Title: Genetic evidence that the acetylation of the Smc3p subunit of cohesin modulates its ATP-bound state to promote cohesion establishment in *Saccharomyces cerevisiae*

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Abstract:
Sister chromatid cohesion refers to the process by which sister chromatids are tethered together until the metaphase to anaphase transition. The evolutionarily conserved cohesin complex mediates sister chromatid cohesion. Cohesin not only insures proper chromosome segregation, but also promotes high fidelity DNA repair and transcriptional regulation. Two subunits of cohesin (Smc1p, Smc3p) are members of the Structural Maintenance of Chromosomes (SMC) family. The SMC family is recognized by their large coiled coil arms and conserved ATP binding cassette (ABC)-like ATPase domain. While both Smc1p and Smc3p ATP binding and hydrolysis are essential for cohesin function in vivo, little is known about how this core enzymatic activity is regulated to facilitate sister chromatid cohesion. Here we use SMC mutant proteins to block specific steps in cohesin’s ATPase cycle in *Saccharomyces cerevisiae*. We show that blocking Smc3p-mediated ATP binding or Smc3p ATP hydrolysis traps unique functional states in
cohesion. Finally, we provide evidence that Smc3p acetylation, which has an essential role in cohesion establishment, modulates the Smc3p ATP-bound state.
INTRODUCTION

Structural Maintenance of chromosomes (SMC) complexes facilitate higher order chromosome structure important for sister chromatid cohesion, condensation, transcription, as well as DNA repair. These complexes tether two regions of chromatin either within the same chromatin strand, or between two separate strands. All SMC complexes contain two SMC protein subunits. Each SMC protein folds back on itself through a ‘hinge’ domain to bring together the walker A motif in the N terminus and the walker B motif in the C terminus, thereby generating a globular head domain with an ABC (ATP-binding cassette) ATPase domain (reviewed in O’Neill et al. 2008). The hinge and head domains are connected by a 45nM long anti-parallel coiled coil arm. In solution, the two SMC proteins dimerize at their hinge and their head domains forming a large ring (reviewed in O’Neill et al. 2008). The molecular role of ATP in SMC function is poorly understood.

Cohesin, the SMC complex required for sister chromatid cohesion, provides an excellent experimental system to elucidate the role of ATP in SMC function. In *Saccharomyces cerevisiae*, cohesin consists of Smc1p and Smc3p, as well as two other core components, Mcd1p (also known as Scc1p or Rad21p) and Scc3p/Irr1p. Cohesin generates sister chromatid cohesion through at least two distinct steps. First, cohesin binds to chromosomes at the pericentric regions and at some intergenic regions along chromosome arms, coined cohesion associated regions (CAR) (Blat and Kleckner 1999; Glynn et al. 2004; Laloraya et al. 2000; Megee et al. 1999; Weber et al. 2004). Cohesin binding to chromosomes is dependent upon the Scc2p/Scc4p loading complex (Ciosk et
al. 2000). In a second step called cohesion establishment or generation, the chromatin-associated cohesin is stimulated into a chromatin tethering form (SKIBBENS et al. 1999; TOTH et al. 1999). This conversion step is regulated by the conserved Eco1p (Ctf7p) acetyltransferase which acetylates cohesin, thereby antagonizing an inhibitor complex containing Wpl1p (Rad61p) and Pds5p (ROWLAND et al. 2009) (SUTANI et al. 2009) (BEN-SHAHAR et al. 2008; UNAL et al. 2008) (ZHANG et al. 2008a) (HEIDINGER-PAULI et al. 2008).

The models that explain how cohesin binds to chromosomes and tethers sister chromatids all invoke a large conformational change mediated by ATP binding and/or hydrolysis. For example, the embrace model posits that ATP binding or ATP hydrolysis causes the cohesin ring to open at the hinge region to allow DNA to enter the ring. Another round of ATP binding and/or hydrolysis reopens the cohesin ring to acquire the replicated sister chromatid and establish cohesion (ROWLAND et al. 2009; SUTANI et al. 2009). Alternatively, oligomerization-based models propose that cohesion binding and establishment occur by two distinct biochemical steps similar to what has been proposed for condensin. In these models, the first round of ATP binding/hydrolysis promotes cohesin’s chromatin binding, while a second subsequent ATP or ADP dependent conformational change allows oligomerization of two or more cohesin molecules, each bound to a separate chromatid (HIRANO and HIRANO 2006; HUANG et al. 2005; MILUTINOVICH and KOSHLAND 2003; ZHANG et al. 2008b).

