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An SMC ATPase mutant disrupts chromosome segregation in *Caulobacter*

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Summary

Accurate replication and segregation of the bacterial genome are essential for cell cycle progression. We have identified a single amino acid substitution in the *Caulobacter* Structural Maintenance of Chromosomes (SMC) protein that disrupts chromosome segregation and cell division. The E1076Q point mutation in the SMC ATPase domain caused a dominant-negative phenotype in which DNA replication was able to proceed, but duplicated *parS* centromeres, normally found at opposite cell poles, remained at one pole. The cellular positions of other chromosomal loci were in the wild-type order relative to the *parS* centromere, but chromosomes remained unsegregated and appeared to be stacked upon one another. Purified SMC-E1076Q was deficient in ATP hydrolysis and exhibited abnormally stable binding to DNA. We propose that SMC spuriously links the duplicated chromosome immediately after passage of the replication fork. In wild type cells, ATP hydrolysis opens the SMC dimer, freeing one chromosome to segregate to the opposite pole. The loss of ATP hydrolysis causes the SMC-E1076Q dimer to remain bound to both chromosomes, inhibiting segregation.

Keywords

ATP hydrolysis; *Caulobacter*; chromosome segregation; SMC

Introduction

The bacterial genome must be completely replicated and segregated prior to the completion of cell division in order to ensure that each daughter cell receives one complement of the genome. The Structural Maintenance of Chromosomes (SMC) protein is conserved from bacteria to humans and is thought to play important roles in chromosome organization and segregation (Nasmyth and Haering, 2005). Eukaryotes have multiple SMC homologues that form heterodimers to perform specific functions. In contrast, bacteria generally have a single SMC homologue that forms homodimers (Nasmyth and Haering, 2005). *E. coli* and other γ -proteobacteria have a related protein, MukB, which has weak sequence homology and the dimeric structure common to SMCs (Melby et al., 1998; Niki et al., 1992). However, the exact role of SMC in bacterial chromosome compaction, organization and segregation is not well understood.

In the α -proteobacterium *Caulobacter crescentus*, SMC accumulates in multiple foci throughout the wild type cell and the number of foci varies over the cell cycle (Jensen and Shapiro, 2003). In 30 – 40% of predivisional cells, bright foci are localized near the cell poles. Both *Bacillus subtilis* SMC (BsSMC) and MukB exhibit more specific subcellular

localization patterns. BsSMC accumulates at *parS* sequences, where it interacts with Spo0J (ParB) at the cell poles (Gruber and Errington, 2009; Sullivan et al., 2009). In *E. coli*, MukB accumulates in foci at the origin region, located at midcell or the quarter cell positions depending on the stage of the cell cycle (Danilova et al., 2007).

Deletion of *Caulobacter smc* causes temperature sensitivity (Jensen and Shapiro, 1999), as do deletions of *B. subtilis smc* (Britton et al., 1998) and *E. coli mukB* (Niki et al., 1991). In the wild type non-replicating swarmer cell, the *parS* centromere is positioned at the flagellated pole while the chromosomal terminus is at the opposite pole (Fig. 3A) (Jensen and Shapiro, 1999; Mohl and Gober, 1997; Viollier et al., 2004). The average subcellular position of the intervening loci reflects their position on the genetic map (Viollier et al., 2004). For example, a genetic locus midway between *parS* and the terminus is positioned at midcell. After replication, chromosomal loci are rapidly segregated to the same relative position in the incipient daughter cell, thereby maintaining the organization of the chromosome. 10 – 15% of cells lacking SMC have aberrantly localized origins or termini, indicating anomalous chromosome organization (Jensen and Shapiro, 1999). However, it is not known if the altered chromosome organization in the *smc* deletion is due to a defect in segregation or compaction.

In vivo BsSMC forms a complex with two other proteins, ScpA and ScpB that are thought to play a role in modulating the interaction of SMC with DNA (Hirano and Hirano, 2004; Mascarenhas et al., 2002; Soppe et al., 2002). ScpA belongs to the kleisin family of proteins along with subunits of eukaryotic SMC complexes (Schleiffer et al., 2003). ScpA interacts directly with BsSMC and ScpB, while ScpB only interacts directly with ScpA (Hirano and Hirano, 2004; Mascarenhas et al., 2002). *Caulobacter* contains homologues of both ScpA (CC2005) and ScpB (CC2004).

