HA-Ulp1	pTR921	YCplac111	Torres-Rosell, unpublished

Table 9: Plasmids used in this study

8.2.3 Media

Yeast extract peptone (YP) and synthetic drop out media lacking various amino acids were prepared according to standard recipes. 2% agar was added to create solid media (plates). Yeast were also grown on media containing 100µg/ml nourseothricin, 300µg/ml hygromycin B or 200µg/ml geneticin to select for strains carrying *NAT*, *HPH* or *KAN* markers respectively.

Yeast were grown on different carbon sources including glucose, galactose, raffinose, and lactate, all at a final concentration of 2% (stock solutions prepared and sterilized by filtration).

5-FOA plates consisted of complete synthetic media supplemented with 3% uracil and 1g/l 5-FOA.

For plates containing genotoxins, MMS, HU or benomyl (in DMSO) were added to warm YPD.

8.2.4 General growth conditions

Liquid cultures were inoculated from freshly streaked plates and grown until the culture reached mid-log phase (generally overnight, until OD_{600} 0.3-1). Liquid cultures were grown at 30°C (temperature sensitive strains at 25°C), in an in a SM1003 shaking incubator (Kuhner) at 150 rpm. The culture density was determined photometrically (OD_{600} of 1 is approximately equal to $1.5x10^7$cells/ml). Yeast on agar plates were stored at 4°C for up to 1-2 months. For long-term storage, stationary cultures were frozen in 15% (v/v) glycerol solutions at –80°C.

8.2.5 Induction of a DSB

DSBs were induced in strains containing stably integrated *GAL10::HO* and the fusions on centromeric plasmids (LEU marker). Strains were grown until they reached mid-log phase in a small volume of –leu media with glucose, then diluted into YP lactate overnight. Once mid-log phase had been reached again, HO expression was induced by adding galactose to a final concentration of 2%.

8.2.6 Competent cells

Based on Knop et al. (1999). Cells from an exponentially growing culture (OD_{600} 0.4-0.8) were harvested (4000rpm, 2 minutes), washed in sterile water followed by SORB solution (100mM LiOAc, 10mM Tris-HCl pH8, 1mM EDTA pH8, 1M sorbital; sterilized by filtration). The cell pellet was resuspended in 360µl SORB per 50ml culture. Denatured salmon sperm DNA was added to a final concentration of 1mg/ml. Competent cells were stored in 50µl aliquots at -80°C until use.

8.2.7 Transformation

Based on Knop et al. (1999). 0.2µg of circular or 1µg of linearized plasmid or PCR product were added to 50µl competent cells. 6 volumes of PEG (100mM LiOAc, 10mM Tris-HCl pH8, 1mM EDTA pH8, 40% (w/v) PEG-3350; sterilized by filtration, stored at 4°C) were added and the cell suspension was incubated for at least 30 minutes at room temperature. Immediately prior to heat shock, DMSO was added to a final concentration of 10%, and the cells incubated at 42°C for 15 minutes. Cells were pelleted (2000rpm, 3 minutes), and either resuspended in sterile water and plated on drop-out plates, or resuspended in YPD and outgrown for approximately 4 hours before being plated on antibiotic containing plates.

8.2.8 Genomic integration by homologous recombination

Chromosomal gene deletions or insertions of epitope tags were performed by a PCR strategy (Goldstein and McCusker, 1999; Janke et al., 2004; Knop et al., 1999; Longtine et al., 1998). Cassettes containing a selectable marker, and where appropriate an epitope tag, were amplified with primers that have tails which will direct integration by homologous recombination at a specific locus.

A YIplac vector (Gietz and Sugino, 1988) was used to integrate site specific Scc1 K to R constructs into the genome at the *URA3* locus. The plasmid was linearized by site-specific cleavage in the auxotrophy marker (in this case URA) before transformation. Linearized plasmids integrate at the endogenous locus of the marker gene.

To integrate the GeneCust constructs at the endogenous Scc1 locus, the constructs had a selectable marker at one end and an epitope tag at the other flanked by a 1kb region of homology.

Positive transformants in which the correct recombination event had occurred were identified by colony PCR, western blot, growth tests and/or microscopy (described below).