It has been recently shown that the Smc3p residues, K112 and K113, are acetylated by Eco1p after cohesin binds chromatin, and these acylations are a prerequisite to generate cohesion during S phase (UNAL et al. 2008) (BEN-SHAHAR et al.
2008) (ZHANG et al. 2008a). In the absence of these modifications, cohesin binds to chromatin but fails to tether the sister chromatids. The proximity of these residues to the ATPase domain suggests that the acetylation of these residues might modulate cohesin’s Smc3 ATPase to promote cohesion generation following cohesin binding to chromatin (UNAL et al. 2008) (HEIDINGER-PAULI et al. 2008).

Despite the significant progress in understanding cohesin function, how Smc3p acetylation might affect ATP binding and hydrolysis has been difficult to study both in vivo and in vitro. In vivo, mutations in Smc3p that specifically block ATP binding or ATP hydrolysis give identical phenotypes: the cohesin complex assembles, but is unable to bind chromatin (ARUMUGAM et al. 2003). Hence, the structures generated with or without ATP binding are thought to lead to inert complexes in the nucleoplasm. Because these mutations block cohesin binding to chromatin, the first step in cohesin function, it has not been feasible to use these mutations to assess whether there is a distinct role for ATP in the second step of cohesion generation. Furthermore, testing the impact of acetylation on Smc3 ATPase activity in vitro has not been feasible because Smc3p has extremely poor in vitro ATPase activity and changes in this activity are obscured by the ATPase activity of Smc1p, its heterodimeric partner.

To begin to overcome these technical hurdles we decided to re-investigate the phenotypes of smc3 mutants defective in ATP binding and ATP hydrolysis in chromatin binding and cohesion generation. Surprisingly, we discovered that the ATP binding and hydrolysis mutants yield dramatically different phenotypes upon over-expression. Inhibition of Smc3p’s ATP hydrolysis, but not ATP binding, captures a unique functional
state of cohesin, revealed by an extremely potent dominant negative phenotype. We exploit this genetic distinction to provide evidence that Smc3p acetylation modulates the Smc3p ATP-bound state.

MATERIALS AND METHODS

Yeast Strains and Media: Yeast strains used in this study are listed in Table S1. All strains are derivatives of the A364A genetic background unless noted otherwise. Plasmid information is also given within the strain table, written as a part of the relevant strain containing the plasmid. Yeast strains were grown in SC-LEU or YEP media as described supplemented with 2% glucose (EMD) (GUTHRIE and FINK 1991). Media used for galactose inductions contained YEP supplemented with 3% glycerol (EMD, 30% v/v stock), 2% lactic acid (Fisher, 40% v/v stock pH 5.7).

Cell Synchronization: Cells were arrested in G1 phase by the addition of α-Factor (1.5 x 10^{-8} M final). To release cells from α-Factor induced G1 arrest, cells were washed 3x media containing pronase E(0.1 mg/ml, Sigma) and 1x in media without pronase. Exponentially growing cultures were arrested in G2/M using nocodazole (15 μg/ml final) in the indicated media. If cultures contained a plasmid, cells were grown to exponential phase in selective media, pelleted and then resuspended in YEPD for subsequent arrest.

Chromosome Spreads: Chromosome spreads were performed as described (HARTMAN et al. 2000).

Chromatin Immunoprecipitation: Chromatin Immunoprecipitation was performed as described (HEIDINGER-PAULI et al. 2008). The only exception was that cells assayed by
Chromatin Immunoprecipitation using the 6HA epitope tagged Smc3p were fixed for 15 minutes instead of 2 hours.