SMC is a multidomain protein with an ATPase head domain, a coiled-coil arm and a hinge region (Fig. 1A). The head domain, consisting of a bipartite ATPase domain formed from the N and C termini, connects to the hinge region through an antiparallel coiled-coil domain (Melby et al., 1998). The hinge region acts as a dimerization domain, linking two SMC monomers into a functional V-shaped dimer (Haering et al., 2002; Hirano and Hirano, 2002). The ATPase domain contains motifs homologous to the ATPase binding cassette (ABC) family of ATPases (Löwe et al., 2001). Crystal structures of an ABC ATPase homologue, Rad50, in the presence or absence of ATP, suggest that engagement of the two ATPase heads of dimers occurs in an ATP-dependent manner (Hopfner et al., 2000). Because of its homology to Rad50, SMC was thought to function in a similar manner, where SMC dimers form a ring upon ATP binding by engagement of the two heads (Hopfner and Tainer, 2003). Indeed, a crystal structure of the *P. furiosus* SMC head domain in the presence of ATP suggests that engagement of the two ATPase heads of dimers occurs in an ATP-dependent manner (Lammens et al., 2004), as have crystal structures of the head domain of yeast Smc1 (Haering et al., 2004). ATP hydrolysis would then release this interaction forming an open dimer in the classic V-shape, as shown in Figure 1A. Exactly how this action compacts DNA is an open question. One proposed compaction mechanism for bacterial SMC is that the hinge region of an SMC dimer interacts with DNA (Hirano and Hirano, 2006). DNA can then be condensed either by trapping a different region of the chromosome by intramolecular engagement of the two heads of the dimer or by an intermolecular interaction with a second SMC dimer bound to a separate region of DNA. Other models postulate the interaction of other SMC domains with DNA, such as the head domain and the coiled-coil region (Akhmedov et al., 1998; Akhmedov et al., 1999). The ring model of SMC-DNA interaction proposes that there is no specific binding of SMC to DNA, rather SMC embraces DNA topologically (Haering et al., 2002; Nasmyth and Haering, 2005).

The ATPase activity of SMC has been studied extensively *in vitro* because of its proposed role in SMC function. Wild type BsSMC has been shown *in vitro* to hydrolyze ATP in two different modes: basal ATPase activity and DNA-induced ATPase activity (Hirano and Hirano, 1998, 2004). A mutation in the BsSMC Walker B motif, BsSMC-E1118Q has very low ATPase activity except in the presence of single stranded DNA. This BsSMC mutant exhibited markedly enhanced binding to both double stranded and single stranded DNA, suggesting that ATP hydrolysis is important for the release of DNA. *In vivo*, the analogous MukB ATP hydrolysis mutant (MukB-E1435Q) expressed as the only copy of SMC in the cell, exhibited the same temperature-sensitive phenotype as the MukB deletion (Woo et al., 2009). While the *in vitro* aspects of wild type and mutant SMC ATPase activity have been studied, the effects of ATPase mutants on cell growth, subcellular organization of the chromosome and chromosome segregation have not been well characterized.

Here, we explore the role of an ATP hydrolysis mutant of *Caulobacter* SMC *in vivo* and *in vitro*. Expression of the Walker B ATPase mutant SMC-E1076Q in a merodiploid strain caused a dominant negative phenotype. Upon induction of SMC-E1076Q, cells became filamentous, formed constrictions that sometimes failed to complete cell division, and produced DNA-free minicells. In these cells, mCherry-ParB bound to *parS* centromeres localized in multiple foci that remained at one pole, while the DNA was deployed throughout the entire cell. Other chromosomal loci also formed multiple foci that clustered at approximately the same relative distance from the pole as observed in wild type cells, indicating that the subcellular organization of the chromosome remained intact. The appearance of multiple chromosomes by FACS analysis and the visualization of multiple foci for each locus suggest that chromosome replication is maintained. We confirmed that SMC-E1076Q was ATP hydrolysis deficient *in vitro*. The loss of ATP hydrolysis, both by mutation of the ATPase domain and by use of a non-hydrolyzable analogue of ATP, caused SMC to bind DNA very stably. We propose that immediately after passage of the replication fork, SMC dimers can spuriously embrace both newly replicated chromosomes. In cells expressing the SMC-E1076Q ATP hydrolysis mutant these dimers cannot open, thereby holding together the newly replicated chromosomes and inhibiting segregation. In wild type cells, however, ATP hydrolysis results in the opening of the SMC arms allowing the release of the DNA and chromosome segregation.

Results

A dominant-negative point mutation in the ATPase domain of SMC causes a cell division defect

To explore the function of *Caulobacter* SMC, we made several point mutations in conserved residues of the ATPase domain (Fig. 1A). In order to avoid the accumulation of suppressors commonly observed in our *smc* deletion strains, we expressed these mutations as inducible constructs in the presence of wild-type *smc* at its native locus and looked for a dominant-negative phenotype. In these merodiploid strains, the wild-type *smc* gene was at its native chromosomal locus, and the second *smc* allele was integrated at the *xylX* locus under the control of the xylose inducible promoter. Three mutations in the SMC ATPase domain that were characterized *in vitro* in *B. subtilis* were constructed in *Caulobacter*: the Walker A ATP binding mutant SMC-K37I (Rao et al., 1988), the C-motif mutant SMC-S1048R that reduces engagement of the ATPase heads (Hirano et al., 2001) and the Walker B ATP hydrolysis mutant SMC-E1076Q (Moody et al., 2002; Smith et al., 2002) (Fig. 1A). Cells expressing wild-type SMC (SMC) from the *xylX* chromosomal locus were indistinguishable from wild type, indicating that xylose induced expression of SMC, in the presence of SMC expressed from its native promoter, did not cause a growth defect (Fig. 1B). Similarly, strains expressing SMC-K37I or SMC-S1048R did not exhibit a dominant-negative phenotype (Fig. S1). However, we found that expression of SMC-E1076Q caused a severe

dominant-negative phenotype (Fig. 1B). After 4–6 hours of induction, cells expressing SMC-E1076Q exhibited aberrant cell division in which cells initiated misplaced constrictions and often did not complete cell division. After 6–10 hours of expression, cells formed long filaments and mini-cells were observed (see inset, Fig. 3C; arrow, Fig. 4A). An SMC-K37I-E1076Q double mutant, however, exhibits wild type morphology, suggesting that the dominant-negative phenotype of SMC-E1076Q was suppressed by a second mutation at K37I (Fig. S1). The cell mass increase of SMC-E1076Q expressing cells as measured by absorbance did not differ greatly from that of SMC until 8–10 hours of induction (Fig. 1C). However, measurement of colony forming units revealed a loss in viability as early as two hours of SMC-E1076Q expression (Fig. 1C).