8.2.9 Drops/phenotype analysis by growth tests

10-fold dilutions of fresh cells were made in PBS. Cells were spotted (as 2µl drops) onto solid media, incubated at the appropriate temperature for 3-5 days and then photographed.

8.2.10 Cell cycle synchronization

Alpha factor was added to exponentially growing MATa cultures (OD600≈ 0.5) to a final concentration of $1.5x10^{-8}$M for *bar1Δ* strains and $3x10^{-6}$M for BAR1 strains. Cultures were monitored by microscopy until ≥90% cells has arrested in G1 with a 'shmoo' (typically 2-3 hours). To inactivate temperature sensitive alleles in G1, cells were shifted to 37°C for 30 minutes prior to release. To release cells, the culture was spun (4000rpm, 2 minutes) and the pellet washed three times in a large volume of prewarmed medium containing 0.1mg/ml pronase. The pellet was then resuspended in fresh medium and samples taken every 15 minutes.
HU was used to arrest cells in S-phase (0.2M final concentration). Nocodazole was used to arrest cells in G2/M, (15µg/ml final concentration, from stocks of 1.5mg/ml in DMSO).

8.2.11 Propidium Iodide staining for flow cytometry.

Cells were fixed in 70% ethanol and incubated at 4°C for at least one hour and then stored at 4°C for up to two weeks. Cells were pelleted (2 minutes at 5000rpm), and the cell pellet resuspended in saline-sodium citrate (SSC) containing either 0.1mg/ml RNase A (Qiagen) for incubation overnight or 0.8mg/ml RNase A for 2 hours at 37°C. Proteinase K (Qiagen) to a final concentration of 0.1mg/ml was then added and the cells incubated for 1 hour at 50°C. The cells were sonicated, then Propidium Iodide in SSC was added to a final concentration of 1µg/ml and the cells were incubated for 1 hour at room temperature in the dark, before being diluted five-fold in SSC and analysed using a FACScan cytometer (Becton Dickinson) and CellQuest Pro (Becton Dickinson) software.

8.3 General bacterial methods

8.3.1 General growth conditions

Liquid cultures were grown in LB media at 37°C with shaking at 200-250rpm. Solid cultures were grown on LB agar plates at 37°C. The selection of transformed bacteria was performed by the addition of either ampicillin (50µg/ml) or kanamycin (30µg/ml) to the media. Bacteria on plates were stored at 4°C for up to 7 days. For long term storage, stationary cultures were frozen in 15% (v/v) glycerol solutions at –80°C.

8.3.2 Transformation

One Shot Chemically Competent Cells (Invitrogen) were routinely used for bacterial transformation, according to the manufacture's instructions. 50µl cells were mixed with 10ng plasmid DNA and incubated on ice for 30 min. Cells were heat shocked for 30s at 42°C, followed by a 2 minute incubation on ice. 1ml pre-warmed S.O.C medium was added, and the cells were incubated at 37°C for 1h with shaking. Transformed cells were selected by plating the cell suspension on antibiotic-containing LB agar plates and incubating the plates overnight at 37°C.

8.4 Molecular biology and biochemical methods

8.4.1 Isolation of plasmid DNA from E. coli

Colonies were cultured in either 5 ml or 50 ml LB with selection. The QIAprep Spin Miniprep kit (Qiagen) or the Plasmid Midi Kit (Qiagen) was used to extract plasmid DNA from the small and large cultures respectively, according to the manufacturers instructions.

8.4.2 Isolation of genomic DNA from S. cerevisiae

Approximately 5 OD_{600} of cells were washed in water and resuspended in 500µl lysis buffer (100 mM Tris pH 8, 50 mM EDTA, 1% SDS). An equal volume of acid washed glass beads were added, and the cells lysed by 1 20s cycle, power 6 in a FastPrep™ FP120 (BIO 101) machine. The base of the tube was punctured with a hot 21-gauge needle and the suspension was centrifuged into a new tube, minus the glass beads. 300µl 6.2 M ammonium acetate (pH 7) was added and the mixture incubated for 5 minutes at 65°C. After cooling on ice for 5 minutes, the sample was spun (13000rpm, 5 minutes), and the supernatant transferred to a fresh tube. DNA was precipitated with 1 ml isopropanol (5 minutes at room temperature followed by centrifugation, 13000rpm, 5 minutes). The DNA pellet was washed with 70% ethanol, dried and then redissolved in 50µl distilled water with 1 mg/ml RNase A.