**Immunoprecipitation and Western Blotting Analysis:** Cells were pelleted and washed with dH$_2$O and frozen in liquid nitrogen. Pellets were resuspended in 350µL IPH150 buffer (50mM Tris pH 8, 150 mM NaCl, 5mM EDTA, 0.5% NP-40, 1mM DTT) containing 100µM sirtonol, 50mM sodium butyrate, and protease mini tablets (Roche). The experiment shown in Figure 1 also contained 12mg/ml trichostatin. Cells were lysed by adding 200-450µM glass beads (Sigma) to the resuspended pellets followed by bead beating for 90 seconds using a Biospec™ mini bead beater. The soluble fraction was separated from the insoluble fraction by two centrifugations (6 and 10 minutes respectively) at 14K rpm at 4°C. Immunprecipitations were performed at 4°C using 60µL anti-HA matrix (Roche) for 3 hours, followed by 3 washes with IPH150 and resuspension in 2x Laemmlli buffer. Standard procedures for Sodium-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were followed (SAMBROOK and RUSSELL 2001) to transfer proteins from gels to a polyscreen PVDF membrane (PerkinElmer). Membranes were blotted with primary antibodies anti-V5 (Invitrogen), anti-Mcd1p (antibody 559, kindly provided by V. Guacci), Smc1(kindly provided by J. Stray), or anti-HA (16B12, Roche). Antibodies were detected using SuperSignal West Dura extended duration substrate (Pierce).

**Microscopy:** Fluorescence was observed using a Zeiss Axioplans 2 microscope (100x objective, NA=1.40) with a Quantix CCD camera (Photometrics).
RESULTS

In order to investigate the role(s) of the Smc3p ATPase in cohesin function, we introduced mutations into Smc3p that either abolish ATP binding (Smc3-K38I, a mutation in the Walker A motif) or ATP hydrolysis (Smc3-E1155Q, a mutation in the Walker B motif) (Arumugam et al. 2003; Arumugam et al. 2006). Both Smc3p ATP binding and hydrolysis are essential for cell viability as reported previously (Figure 1A) (Arumugam et al. 2003). While previous work suggested that Smc3p ATP binding and hydrolysis were required for cohesin binding to chromosomes (Arumugam et al. 2003), this conclusion was drawn exclusively from the failure of the Mcd1p subunit to localize to chromosomes in Smc3p ATP binding (smc3-K38I) and hydrolysis (smc3-E1155Q) mutants. Since in vitro studies show that Smc heterodimers can bind to DNA without the non-Smc subunits (Losada and Hirano 2001), it was important to directly monitor the localization of the Smc3p ATP binding and hydrolysis mutants.

We engineered these mutations into a copy of Smc3p where a 6-hemagglutinin (HA) tag was inserted after residue N250, located in a predicted break in the coiled coil (Gruber et al. 2003). The N250 tagged allele completely supports viability, even at high temperatures (Figure S1). More importantly, the N250 tagged allele of SMC3 yields a stronger signal in both chromatin immunoprecipitation (ChIP) and in chromatin spreads than C terminal tagged SMC alleles. Using our N250 tagged alleles, we found that Smc3p ATP binding or hydrolysis mutants are proficient for complex assembly but not for chromatin association (Figure 1B, C, D, Figure S2). Thus, both ATP binding and hydrolysis are required for robust localization of Smc3p to chromosomes generally, and
to CARs specifically (Figure 1C, D, Figure S2), consistent with previous results monitoring Smc3p chromatin indirectly through Mcd1p (ARUMUGAM et al. 2003).

Expression of Smc3p defective for ATP hydrolysis dominantly inhibits cohesion by wild-type cohesin

In order to further characterize the importance of Smc3p ATPase, we attempted to integrate a single copy of either the ATP-binding or ATP-hydrolysis defective smc3 allele at the URA3 locus in a haploid smc3-42 temperature sensitive (ts) strain at the endogenous smc3 locus. We were unable to obtain integrants of smc3-E1155Q, the ATP-hydrolysis defective allele, in this smc3-42 strain growing at the permissive temperature. This was surprising, because we obtained integrants of the smc3-E1155Q allele when the wild-type SMC3 allele was at the endogenous locus. This observation suggested that the ATP-hydrolysis defective mutant protein is toxic to cells, and this toxicity leads to cell death when the ratio of smc3p-E1155Q activity to Smc3p activity is increased. This ratio is increased in the smc3-42 strain background because the smc3-42 protein has presumably reduced Smc3p activity even at permissive temperature.

To test this idea further, we increased the ratio of smc3p-E1155Q to wild-type Smc3p. We introduced a galactose inducible smc3-E1155Q allele at the URA3 locus in a strain containing SMC3 at the endogenous locus. This strain fails to grow on galactose containing media, indicating that the ATP hydrolysis defective smc3p-E1155Q acts as a dominant negative, interfering with the activity of cohesin containing functional Smc3p molecules (Figure 1E).
If the loss of viability of cells expressing smc3p-E1155Q results from smc3p being trapped in an ATP-bound state, then this dominant negative phenotype of smc3p-E1155Q should be mitigated by blocking its ATP binding. Therefore, we introduced the K38I ATP binding mutation into the galactose inducible smc3-E1155Q allele. Wild-type cells over-expressing smc3p-K38I, E1155Q grow well, exactly like strains over-expressing smc3p-K38I (Figure 1E). Thus the ATP-bound form of Smc3p is required for the dominant negative phenotype.