Purified SMC-E1076Q is deficient in ATP hydrolysis

The SMC-E1076Q point mutation was predicted to be ATP hydrolysis deficient based on previous work on a model ATPase-binding cassette (MJ0796, from *Methanococcus jannaschii*) and on BsSMC (Hirano and Hirano, 2004; Moody et al., 2002; Smith et al., 2002). To confirm that the *Caulobacter* SMC-E1076Q mutant was defective in ATP hydrolysis, we measured the ATPase activity of purified preparations (Fig. 2A) of both SMC and SMC-E1076Q. We measured an ATPase rate of 0.37 ATP/min/monomer for purified SMC in the absence of DNA (Fig. 2B). As predicted, SMC-E1076Q failed to appreciably hydrolyze ATP, with an ATPase rate comparable to the control containing no protein. The rate of ATP hydrolysis by wild type *Caulobacter* SMC was strongly enhanced by double stranded DNA (0.98 ATP/min/monomer; 2.6 fold increase), and mildly stimulated by single stranded DNA (0.47 ATP/min/monomer; 1.3 fold increase). ATP hydrolysis by the SMC-E1076Q ATPase mutant was not stimulated even in the presence of either single stranded or double stranded DNA. These results demonstrate that, as predicted, *Caulobacter* SMC-E1076Q is deficient in ATP hydrolysis.

Native SMC and induced SMC-E1076Q interact in vivo

The strong phenotype of cells expressing SMC-E1076Q in the presence of the wild type protein suggests that these two proteins may interact. In order to determine if the induced SMC and SMC-E1076Q were capable of associating with the native SMC in the merodiploid strains, we tagged the *smc* gene at its native chromosomal locus with the sequence encoding the FLAG-M2 peptide and the *smc* or *smc-E1076Q* gene at the *xyiX* locus with the sequence encoding the His6 peptide. We immunoprecipitated the FLAG-M2 labeled native protein with anti-FLAG-M2 beads and probed for co-immunoprecipitation of induced His6 labeled proteins using anti-His antibodies (Fig 2C). We found that both SMC-His6 and SMC-E1076Q-His6 efficiently co-immunoprecipitated with the native SMC-FLAG protein, demonstrating that the induced proteins are competent to associate with the native SMC *in vivo*.

Replicated centromeres are unable to segregate to the opposite pole

SMC has been proposed to play a role in chromosome condensation and subcellular organization. In order to examine the role of the ATPase activity of SMC in these functions, we studied the organization of the *Caulobacter* chromosome upon induction of SMC or SMC-E1076Q in the merodiploid. We visualized the centromere (*parS*) by tagging its binding protein ParB with mCherry. In the absence of induction of either SMC or SMC-E1076Q, mCherry-ParB was found at the flagellated pole in swarmer cells and at both poles of the dividing cell, as observed in wild type cells (Fig. 3A and C) (Mohl and Gober, 1997; Viollier et al., 2004). This localization pattern was maintained even after 6 hours of SMC induction in the merodiploid. However, upon induction of SMC-E1076Q, multiple mCherry-ParB foci were observed at one pole (Fig. 3B and C). To determine if the chromosome is asymmetrically deployed near these *parS*/ParB clusters leaving a DNA-free area in the cell,

we stained the cells with DAPI. In both uninduced and SMC induced merodiploid cells, the DAPI staining was homogenous throughout the cell (Figure 3C). Surprisingly, despite having multiple *parS*/mCherry-ParB foci at one pole, the DAPI stain filled the SMC-E1076Q expressing cells, indicating that there is DNA throughout these long filamentous cells. DAPI staining also revealed DNA-free mini cells (inset, Fig. 3C), indicating that aberrant cell division had occurred.