8.4.3 PCR

8.4.3.1 For HR integration, cloning or sequencing

The Expand High Fidelity System (Roche) was used (primers at 0.3µM and dNTP mix at 300µM). For products with a high GC content, DMSO was added to a final concentration of 5%.

PCR conditions: initial denaturation step of 95°C for 3 minutes; amplification cycle of 94°C for 1 minute, 52°C for 30s, 72°C for 1minute/kb repeated 10 times then 94°C for 1 minute, 55°C for 30s, 72°C for 1minute/kb repeated 20 times; and a final extension step of 72°C for 10 minutes.

8.4.3.2 For colony PCR

A small amount of yeast from a colony was resuspended in 25µl Go Taq Flexi DNA polymerase (Promega) PCR reaction mix (primers at 0.3µM and dNTP mix at 300µM).

PCR conditions: initial denaturation step of 95°C for 5 minutes; amplification cycle of 94°C for 30s, 55°C for 30s, 72°C for 1minute/kb repeated 30 times; and a final extension

step of 72°C for 10 minutes.

8.4.4 Site directed mutagenesis

Site directed mutagenesis was performed using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturers instructions. Briefly, the desired mutation was incorporated into primers (designed using the QuikChange Primer Design programme available at http://www.stratagene.com/qcprimerdesign) then used for PCR. Incorporation of the primers during PCR generates a mutated plasmid containing staggered nicks. PCR was performed using the standard reaction mix containing 50ng template DNA and 125ng of each primer. The PCR conditions used were: initial denaturation step of 95°C for 1 minute; amplification cycle of 95°C for 50s, 60C for 50s, 68°C for 10 minutes for 18 cycles; and a final extension step of 68°C for 7 minutes. Following PCR, parental DNA (not containing the mutation) was digested by the addition of 1µl *DpnI* and the mixture incubated for 1-2 hours at 37°C. *DpnI* specifically digests methylated and hemimethylated DNA. The nicked vector containing the desired mutation was then transformed into XL10-Gold Ultracompetent cells. 2µl of the digested PCR mix was added to 45µl of competent cells that had been pre-treated with β-mercaptoethanol. The mixture was incubated on ice for 30 minutes, and then heat shocked (30s, 42°C). The cells were immediately chilled on ice before being resuspended in 250µl S.O.C medium and allowed to outgrow for 1h (37°C with shaking at 200-250rpm). Cells were plated onto LB plates containing ampicillin (50µg/ml).

8.4.5 Molecular cloning

8.4.5.1 Restriction digest of DNA

Restriction enzymes were employed for sequence-specific cleavage of DNA according to the manufacturer's instructions (New England Biolabs). For the digestion of 1µg DNA, 5 to 10 units of restriction enzyme were usually used. Reaction samples were incubated at the recommended temperature for between 1h and overnight.

8.4.5.2 Separation of DNA fragments by gel electrophoresis

DNA samples in loading buffer (Promega) were run on 0.8-2% agarose gels, containing 0.5µg/ml ethidium bromide at 100V in TBE buffer. DNA could be visualized using an UV transilluminator (324nm).

8.4.5.3 Purification of DNA fragments from agarose gels

The DNA fragment was excised from the gel using a clean razor blade. DNA was purified using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

8.4.5.4 Ligation of DNA fragments

Digested fragments were ligated into dephosphorylated digested vector (dephosphorylated using Shrimp Alkaline Phosphatase (Promega)) using the Rapid DNA ligation kit (Roche), according to the manufacturer's instructions.
PCR products were cloned into the Zero Blunt TOPO PCR Cloning vector (Invitrogen), according to the manufacturer's instructions.

8.4.5.5 DNA sequencing

DNA sequencing reactions were carried out by the MRC Genomics Core Facility, CSC, Hammersmith Hospital, London.

8.4.6 SDS-PAGE and Western blotting

8.4.6.1 SDS PAGE

The Bio Rad Mini-PROTEAN 3 system or the Invitrogen NuPAGE system was used for SDS-PAGE.