We next asked whether the loss of viability caused by smc3p-E1155Q overexpression was due to a defect in cohesion maintenance or establishment. To distinguish between these possibilities, we first induced the ATP hydrolysis mutant protein smc3p-E1155Q in G2/M arrested cells and assayed for cohesion. No loss of cohesion is observed, indicating that the smc3p-E1155Q does not perturb the ability of wild-type cohesin to maintain cohesion (Figure 2A). Next we induced smc3p-E1155Q such that the mutant protein would be present during cohesion establishment. We arrested cells in G1, induced smc3p-E1155Q, and subsequently allowed cells to proceed into G2/M. Under these conditions, cohesion is dramatically reduced (Figure 2A). Taken together these two results suggest that smc3p-E1155Q blocks the ability of wild-type cohesin from establishing cohesion.

smc3p-E1155Q could inhibit cohesion establishment by wild-type cohesin by either preventing wild-type cohesin binding to chromosomes, or by inhibiting its conversion to the cohesive state. We tested the total level of cohesin binding to a CAR by ChIP. ChIP was preformed on wild-type cells over-expressing wild-type or mutant
Smc3p from an allele integrated at the URA3 locus. Overexpression of Smc3p-E1155Q dramatically reduces the total cohesin binding, but overexpression of wild-type Smc3p has no effect (Figure 2B). Since cohesin with smc3p-E1155Q does not bind CARs (Figure 1C, S2), the loss of binding indicates that the wild-type cohesin is compromised for chromatin binding in the presence of excess smc3p-E1155Q. This conclusion is further supported by the fact that the absolute level of cohesin binding in the cells overexpressing smc3p-E1155Q is below the level observed in cells only expressing Smc3p from its endogenous locus. Thus the ATP-trapped form of Smc3p not only fails to bind chromatin, but also reduces the ability of wild-type cohesin binding to CARs, providing a biochemical explanation for both the loss of cohesion and the cell inviability.

From these observations we draw three conclusions. First, the forms generated by the ATP-bound and ATP-unbound Smc3p define two distinct functional states of cohesin, as only the ATP hydrolysis defective smc3p is dominant negative. Second, the functional state generated by the ATP-trapped smc3p is not inert, but rather interferes with cohesion establishment of wild-type cohesin. Finally, the observation that the ATP-bound smc3p-E1155Q disrupts wild-type cohesin from loading onto chromosomes and establishing cohesion implies that the accumulation of ATP-bound Smc3p without subsequent ATP hydrolysis does not normally occur prior to cohesin loading onto chromosomes.

**Smc3p acetylation modulates the Smc3p ATPase**

The ability to genetically distinguish the Smc3p ATP-bound and ATP-unbound structures allowed us to investigate how Smc3p acetylation might modulate the Smc3p
ATPase. If Smc3p acetylation reduces ATP binding, then acetylated Smc3p should
generate phenotypes like the smc3p ATP binding mutant. Acetylated Smc3p should not
be very toxic to wild type cells upon over-expression. Furthermore, acetylation of the
smc3p-E1155Q ATP hydrolysis mutant should reduce its dominant negative effect on
cohesin binding and cohesion. Alternatively if Smc3p acetylation inhibits ATP hydrolysis,
acetylated Smc3p should have phenotypes like the smc3p ATP hydrolysis mutant.
Overexpression of the acetylated Smc3p should be very toxic to cells and block
cohesion.

While these predictions seem straightforward to test, they are complicated by
the fact that acetylation of Smc3p by Eco1p is restricted to the chromatin-bound
cohesin and only a small subset of cohesin is normally acetylated (Ben-Shahar et al.
2008; Unal et al. 2008) (Zhang et al. 2008a). To circumvent this problem, we exploited
the acetyl-mimic alleles (where residue Lysine (K) 113 is converted to Asparagine (N), or
K112 and K113 are converted to Glutamine(Q)) to effectively force an acetylation-like
state independent of chromatin binding. Like the ATP binding and hydrolysis mutants,
the acetyl-mimic alleles are proficient for assembly into the cohesin complex (Figure
3A). These alleles, combined with our ability to genetically distinguish the Smc3p ATP-bound and ATP-unbound structures, allowed us to investigate the potential role of
Smc3p acetylation in regulating Smc3p ATP binding and/or hydrolysis.