Chromosome segregation, but not replication is impaired in the SMC-E1076Q mutant

As DNA was found to be deployed throughout the filamentous cells expressing the dominant-negative SMC ATPase mutant, we determined the overall organization of the chromosome by analyzing the subcellular position of several chromosomal loci. It was previously shown that the position of individual loci on the *Caulobacter* chromosome map reflects their subcellular position within the swarmer cell: those that map close to the *parS* locus appear near the flagellated cell pole and those with more distal map positions appear farther from the pole (Fig. 3A) (Viollier et al., 2004). We used the native *parS*-eCFP-ParB system to label the centromere and in the same cells we labeled a second chromosomal locus using the *parS*-mCherry-ParB system from the plasmid pMT1 (Nielsen et al., 2006; Toro et al., 2008). These two systems are specific and the two different ParB proteins interact only with their cognate *parS* sequences. We labeled four loci spread out on the left arm of the chromosome by inserting *parS*(pMT1) at those positions (Fig 4A, top). In uninduced merodiploid cells and cells with xylose-induced expression of SMC for six hours, *parS* and all four other loci formed one or two foci, reflecting the state of chromosome replication. The relative distance between *parS* and all four loci was proportional to their genetic distance from *parS*, indicating wild type chromosome organization. Upon induction of SMC-E1076Q, however, we observed that like *parS*, all four loci formed clusters of multiple foci as shown in Figure 4A and diagrammed in Figure 3B. The *parS*(pMT1) insertion at *MPO7* formed clusters of foci immediately adjacent to the ParB clusters (Fig 4A). Foci of *parS*(pMT1)/ParB(pMT1) at *pilA*, *pleC* and *podJ* formed clusters at increasing distances from the ParB clusters. While the absolute distance between the two clusters of labeled loci in each cell was increased in the filamentous cells, the relative distance between them in relation to cell length was approximately the same as in wild type cells for all loci tested. Thus, we observed multiple copies of all chromosomal loci in cells expressing SMC-E1076Q and all the loci formed clusters that were arrayed in the wild type order within the cell. This experiment suggests that chromosomes were fully replicated, since even the locus near the terminus (*podJ*) was present in multiple copies in the cell.

To confirm that the chromosomes were fully replicated in SMC-E1076Q expressing cells, we used Fluorescence Activated Cell Sorting (FACS). We found that uninduced cells or cells expressing induced SMC had either one or two chromosomes (Fig. 4B). However, induction of SMC-E1076Q led to accumulation of three or more chromosomes in most cells, with a corresponding reduction in the fraction of cells with both one and two chromosomes. These data, and the results demonstrating that multiple copies of chromosomal loci were present (Fig. 4A), suggest that chromosome replication is being maintained in merodiploid cells expressing SMC-E1076Q, and we propose that a defect in segregation resulted in multiple chromosomes lining up along the length of the cell (Fig. 3B).

Caulobacter SMC co-immunoprecipitates in complex with ScpA and ScpB, but not ParB

Fluorescence microscopy of SMC-eYFP (Fig. 5A) confirmed that *Caulobacter* SMC forms several foci in the cell with the average number of foci varying over the cell cycle (Jensen and Shapiro, 2003). We often observed a focus near the pole, as had been previously described (Jensen and Shapiro, 2003), but these foci only rarely colocalized with the eCFP-ParB focus (Fig. 5A). We performed co-immunoprecipitation experiments to determine if

ParB and SMC interact *in vivo*. Native SMC was tagged with a FLAG-M2 epitope. Pulldown of SMC-FLAG co-immunoprecipitated the *Caulobacter* homologues of the SMC complex proteins ScpA (CC2005) and ScpB (CC2004), but not ParB (Fig. 5B). Thus, it appears that *Caulobacter* SMC does not interact with the centromere.

To determine if the mutation in the ATPase domain alters the localization pattern and interaction partners of SMC, we labeled the xylose inducible copy of *smc* or *smc-E1076Q* with eCFP and a vanillate inducible copy of *parB* with mCherry. The induction of SMC-eCFP resulted in more diffuse signal, but very little co-localization with mCherry-ParB (Fig. 5C). Induction of SMC-E1076Q-eCFP resulted in a wild type-like localization pattern of several foci within the cell, but more diffuse signal due to the higher level of expression (Fig. S3). And as found in wild type cells, these foci only rarely co-localize with mCherry-ParB (Fig. 5C). However, this low level co-localization may be spurious, since SMC-E1076Q-FLAG-M2 did not interact with ParB in a co-immunoprecipitation experiment (Fig. 5D). Inducible SMC or SMC-E1076Q was tagged with a FLAG-M2 epitope. Just as found for native SMC, the pulldown of FLAG-M2 labeled SMC or SMC-E1076Q co-immunoprecipitated the positive control ScpA, but not ParB.

Aberrant localization pattern of proteins involved in chromosome replication, segregation and cell division in SMC-E1076Q expressing cells

Cells expressing SMC-E1076Q have defects in chromosome segregation and cell division, while replication proceeds. To determine the effect of the SMC-E1076Q ATPase mutant on the subcellular localization of proteins central to these processes, we observed the distribution of fluorescently tagged DnaN, ParA and FtsZ (Fig. 6). We labeled the replisome component DnaN (β clamp) with mCherry at its native chromosomal locus (Collier and Shapiro, 2009). In uninduced cells, DnaN-mCherry formed a single focus as the two replisomes replicate the DNA in concert, only occasionally splitting and forming two neighboring foci (Fig. 6A), as has been seen in wild type cells with other *Caulobacter* replisome components (Jensen et al., 2001). Upon induction of SMC, the same localization pattern was observed. In cells expressing SMC-E1076Q, however, we observed multiple replisomes along the length of the filamentous cells. Some co-localized with ParB foci, suggesting that replication had just initiated, others were positioned farther along the cell, and were likely in the process of replicating the rest of the chromosome. These results are consistent with the previous observation in *Caulobacter* in which the replisome forms at the origin of replication and moves towards mid-cell as replication proceeds (Jensen *et al.*, 2001).