8% acrylamide gels (resolving casting solution: 26.7% ProtoGel Acrylamide solution (National Diagnostics), 26% ProtoGel Resolving Buffer, 0.1% ammonium persulphate, 0.1% N,N,N1,N1 tetramethylethylenediamine (TEMED; National Diagnostics); stacking casting solution: 13% ProtoGel Acrylamide solution, 25% ProtoGel Stacking Buffer, 0.1% APS, 0.1% TEMED) were used with the Bio-Rad system. Gels were run at 200 V in Tris-glycine-SDS running buffer (National Diagnostics).
Pre-cast 4-12% Bis-Tris or 3-8% Tris-Acetate NuPAGE gels were run in NuPAGE MOPS buffer or in NuPAGE Tris-Acetate buffer respectively, according to the manufacturer's instructions. 4-12% gels were run at 200V, 3-8% gels were run at 150V.

8.4.6.2 Coomassie staining

Gels were stained with Coomassie (1.25% w/v Coomassie Brilliant Blue, 50% methanol, 10% acetic acid), destained with destaining solution (10% acetic acid, 30% methanol) then air-dried. For staining prior to mass spectrometry, SimplyBlue SafeStain (Invitrogen) was used, according to the manufacturer's instructions.

8.4.6.3 Western blot

SDS-PAGE gels were transferred to polyvinylidene fluoride transfer membrane (Hybond-P, Amersham Biosciences) in either the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell or by using the XCell SureLock Mini Cell Transfer module (Invitrogen). The Bio-Rad system was used in conjunction with Tris-Glycine blotting buffer (National Diagnostics) containing 20% methanol, and run for 1h at 200V or overnight at 30V. The NuPAGE system was used with NuPAGE transfer buffer containing 20% methanol and run for 1h at 30V.

8.4.6.4 Immunological detection

Membranes were blocked in 5% skimmed milk powder in PBS with 0.1% Tween 20 (PBS-T) for 1h or overnight at 4°C, then incubated with either mouse monoclonal anti-c-myc IgG1κ antibody 9E10 (Roche), anti-HA IgG1 antibody 12CA5 (Roche), or anti-FLAG IgG2 antibody M2 (Sigma) at a 1/5000 dilution in blocking solution for

between 1 hour at room temperature to overnight at 4°C. Following several washes in PBS-T, membranes were incubated with the sheep anti-mouse IgG Horseradish Peroxidase-linked antibody (GE Healthcare) at a 1/10000 dilution in blocking solution. After several further washes in PBS-T, membranes were incubated with the ECL Plus Western Blotting Detection System (GE Healthcare) followed by exposure to ECL Hyperfilm (GE Healthcare), to detect the secondary antibody.

8.4.7 Preparation of extracts

8.4.7.1 Post alkaline extraction

Based on Kushnirov (2000) (Kushnirov, 2000). 2.5OD_{600} of cells were harvested by centrifugation from liquid culture or scraped off an agar plate and resuspended in 100µl sterile water. 100µl 0.2M NaOH was added, and the mixture was incubated for 5 minutes at room temperature, following which the cells were pelleted (13000rpm, 30s) and resuspended in 50µl sample buffer (0.06M Tris-HCl pH6.8, 5% glycerol, 2% SDS, 4% β-mercaptoethanol, 0.0025% bromophenol blue). Samples were boiled for 3 minutes (95°C) and pelleted again. The supernatant was loaded onto a SDS-PAGE gel or stored at -20°C.

8.4.7.2 TCA extraction

TCA extracts were prepared as in Keogh et al. (2006) (Keogh et al., 2006). Cells were collected by centrifugation (4000rpm, 2 minutes) and washed with 20% TCA. The TCA was aspirated and the pellets frozen at -80°C. All of the following purification steps were performed on ice with pre-chilled solutions. Cells were resuspended in 250µl 20% TCA, glass beads were added and the cells broken by 1 40s cycle, power 5.5 in a FastPrep™ FP120 (BIO 101) machine. Tubes were pierced with a hot needle and placed onto of fresh eppendorfs and spun (1000rpm, 2 minutes) to collect lysate minus glass beads. The glass beads were washed with 1ml 5% TCA and this was added to the lysate, and mixed by pipetting. The precipitated proteins were collected by centrifugation (14000rpm, 10 minutes 4°C) and then pellets were washed with 750µl 100% ethanol. Proteins were

solubilized in 50µl 1M Tris pH 8 and 100µl x2 SDS-PAGE loading buffer (60mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.2% bromophenol blue) and boiled for 5 minutes at 95°C. Insoluble material was removed by centrifugation (14000rpm, 5 minutes, room temperature) and the supernatant either stored at -20°C or loaded immediately onto a SDS-PAGE mini-gel.