Galactose inducible expression of the Smc3 acetyl-mimic allele, smc3p-K112Q,
K113Q, only marginally slows cell growth similar to that of the Smc3p ATP binding
mutant, smc3p-K38I (Figure 3B). We next asked whether the introduction of the acetyl-
mimic residues (K112Q, K113Q) into the ATP hydrolysis mutant smc3p-E1155Q might similarly rescue the cohesion defect caused by the E1155Q substitution. Indeed, expression of the smc3p-K112Q,K113Q,E1155Q had almost no cohesion defect, similar to smc3p-K38I, E1155Q (Figure 3C). From the absence of a cohesion defect we could infer that the introduction of K112Q,K113Q into smc3p-E1155Q must eliminate the dominant negative effect of E1155Q on wild-type cohesin binding to chromatin (Figure 2B). To test this inference we induced smc3p-E1155Q or smc3p-K112Q, K113Q, E1155Q in a strain containing epitope tagged Smc3p, and monitored Smc3p binding at CARC1 on chromosome III by ChIP. Consistent with our results in figure 2B, over-expression of smc3p-E1155Q greatly diminishes the amount of wild type Smc3p to CARs (Figure 4, Figure S3) while overexpression of smc3p-E1155Q K112Q, K113Q does not significantly interfere with the localization of the wild type Smc3p to chromosomes (Figure 4, Figure S3). Intriguingly, since smc3p-K112Q, K113Q, E1155Q cannot bind to CAR sites, the K112Q, K113Q residues must be preventing the formation of the toxic intermediate prior to cohesin loading at CAR sites. The phenotypic similarities between the ATP binding and acetyl mimic mutants individually and in combination with the hydrolysis mutant are consistent with the acetylation of Smc3 K112 and K113 modulating the ATP-bound state of Smc3p to mimic functionally the ATP-unbound state.

However, the phenotypes of cohesin complexes with the smc3p-K38I or smc3p-K112Q K113Q are not exactly identical. The dominant negative effect of smc3p-E1155Q expression on cell growth is almost completely abolished by the introduction of K38I while only partially abolished by introduction of the acetyl-mimics (compare growth of
cells expressing smc3p-K38I, E1155Q and smc3p-K112Q K113Q, E1155Q or smc3p-K113N,E1155Q; Figure 1E and S4). Similarly cohesin with smc3p-K38I is unable to bind chromatin while cohesin with smc3p-K112Q K113Q binds chromatin poorly (compare Figure 5A to Figure 1C, Figure 5B, S3). This poor binding is similar to the binding of cohesin with smc3p-D1154A, a mutant version of Smc3p which is partially impaired for ATP binding (ARUMUGAM et al. 2003) (Figure S5). Hence, our observations are consistent with the acetyl-mimic residues modulating an ATP-dependent biochemical structure of Smc3p, potentially by reducing but not eliminating its ability to bind ATP.

DISCUSSION

Our mutant analyses show that the ATP-bound Smc3p generates a distinct functional state from Smc3p lacking ATP. This functional distinction is revealed by the different phenotype of cells expressing smc3p-K38I (ATP-binding defective) or smc3p-E1155Q (ATP-hydrolysis defective, trapping the ATP-bound state). Only expression of smc3p-E1155Q disrupts the chromosome localization of cohesin with wild-type Smc3p. Both the ring and oligomerization models for cohesin function propose distinct functional states of cohesin governed by ATP binding. The unique dominant phenotype of smc3p-E1155Q provides the first in vivo evidence for these distinct functional states. Furthermore, our work suggests that biochemical studies of the ATP-bound Smc3p may yield important mechanistic insights into how cohesin tethers sister chromatids together and provides a genetic handle to investigate modulators of Smc3p ATP binding.
Finally, our intriguing observations with Smc3p underscore the importance of extending these studies to the Smc1p ATPase.