Because SMC-E1076Q expressing cells are deficient in chromosome segregation, we examined the localization pattern of the ParA partitioning protein, shown previously to form a linear structure that facilitates the movement of a newly replicated *parS*/ParB complex to the opposite pole (Ptacin et al., 2010). ParA-eYFP was expressed from a vanillate inducible promoter in cells also containing the native copy (Ptacin et al., 2010). Prior to xylose induction, we observed ParA-eYFP extending from the pole opposite the ParB focus (Fig. 6B, arrow) or a segregating ParB focus on the trailing edge of ParA-eYFP (Fig. 6B, double arrow). The same localization pattern was observed upon expression of SMC. However, after 6 hours of SMC-E1076Q induction, we observed a cluster of ParB foci at one pole and ParA-eYFP signal extending from the opposite pole. These results suggest that segregation is inhibited in cells expressing SMC-E1076Q despite the presence of the segregation machinery.

Upon induction of SMC-E1076Q, filamentous cells with multiple constrictions are formed. In order to determine the position of the FtsZ ring, which is thought to mark the division site and generate the force necessary for cell division (Margolin, 2005), we expressed a vanillate

inducible copy of *ftsZ-eyfp* in the presence of a native copy of *ftsZ* (Thanbichler and Shapiro, 2006). Prior to induction of SMC or SMC-E1076Q and in cells expressing SMC, FtsZ-eYFP formed a polar focus in swarmer cells (Fig. 6C, double arrow) or a tight band at midcell in stalked and predivisional cells (Fig. 6C, arrow), as is seen in wild type cells. In cells expressing SMC-E1076Q we observed multiple tight bands of FtsZ, but they were often found separated from constrictions (Fig. 6C, black arrowhead). In addition, faint bands or foci were present that appear to be incomplete FtsZ rings (Fig. 6C, white arrowhead). These results suggest that in SMC-E1076Q expressing cells FtsZ can form rings and initiate constrictions, but cannot always successfully complete cell division.

Loss of ATP hydrolysis by SMC leads to stable interaction of SMC and DNA

To gain insight into the molecular mechanisms underlying the chromosome segregation defect observed in the SMC-E1076Q mutant, we ascertained the ability of wild type and mutant SMC to bind to DNA. We performed gel shift assays with supercoiled plasmid DNA and purified protein to determine the DNA binding efficiency of SMC and SMC-E1076Q (Fig. 7A). DNA binding failed to occur in the absence of ATP (Fig. 7A, lanes 1–5, 11–15). In the presence of ATP, both purified SMC and SMC-E1076Q bound DNA as indicated by the appearance of apparent higher molecular weight products (Fig. 7A, lanes 9–10, 17–20). SMC was observed to shift DNA at concentrations of 0.5 – 1 μ M (Fig. 7A, lanes 9–10). However, significantly lower concentrations of SMC-E1076Q (125 nM) were sufficient to dramatically shift DNA (Fig. 7A, lane 17) and higher concentrations (250 nM – 1 μ M) resulted in complete loss of free DNA and a shift to very high apparent molecular weight complexes (Fig. 7A, lanes 18–20), indicating that SMC-E1076Q binds DNA significantly more stably than SMC. The level of the protein-DNA complex observed was proportional to the amount of protein added to the reaction. In addition to examining gel shifts of supercoiled DNA with SMC-E1076Q, we tested nicked and linear plasmid DNA and the results were comparable (data not shown).

To determine if reduction of ATP hydrolysis is sufficient to cause more stable binding of SMC to DNA, we performed gel shift assays with the poorly hydrolyzable ATP analog ATP γ S (Fig. 7B). Addition of ATP γ S to SMC caused a visible shift in DNA mobility at SMC concentrations where no discernable shift was observed upon addition of ATP (Fig. 7B, lanes 3 and 4). The shifted band was diffuse, possibly due to slow hydrolysis of ATP γ S (Leonard et al., 2005; Yasuoka et al., 1982) by SMC as the protein-DNA complex was running through the gel. Addition of ATP γ S to higher concentrations of SMC caused a complete loss of free DNA and a shift to high apparent molecular weight (Fig. 7B, lane 6). Interestingly, SMC-E1076Q also showed a decrease in DNA mobility in the presence of ATP γ S, as compared to ATP (Fig. 7B, lanes 7 and 8, 9 and 10), suggesting that this mutant was capable of hydrolyzing ATP at very low rates. However, these low rates were not detectable by the colorimetric ATPase assay used to determine the ATPase rate of SMC-E1076Q (Fig. 2B). These results suggest that the loss of ATP hydrolysis results in more stable interaction of SMC with DNA.

Discussion

We have identified a *Caulobacter* SMC mutant with a single amino acid substitution (SMC-E1076Q) that exhibits dominant-negative disruption of chromosome segregation, but not DNA replication. In order to avoid the rapid accumulation of our found in *smc* deletion strains, we generated several point mutations in the ATPase domain of SMC and screened for a dominant-negative phenotype. SMC-E1076Q is a gain-of-function mutant which binds to DNA more stably than wild type SMC (Fig. 7A), causing a segregation phenotype even in the presence of the wild type protein. In addition to *smc-E1076Q*, we tested two other mutations in the ATPase domain of SMC, *smc-K37I* and *smc-S1048R* (Fig. S1). The

analogous mutants in BsSMC, K37I and S1090R, had reduced ATPase activity *in vitro*. BsSMC-K37I, cannot bind ATP (Hirano and Hirano, 1998) and the C-motif mutation BsSMC-S1090R reduced engagement of the two ATPase heads of a dimer (Hirano et al., 2001). We found that *Caulobacter* SMC-K37I and SMC-S1048R did not cause a phenotype when expressed in the presence of wild type SMC. In these cells, homo or heterodimers containing these loss-of-function mutants may be incapable of adopting the closed conformation thought to bind DNA. Nevertheless, the wild type SMC dimers in these merodiploids may be sufficient to allow DNA compaction and segregation to proceed normally. Furthermore, as expected, expression of a SMC-K37I-E1076Q double mutant did not cause a phenotype (Fig. S1), indicating that a mutation causing the loss of ATP binding activity suppressed the dominant-negative phenotype of SMC-E1076Q.