8.4.8 Protein purification

8.4.8.1 Purification of His-tagged proteins

Based on protocols from Denison et al. (2005) and Sacher et al. (2005) (Denison et al., 2005; Sacher et al., 2005). 100 OD of cells were harvested (4000rpm, 2 minutes), washed once with water, and the cell pellet frozen at -80°C. The cell pellet was resuspended in 500µl Buffer A (8M Urea, 100mM NaH_2PO_4, 10mM Tris HCl, 0.05% Tween pH 8), an equal volume of glass beads were added, and the cells lysed by 1 45s cycle, power 6 in a FastPrep™ FP120 (BIO 101) machine. Tubes were pierced with a hot needle and placed onto of fresh eppendorfs and spun (1000rpm, 2 minutes) to collect lysate minus glass beads. Cell lysate was clarified by centrifugation (14000rpm, 15 minutes, 4°C) Protein concentration was determined using a Bradford Assay, and 15mg of protein in 1ml was added to 50µl of a 50:50 slurry of Ni-NTA beads (Qiagen) (prewashed in Buffer A). Imidazole was added to a final concentration of 20mM, to reduce non-specific binding. Proteins were bound for 2-3 hours at 4°C on a rotating platform, before the beads were washed 3 times in Buffer A containing 2mM imidazole, followed by 5 washes in Buffer B (8M Urea, 100mM NaH_2PO_4, 10mM Tris HCl, 0.05% Tween pH 6.3). Bound proteins were eluted off the beads using 30µl x2 NuPAGE loading buffer (Invitrogen) supplemented with 4% β-mercaptoethanol and 200mM EDTA. Eluates were loaded onto 3-8% Tris-Acetate or 4-12% Bis-Tris pre-cast NuPAGE gels (Invitrogen) and analysed by western blotting.

8.4.8.2 Immunopreciptiation

Immunoprecipitation was performed using a μMACS™ Epitope Tag Protein Isolation Kit (MACS Molecular). 100 OD of cells were harvested (4000rpm, 2 minutes), washed once with water, and the cell pellet frozen at -80°C. The cell pellet was resuspended in 500μl pre-chilled lysis buffer (150mM NaCl, 1% Triton X-100, 50mM Tris HCl pH 8) supplemented with 1mM PMSF and 10mM NEM, and an equal volume of glass beads were added. Cells were lysed by 1 40s cycle, power 5.5 in a FastPrep™ FP120 (BIO 101) machine. Tubes were pierced with a hot needle and placed onto of fresh eppendorfs and spun (1000rpm, 2 minutes) to collect lysate minus glass beads. Cell lysate was clarified by centrifugation (14000rpm, 15 minutes, 4°C). Protein concentration was determined using a Bradford Assay. 50μl Anti-Tag MicroBeads were added to the lysate (containing 15mg of protein in a volume of 1ml), and the suspension mixed well before being incubated on ice for 30 minutes. The cell lysate was then added to a pre-equilibrated μ Column. The column was then washed four times with Wash Buffer 1 (150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS 50mM Tris HCl pH 8) and then twice with Wash Buffer 2 (20mM Tris HCl pH 7.5). Bound proteins were eluted from the column with hot (95°C) Elution Buffer (50mM Tris HCl pH 6.8, 50mM DTT, 1% SDS, 1mM EDTA, 0.005% Bromophenol blue, 10% Glycerol). Eluted proteins were analysed directly by SDS-PAGE, or stored at -20°C.