The toxicity of the ATP-trapped Smc3p can be explained in two ways. One, it may function in a prion-like manner, promoting the oligomerization of cohesins in the nucleoplasm that is normally restricted to chromosomes. Alternatively, the ATP-trapped state of Smc3p might tightly bind a limiting factor for cohesin loading, thereby sequestering it from wild-type Smc3p and/or cohesin. The putative limiting factor is unlikely to be one of the other cohesin subunits. The non-functional smc3p-K38I or smc3p- K112Q K113Q, E1155Q appear to bind the other cohesin subunits as well as smc3p-E1155Q, yet only smc3p-E1155Q impairs wild-type cohesin binding to chromosomes. The limiting factor could be Scc2p/Scc4p, the sole known complex essential for cohesin loading. Only very weak associations between Scc2p/Scc4p and cohesin have been reported (Bernard et al. 2006; Ciosk et al. 2000; Watin et al. 2006) perhaps because the potential role of ATP in this interaction was unappreciated. So far our attempts to address the impact of ATP on the association of cohesin with Scc2p, either by adding excess of ATP to in vitro extracts or by expressing smc3p-1115Q, have proved technically challenging due to the instability of our extracts. Nonetheless, our genetic observations highlight the importance of overcoming these technical challenges in order to study the composition of cohesin in the presence and absence of ATP.

We also link the Smc3p ATPase with the Eco1p-dependent acetylation of Smc3p that promotes cohesion establishment. The dominant negative phenotype of the smc3p-E115Q expression is mitigated by changing smc3p-E1155Q to block its ability to
bind ATP (K38I, E1155Q) or to mimic its acetylation state (K112Q K113Q, E1155Q). This similarity implies that Smc3p acetylation promotes cohesion establishment by modulating the binding of ATP to Smc3p directly, or the interaction of the ATP-bound Smc3p with an ATP-regulated binding partner. This inferred role of the Smc3p ATPase in cohesion establishment coupled with its established role in cohesin binding to chromatin ([ARUMUGAM et al. 2003], this study) may explain why mutations in auxillary factors or cohesin modifications necessary for establishment and maintenance also often impact cohesin binding (ROWLAND et al. 2009) (SUTANI et al. 2009) (BEN-SHAHAR et al. 2008; UNAL et al. 2008) (ZHANG et al. 2008a)(Gandhi et al. 2006)(KUENG et al 2006).

In conclusion, we present a working model for how Smc3p binding to ATP might regulate cohesin binding and cohesion establishment. We propose, as suggested previously, that a single round of ATP binding and hydrolysis governs the association of cohesin with chromatin. Scc2p/Scc4p facilitates this step, potentially by binding to the ATP-bound cohesin and stimulating ATP hydrolysis. Smc3p acetylation is then temporally regulated to occur only after Scc2p facilitates cohesin loading onto chromatin (UNAL et al. 2008). At this step, Eco1p mediated Smc3p acetylation prevents the rebinding of ATP and/or Scc2p/Scc4p stimulating either capture of the second sister chromatid or nucleation of cohesin oligomerization (Figure 6). This model is attractive because it provides a framework to investigate how cohesin’s ATPase, and additional cohesin regulators such as Pds5p and Wpl1p, facilitate the initial tethering of sister chromatids, the maintenance of the tethered form until anaphase, and separase independent cohesion removal.
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FIGURE 1: THE SMC3 ATP HYDROLYSIS MUTANT GENERATES A TOXIC INTERMEDIATE

A. EU3435 (Δsmc3:CLONAT, pEU42(SMC3, URA3, CEN/ARS)) containing pEU41 (SMC3, LEU2, CEN/ARS) or its derivative pEU41-K38I(smc3-K38I, LEU2, CEN/ARS) or pEU41-E1155Q(smc3-E1155Q, LEU2, CEN/ARS) were grown to saturation in –Leu media. Five fold serial dilutions of the strains were plated on control plates (YEPD) or 5-FOA (5-Fluoroorotic Acid) plates and incubated at 30°. An absence of growth on 5-FOA indicates an obligate retention of the wild-type SMC3 plasmid (pEU42(SMC3, URA3, CEN/ARS)).

B. Parent strain VG3178-13A (Scc3-V5) bearing pJH26 (SMC3 (N250-6HA), CEN, LEU2) containing mutations that impair either Smc3p ATP binding (K38I) or ATP hydrolysis (E1155Q) were grown to mid-log phase in –LEU media. Cells were broken open and subjected to immunoprecipitation against the HA tag as described in Materials and Methods. N=at least two, a representative experiment is shown. The indicated bands were all run out on a single gel per antibody, so relative co-IP efficiency can be directly compared.