Expression of SMC-E1076Q results in the production of filaments with constrictions that often do not complete cell division, DNA-free mini-cells, and ultimately the loss of viability. In the wild type swarmer cell, the ParB partitioning protein bound to the *parS* centromere is located at one cell pole (Fig. 2A). Upon replication initiation, one copy the *parS*/ParB complex is moved to the opposite cell pole (Toro *et al.*, 2008). The polar localization of the replicated centromeres directs the positioning of the FtsZ cell division ring to midcell via the MipZ ATPase, which mediates this spatio-temporal control by inhibiting FtsZ polymerization (Thanbichler and Shapiro, 2006). MipZ binds to ParB and forms a cellular gradient with its highest concentration at the cell poles, thereby restricting FtsZ polymerization to midcell, the site of lowest MipZ concentration. In cells expressing SMC-E1076Q, MipZ-eYFP (Thanbichler and Shapiro, 2006) foci formed a cluster at one pole (Fig. S2), leaving most of the filamentous cell without the inhibitory action of MipZ and thus allowing multiple FtsZ rings to form along the length of the filament (Fig. 6C). In these cells, many of the FtsZ rings form constrictions that do not complete cell division, possibly due to the trapping of the chromosome at the division site. This is supported by the observation that the multiple chromosomes present upon SMC-E1076Q expression fill the entire cell (Fig. 3C). The generation of DNA-free minicells (inset Fig. 3C, arrow Fig. 4A) may be the result of cell division near the pole opposite the *parS*/ParB/MipZ complex when the DNA is transiently not present.

Examination of the subcellular localization of several chromosomal loci revealed that their position relative to *parS* was maintained in cells expressing SMC-E1076Q. We found that all chromosomal loci tested formed multiple foci indicating the presence of several chromosomes (Fig 4A), which was confirmed by FACS analysis (Fig. 4B). Thus, replication was able to proceed in the absence of segregation. Because the foci were grouped into clusters, we propose that the chromosomes stack up on each other and expand lengthwise to fill the cell (Fig. 3B). The mutant cells were approximately the same width as wild type cells, thus the only direction of expansion for the multiple chromosomes in the cell was along its length. The expansion of the chromosomes seems to be linear, since the relative subcellular organization of the chromosomal loci is maintained. However, this expansion may not be uniform, resulting in chromosomal loci forming a cluster and not aligning completely.

Biochemical analysis of SMC revealed several differences between *Caulobacter* and *B. subtilis* SMC. Analysis of the *in vitro* ATPase activity of SMC revealed that the ATPase rate for wild type *Caulobacter* SMC (Fig. 2B) was an order of magnitude below the rate reported for BsSMC (Hirano and Hirano, 1998, 2004), and falls at the low end of the range ATPase rates of other ABC ATPases (Holland and Blight, 1999). The SMC ATPase rate was also less sensitive to MgCl₂ concentration than BsSMC (Figure S4). Furthermore, in *Caulobacter*, the basal rate of ATPase activity was more strongly enhanced by double stranded DNA than single stranded DNA (Fig. 2B), as is predicted by the proposed role of

SMC in condensing the double stranded chromosome. *Caulobacter* SMC-E1076Q does not have any appreciable ATPase activity (Fig. 2B), thereby behaving like the model ABC ATPase (MJ0796, from *Methanococcus jannaschii*) with which the mutation was originally characterized (Moody et al., 2002).