8.4.8.3 Purification for mass spectrometry

1 litre of culture was grown to OD_{600} 1 in YPD at 30°C, MMS was then added to a final concentration of 0.3% for a further 2 hours. Cells were harvested, washed twice in water, frozen in liquid N_2, then broken using a Freezer Mill (Spex CertiPrep). The cell powder was resuspended in IP buffer (50mM Hepes-NaOH pH7.5, 150mM $NaCl_2$, 5mM EDTA, 0.1%NP-40, 5mM DTT, 0.2mM PMSF, EDTA-free Complete protease inhibitor cocktail tablets (Roche)). The lysate was cleared by centrifugation (2 cycles of 15000rpm for 1h, 4°C). The lysate was then bound in batch to 500μl of α-HA beads (Roche) overnight at 4°C. The beads were washed twice in batch with IP buffer containing protease inhibitors

(10mM NEM and protease inhibitor cocktail tablets (Roche)) and 0.2µM PMSF and 5mM DTT. They were then loaded onto a column (BioRad Poly-Prep Column) and then washed with a further 50ml lysis buffer. Bound proteins were eluted in 5 fractions with 100µl IP buffer containing 5xHA peptide. Eluate was TCA precipitated, resuspended in Tris-base pH8 and NuPAGE LDS Sample buffer (Invitrogen) containing 4% ß-ME and boiled. Proteins were then TCA precipitated.

The eluate was fractionated on a 4-12% gradient NuPAGE gel (Invitrogen) and stained using SimplyBlue™ Safe Stain (Invitrogen). Individual gel-lanes were cut manually into approximately 60 x 1.0mm bands with a razor blade, and each band was transferred to a 96-well plate (Genomic Solutions) and sent to Richard Jones (NextGen Sciences Inc, Ann Arbor, USA) for mass spectrometry, using an Orbitrap-LTQ machine.

8.4.9 Chromatin Immunoprecipitation (ChIP)

Based on Nelson et al. (2006) (Nelson et al., 2006).

For ChIP analysis, 50ml of culture (at OD_{600}~0.5) was fixed with formaldehyde (final concentration 1.42%) for 15 minutes at room temperature. The formaldehyde was quenched with glycine (final concentration 125mM) for 5 minutes at room temperature and the cells harvested by centrifugation (4000rpm, 2 minutes). The pellet was washed with PBS, transferred to a screw cap tube, and snap frozen in liquid nitrogen. Pellets were stored at -80°C.

Pellets were resuspended in 100µl IP buffer (150mM NaCl, 50mM Tris-HCl (pH 7.5), 5mM EDTA, NP-40 (0.5% v/v), Triton X-100 (1.0% v/v)) containing PMSF (final concentration 1mM) and Complete protease inhibitor cocktail (Roche), and 500µl glass beads were added. Cells were lysed by two 20s cycles, power 6.5 in a FastPrep™ FP120 (BIO 101) machine, with 5 minutes on ice in between cycles. 300µl IP buffer containing PMSF and protease inhibitors was then added. Tubes were pierced with a hot needle and placed on top of fresh eppendorfs and spun (1000rpm, 2 minutes) to collect lysate minus glass beads. Samples were pelleted (13000rpm, 1 minute, 4°C). The nuclear pellet was resuspended thoroughly in 1ml IP buffer containing PMSF and protease inhibitors. The

chromatin was sonicated for 1 hour (15s ON 15s OFF at high power at 4°C) (Diagenode Biorupter). At this point, sonicated chromatin could be stored at -80°C.
120µl (30% of sample) was taken for input and 400µl for IP. After clarification (13000rpm, 1 minute) input DNA was precipitated from the supernatant by the addition of KaOAc to a final concentration of 0.3M and 2.75 volumes of ethanol. The mixture was incubated at -20°C for 2 hours-overnight, then spun (13000rpm, 5 minutes) and the supernatant discarded. 100µl 10% (w/v) Chelex 100 suspension was added to the dried pellet and the sample boiled for 20 min. The supernatant was cleaned using the PCR purification kit (Qiagen) according to the manufacturers instructions, and the DNA eluted in 250µl water. DNA was stored at -20°C.
2µg α-HA antibody (Roche) was added to 400µl chromatin for the IP, and the samples were incubated in an ultrasonic water bath for 30 min at 4°C. After clarification (13000rpm, 2 minutes, 4°C) the supernatant was added to 100µl of a 50:50 slurry of Protein A and Protein G beads (Roche), which had been pre-equilibrated in IP buffer. The sample and beads were incubated for 2 hours-overnight on a rotating platform at 4°C. The beads were then washed 3 times in IP buffer, and then 250µl 10% (w/v) Chelex 100 suspension added and the sample boiled for 20 minutes. After spinning down (13000rpm, 1 minute) the supernatant was transferred into a fresh tube and stored at -20°C.