C. Wild-type strain VG3349-1B containing pJH26 (SMC3(N250-6HA), CEN, LEU2) containing mutations that impair either Smc3p ATP binding (K38I) or ATP hydrolysis (E1155Q) were grown to mid-log phase in –Leu media. Cells were pelleted and resuspended in YEPD containing nocodazole to arrest cells in G2/M phase. Upon arrest, cells were fixed and processed for Chromatin Immunoprecipitation against HA epitope proteins (See materials and methods). N=at least two, a representative experiment is shown.

D. The same strains as in (C) were arrested in G2/M in YEPD using nocodazole and processed for chromatin spreads as described in materials and
methods. E. Strains of JH5227 (pGAL:SMC3), JH5267 (pGAL:smc3-K38I), JH5228 (pGAL:smc3-E1155Q), and JH5268 (pGAL:smc3-K38I, E1155Q) were grown to saturation in YEPD were plated with 5X serial dilutions on plates containing YEP supplemented with 3% glycerol, 2% lactic acid, with or without 2% galactose.

FIGURE 2: OVER-EXPRESSION OF THE SMC3 ATP HYDROLYSIS MUTANT REDUCES WILD-TYPE COHESIN LEVELS ON CHROMOSOMES AND DISRUPTS COHESION ESTABLISHMENT DURING S PHASE

A. Strains JH5227 (pGAL:SMC3) and JH5228 (pGAL:smc3-E1155Q) were grown to mid-log phase in YEP supplemented with 3% glycerol, 2% lactic acid. Cells were arrested in G1 phase by the addition of α-Factor (1.5 x 10⁻⁸ M final) and galactose (2% final) was introduced into the media for one hour to induce production of wild-type Smc3p or mutated smc3p with the indicated mutation. Cells were released from α-Factor arrest and re-suspended in media containing nocodazole for 2 hours to arrest the cells in G2/M phase. Cohesion at LYS4 was assayed by GFP dots. N=three, at least 500 cells were counted for each experimental condition. B. Strains JH5227 (pGAL:SMC3) and JH5228 (pGAL:smc3-E1155Q) were grown as described in A. Cells were fixed for 2 hours and with formaldehyde, washed 2x with cold PBS, and isolated for Chromatin Immunoprecipitation (See materials and methods). N=two, a representative experiment is shown.

FIGURE 3: THE SMC3P ACETYL-MIMIC PHENOCOPYS THE ATP BINDING MUTANT
A. Parent strain VG3178-13A (Scc3-V5) bearing pJH26 ((N250-6HA), CEN, LEU2) containing mutations (K112Q, K113Q) or (K112Q, K113Q, E1155Q). Cells grown to mid-log in –LEU media, broken open, and subjected to immunoprecipitation against the HA tag as described in Materials and Methods. N=at least two, a representative experiment is shown. The indicated bands were all run out on a single gel per antibody, so relative co-IP efficiency can be directly compared. B. Strains JH5227 (pGAL:SMC3), JH5267(pGAL:smc3-K38I), JH5228 (pGAL:smc3-E1155Q), and JH5228 (pGAL:smc3-E1155Q) grown to saturation in YEPD were plated as described in Figure IE. C. Strains JH5227 (pGAL:SMC3), JH5228 (pGAL:smc3-E1155Q), VG3371-11A (pGAL:smc3-K112Q, K113Q), and JH5239 (pGAL:smc3-K112Q,K113Q, E1155Q) were grown as described in Figure 2A and cohesion was assayed by GFP dots. N=three, at least 500 cells were counted for each experimental condition.

FIGURE 4. THE SMC3P ACETYL-MIMIC PREVENTS THE SMC3P ATP HYDROLYSIS MUTANT-INDUCED TITRATION OF WILD-TYPE COHESIN FROM CHROMATIN.