While we have not ruled out an effect of SMC-E1076Q on chromosome topology, our data are consistent with a model put forth by the Hirano group (Hirano and Hirano, 2006) in which *B. subtilis* SMC interacts with the DNA, then traps or gathers separate regions of DNA through an intermolecular or intramolecular interaction of the SMC heads. The *in vitro* characterization of *Caulobacter* SMC-E1076Q and its dominant-negative phenotype *in vivo*, which abrogates segregation but not replication, supports the following model: *Caulobacter* SMC binds to and condenses the entire chromosome, including condensation of the DNA immediately after the passage of the replication fork when the two newly replicated chromosomes are in close proximity (Fig. 8). The SMC protein cannot distinguish between the DNA of the different chromosomes, and so may spuriously link the two newly replicated chromosomes. We propose that the segregation phenotype of SMC-E1076Q mutants initiates at this point, because this is when the two chromosomes are in close proximity. Dimers containing the ATP hydrolysis mutant SMC-E1076Q cannot release the DNA (Fig. 8A). Thus, if the newly replicated DNA bound by SMC-E1076Q is from two different chromosomes, it is now trapped. The segregation machinery is not strong enough to pull one of the newly replicated chromosomal centromeres to the opposite pole when this region is joined by an SMC-E1076Q dimer. In contrast, the replication machinery can replicate chromosomes held together this way, resulting in chromosomes being replicated but not segregated. Several mechanisms have been proposed to explain how the replisome can overcome a replication barrier, such as the polymerase hopping over a barrier and associating with a second sliding clamp (Georgescu et al., 2010). A mechanism such as this may explain how replication can proceed in the presence of tightly bound SMC-E1076Q dimers. Once the two chromosomes are held together by SMC-E1076Q, the linking of the two chromosomes can be reinforced by further mutant dimers binding elsewhere on the adjacent chromosomes. In wild type cells however, the inherent ATPase activity of wild type SMC will open the SMC dimer after a period of time, release the two spuriously linked newly replicated chromosomes and allow segregation to proceed (Fig. 8B). SMC is more likely to trap DNA from a single chromosome once the physical separation of the two chromosomes has occurred, resulting in the separate condensation of each individual chromosome. We propose that chromosome segregation requires dynamic binding of SMC to DNA, which is regulated by its ATPase activity. Further studies are required to determine the role of the SMC complex proteins ScpA and ScpB in chromosome organization and segregation.

Experimental Procedures

Generation of point mutations and strains

SMC fragments (1–1171 bp [NdeI XmaI] or 1166–3444 bp [XmaI XmaI]) from pMS196 were subcloned into pUC19, generating pMS197 and pMS198 respectively. Point mutants were generated by amplifying the entire plasmid by PCR with the forward primer (see Supplemental Table S3) carrying the point mutation and the reverse primer containing a gap at mutation site (both primers were phosphorylated). The PCR products were gel purified (QIAquick Gel Extraction Kit; QIAGEN) and self-ligated. The XmaI fragment (*smc-E1076Q* and *smc-S1048R*) or the NdeI MluI fragment (*smc-K37I*) containing the mutation was subcloned into same sites of pMS196 and the entire SMC ORF was sequence verified. *Caulobacter CB15N* was electroporated with the *smc* plasmids and transformants with the plasmid integrated in the chromosome by single homologous recombination were selected for. A list of all relevant strains and plasmids is given in Supplemental Tables S1 and S2.

Growth conditions

All strains were grown in minimal M2G medium (Ely, 1991) at 28 °C, except for strains for co-immunoprecipitation experiments, which were grown in peptone yeast extract (PYE). Antibiotic concentrations were used as previously described (Thanbichler and Shapiro, 2006). *P_{xyIX::smc}*, *P_{xyIX::smc-E1076Q}* and all other *smc* alleles were induced in early exponential phase with 0.03% xylose. mCherry-ParB was induced with 250 μM vanillate pH 7.5 for 2 hours. FtsZ-eYFP and ParA-eYFP were induced with 500 μM vanillate pH 7.5 for 60 and 90 minutes, respectively. For Figure 3C, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) by incubating cells in 2 mg DAPI/ml culture for 5 minutes.

Fluorescence microscopy

Strains were imaged in mid exponential phase. Cells were immobilized on a 1% agarose-M2G pad (Ely, 1991). Microscopy was performed on a Leica DM 6000 B microscope with a HCX PL APO 100×/1.40 Oil PH3 CS objective, Hamamatsu EM-CCD C9100 camera and KAMS software (Christen et al., 2010) or Metamorph software (Figure S1, SMC-K37I-E1076Q panels). Images for Figure 3C were obtained with a Princeton Instruments PRO-EM 1024 camera and Metamorph software. Images were processed with Adobe Photoshop.

Growth curve and colony forming units assay

Strains were grown in M2G medium and induced in early log phase with 0.03% xylose. At each time point the absorbance was measured at 600 nm and serial dilutions were plated on PYE plates containing spectinomycin and streptomycin. Antibiotic concentrations were used as previously described (Thanbichler and Shapiro, 2006). After two days colonies on two plates were counted and averaged. Data was analyzed in Microsoft Excel and GraphPad Prism Software to determine growth curve and colony forming units.