8.4.10 Real-time PCR

PCR reactions were performed using the Sensimix NoRef Kit (Quantance). Reactions were carried out according to the manufacturers instructions in a total volume of 20µl containing 3µl Input or IP DNA and oligonucleotide primer pairs (final concentration 1.5µM). Amplification was performed in a DNA Engine Opticon2 thermal cycler and analysed using Opticon software (MJ Research) or in a Bio-Rad C1000 thermal cycler in conjunction with the Bio-Rad CFX96 Real-time-system and analysed using CFX manager (Bio-Rad).
The primers used for the analysis of an HO-break at the *MAT* locus have been described (Shroff et al., 2004). The melting curve of each primer pair was analysed to confirm the

absence of contaminant PCR products. The relative occupancy of the immunoprecipitated factor at a locus was estimated using the following equation: $2^{(CtIP - CtInput)}$, where CtIP and CtInput are mean threshold cycles of PCR done in duplicate on Input and IP DNA samples.

8.4.11 Microscopy

For fluorescence microscopy, series of z-focal plane images were collected on Leica IRB using a Hamamatsu D742-95 digital camera and OpenLab™ software (Improvision). A tuneable light source (Polychrome IV (Photonics)) with a Xenon lamp or an ultraviolet mercury lamp (Leica) were used. Images in different z-axis planes were flattened into a two-dimensional projection and processed in OpenLab. To visualize the nuclei of intact cells, cells were resuspended in a final concentration of 1% Triton X-100 and 25ng/ml DAPI/Antifade.

8.5 Additional unlisted apparatus

micro-spectrophotometer (DNA)	Nanodrop® Spectrophotometer ND-1000
PCR machine	Eppendorf Mastercycler
spectrophotometer (cells)	UV1101 Biotech Photometer
water distiller	Elga Maxima Ultra-Pure

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10 Abbreviations

3C	Chromosome conformation capture
5-FOA	5-fluoroorotic acid
APC	Anaphase Promoting Complex
APS	Ammonium persulphate
ATP	Adenosine triphosphate
CAR	Cohesin Associated Region
ChIP	Chromatin Immunoprecipitation
Chr	Chromosome
CTCF	CCCTC-binding factor
DAPI	4,6-Diaminidino-2-phenylindole
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DSB	Double Strand Break
DTT	dithiothreitol, threo-2,3-dihydroxy-1,4-dithiolbutane
EDTA	Ethylenediaminetetraacetic acid
FRAP	Fluorescence recovery after photobleaching
FRET	Forster resonance energy transfer
G1	Growth 1
G2	Growth 2
GCR	Gross chromosomal rearrangement
GFP	Green fluorescent protein
GPS	Group-based phosphorylation scoring
h	hour
HA	Hemagglutinin
HeLa	Henrietta Lacks
HMR	Hidden MAT Right
HO	Homothallic switching
HP1	Heterochromatin protein 1
HR	Homologous recombination
HU	Hydroxyurea
IP	Immunoprecipitation
IR	Ionizing radiation
LB	Luria-Bertani
M	Mitosis
MMS	Methyl methanesulfonate
MOPS	3-(N-Morpholino)-propanesulphonic acid
NEM	N-ethylmaleimide
Nz	Nocodazole
OD	Optical density

OMP	Oroline-5'-monophosphate
PBS	Phosphate-buffered saline
PBS-T	PBS-Tween 20
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulphonylfluoride
PP2A	Protein phosphatase 2A complex
PVDF	Polyvinylidene Difluoride
rDNA	Ribosomal DNA
RF-C	Replication Factor C
RING	Really Interesting New Gene
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
rpm	Rotations per minute
S	Synthesis
s	Seconds
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SMC	Structural Maintenance of Chromosomes
SUMO	Small ubiquitin-like modifier
TAE	Tris-acetate-EDTA
TAP	Tandem Affinity Purification
TBE	Tris-borate-EDTA
TCA	Trichloroacetic acid
TEMED	N,N,N1,N1-tetramethylethylenediamine
TEV	Tobacco etch virus
U	Unit
UFDS	Ubc9 fusion directed sumoylation
UV	Ultraviolet
v	Volume
V	Volt
w	Weight
WCE	Whole cell extract
WT	Wild-type
YPD	Yeast extract peptone dextrose

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