A. Strains JH5252 (pGAL:smc3-E1155Q, pJH26 (SMC3(N250-6HA), CEN, LEU2)) and JH5253 (pGAL:smc3-K112Q, K113Q, E1155Q, pJH26 (SMC3(N250-6HA), CEN, LEU2)) were grown in –LEU media supplemented with 3% glycerol, 2% lactic acid to mid-log phase. Cells were spun down and resuspended in YEP supplemented with 3% glycerol, 2% lactic acid and arrested as described in Figure 2A and isolated for Chromatin Immunoprecipitation (See materials and methods). N=two, a representative experiment is shown.
FIGURE 5. THE SMC3P ACETYL-MIMIC IS ONLY PARTIALLY COMPETENT FOR CHROMOSOME BINDING

A. Chromatin Immunoprecipitation experiment in strains VG3349-1B bearing pJH26 (SMC3(N250-6HA), CEN, LEU2) containing the acetyl-mimic (K112Q, K113Q) mutations or the combination acetyl-mimic ATP hydrolysis mutant (K112Q, K113Q, E1155Q). Cells were prepared as described in Figure 1C. This experiment can be directly compared to Figure 1C, because the experiment was done at the same time with the same batch of HA antibody. N=at least two, a representative experiment is shown. B. Chromosome spreads in VG3349-1B containing pJH26 (SMC3(N250-6HA), CEN, LEU2) containing the acetyl-mimic (K112Q, K113Q mutations) or the combination acetyl-mimic ATP hydrolysis mutant (K112Q, K113Q, E1155Q). Cells were arrested in G2/M and processed for chromatin spreads using and antibody against the internal 6HA tag (See materials and methods).

FIGURE 6. A MODEL FOR HOW ECO1P-MEDIATED ACETYLATION OF SMC3P MODULATES AN ATP DEPENDENT INTERMEDIATE IN COHESION ESTABLISHMENT

For simplicity we have focused only on the Smc3p ATPase and the contribution of ATP to cohesion. At least one round of Smc3p ATP hydrolysis promotes cohesin binding to CAR sites, and this step is mediated by Scc2p/Scc4p. Establishment occurs when Eco1p blocks either subsequent ATP binding and/or Scc2p association. This either stimulates
the association of cohesin with the other sister chromatid, or stimulates cohesion oligomerization.
FIGURE 1:

A

\textbf{sfcm2/SMC3-CEN-URA3}

\begin{tabular}{llll}
  & \textbf{SMC3} & \textbf{smc3-K38I} & \textbf{smc3-E1155Q} \\
\hline
\textbf{control} & & & \\
\hline
\end{tabular}

B

IP: HA (Smc3p)

\begin{tabular}{llll}
  & \textbf{Smc3p:} & WT & K38I & E1155Q \\
\hline
\textbf{WB: V5} & (Scc3p) & & & \\
\textbf{WB: Smc1p} & & & & \\
\textbf{WB: Mcd1p} & & & & \\
\textbf{WB: HA} & (Smc3p) & & & \\
\end{tabular}

C

\begin{tikzpicture}
\begin{axis}[
  xlabel=chromosome III coordinates (kb),
  ylabel=\% chromatin in HA (Smc3p) IP,
  xmin=94.5, xmax=100.5,
  ymin=0.15, ymax=0.45,
]
\addplot+[mark=x,mark options=solid] coordinates {
(95.0,0.15)
(97.5,0.30)
(100.0,0.45)
};
\addplot+[mark=x,mark options=solid] coordinates {
(95.0,0.15)
(97.5,0.30)
(100.0,0.45)
};
\addplot+[mark=x,mark options=solid] coordinates {
(95.0,0.15)
(97.5,0.30)
(100.0,0.45)
};
\end{axis}
\end{tikzpicture}

D

\begin{tabular}{llll}
  & no tag & Smc3p & smc3p-K38I & smc3p-E1155Q \\
\hline
\textbf{Dapi} & & & & \\
\textbf{anti-HA} (Smc3p) & & & & \\
\end{tabular}

E

\textbf{pGAL:SMC3}

\begin{tabular}{llll}
  & \textbf{SMC3} & \textbf{smc3-K38I} & \textbf{smc3-E1155Q} & \textbf{smc3-K38I, E1155Q} \\
\hline
\textbf{control} & & & & \\
\textbf{plus galactose} & & & & \\
\end{tabular}
FIGURE 2:

A

induction in G1  induction in G2/M

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>E1155Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>% cohesion loss</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGal:</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>Smc3:</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

B

WT uninduced
WT induced
E1155Q uninduced
E1155Q induced

% chromatin in Medip IP

96 97 98 99 100 101 102
Chromosome III coordinates (kb)
FIGURE 3:
FIGURE 4:

- E1155Q uninduced
- E1155Q induced
- K112Q, K113Q, E1155Q uninduced
- K112Q, K113Q, E1155Q induced

% chromatin in HA (Snc3p) IP

chromosome III coordinates (kb)