Protein purification

Expression and purification of SMC was performed using an adaptation of methods used for BsSMC (Hirano and Hirano, 2002). SMC-His₆ (pMS239) and SMC-E1076Q-His₆ (pMS240) were overexpressed in 1L *E. coli* (Rosetta (DE3) pLysS) by induction with 50 μM IPTG for 2 hours at 28 °C. Cells were washed in phosphate-buffered saline and frozen at −80 °C. Pellets were thawed and resuspended in lysis buffer (50mM Na-phosphate pH 8.0, 300 mM NaCl, 10% Glycerol, 5 mM β-mercaptoethanol (βME), 1 mM PMSF, Complete protease inhibitor tablet [Roche]). Cells were lysed by passage through a French press at 15,000 psi. Lysate was cleared by pelleting debris at 23000 ×g for 30 minutes. Lysate was incubated with Ni-NTA agarose (QIAGEN) for 1 hour at 4 °C. Beads were washed with 15 ml wash-1 buffer (50 mM Na-phosphate pH 6.0, 300 mM NaCl, 50 mM Imidazole pH 6.0, 10% Glycerol, 5 mM βME, 1 mM PMSF, Complete protease inhibitor tablet [Roche]) and transferred to a column. Then resin was washed with 30 ml wash-2 buffer (50 mM Na-phosphate pH 7.5, 300 mM NaCl, 50 mM Imidazole pH 7.5, 10% Glycerol, 5 mM βME, 1 mM PMSF, Complete protease inhibitor tablet [Roche]). Bound proteins were eluted with 20 ml Elution buffer (50 mM Na-phosphate pH 7.5, 300 mM NaCl, 500 mM Imidazole pH 7.5, 10% Glycerol, 5 mM βME, 1 mM PMSF, Complete protease inhibitor tablet [Roche]). Peak fractions were pooled and dialyzed against buffer M (20 mM K-HEPES pH 7.7, 1 mM EDTA, 10% Glycerol) with 50 mM KCl, 5 mM βME, 0.1 mM PMSF overnight at 4 °C. Dialysate was applied to a 1 ml HiTrap Q HP column (GE Healthcare) and fractionated over 20 column volumes using a 50 – 500 mM KCl gradient in buffer M containing 5mM βME. Peak samples were pooled. Samples were concentrated and applied to a gel filtration column (GE Healthcare Sephadex 200, 10/300 GL) in modified buffer M containing 0.1 mM EDTA and with 50 mM KCl and 1 mM βME. Peak fractions were pooled, concentrated and stored at −80 °C.

ATPase assay

ATPase activity was measured using the SensoLyte MG Phosphate Assay Kit (AnaSpec) according to the manufacturer's protocol. Reactions were performed at room temperature over 180 minutes in the presence of 1 μ M SMC or SMC-E1076Q, 31.2 μ M DNA nucleotides (dsDNA: linearized pBluescript KS+, ssDNA: ϕ X174 virion DNA [NEB]), 2 mM ATP, 5 mM $MgCl_2$, 50 mM HEPES pH 7.7, 50 mM KCl. For Figure S4, the concentration of $MgCl_2$ was varied from 0 to 10 mM. Data was analyzed in Microsoft Excel and GraphPad Prism Software to determine ATPase rates. For Figure 2B measurements were performed in triplicate on two independent protein preparations. Averages and standard deviations of the two independent experiments are depicted. For Figure S4, measurements were performed in triplicate; averages and standard deviations are depicted.

Co-immunoprecipitation

Immunoprecipitations were performed as previously described (Yeh et al., 2010). Strains were grown in PYE medium and induced in early log phase with 0.03% xylose for 6 hours. Cells from a 500 ml culture were washed in co-IP buffer (20 mM HEPES pH 7.5, 100 mM NaCl, 20% Glycerol). Proteins were crosslinked with formaldehyde (1% final concentration; Fisher). Cells were lysed by passage through a French press at 15,000 psi. Lysate was incubated with FLAG-M2 beads (FLAGIPT-1 kit; Sigma) overnight. Beads were washed first with co-IP buffer, then with wash buffer (50 mM Tris-HCl, 150 mM NaCl) and proteins were boiled off the beads with sample buffer (Fig. 2) or eluted by incubating with 3 $\times$ FLAG peptides (Fig. 5).

Western Blotting

Western blots were performed as previously described (Chen et al., 2005). Antibody dilutions were as follows: α -FLAG (Sigma) 1:1000, α -His (C-term; Invitrogen) 1:5000, α -GFP (Roche) 1:2000, α -ParB (Mohl and Gober, 1997) 1:8000, α -SMC 1:4000 (Jensen and Shapiro, 2003). Films were scanned and processed with Adobe Photoshop. Lysates for Figure S3 were generated by boiling cells in sample buffer (normalized by adding 250 μ l sample buffer per 1 ml of cells at $OD_{600} = 1$).

Fluorescence Activated Cell Sorting (FACS)

Flow cytometry was performed as previously described (Collier and Shapiro, 2009; Lesley and Shapiro, 2008). Strains were grown in M2G to early log phase. Cultures were split and half was induced with 0.03% xylose for 6 hours, while half remained untreated. Cells were then treated with 15 μ g ml^{-1} rifampicin for 3 hours, fixed in 70% ethanol and stored at 4 $^{\circ}C$ overnight. Fixed cells were gently pelleted and resuspended in TMS buffer (10 mM Tris pH 7.2, 1.5 mM $MgCl_2$, 150 mM NaCl) and labeled with 10 μ M Vybrant DyeCycle Orange (Invitrogen). Cells were analyzed on a FACScan (BD Biosciences). Data analysis was performed using FlowJo software.

Gel shift assay

Gel shift assay were performed with purified SMC and SMC-E1076Q at various concentrations (0 nM, 125 nM, 250 nM, 500 nM, 1 μ M) and pBluescript KS+ (15.6 μ M nucleotides) in the presence or absence of 1mM ATP or 1mM ATP γ S. Reactions (10 μ l volume) were incubated for 60 minutes at room temperature in reaction buffer (20 mM HEPES pH 7.7, 20 mM KCl, 2.5 mM $MgCl_2$). Experiments performed with addition of MgATP produced identical results (data not shown). Samples were run on a 0.7% agarose gel at 5.5 V cm^{-1} for 4 hours at 4 $^{\circ}C$ in 0.5 $\times$ TB buffer with 1mM $MgCl_2$. Gels were soaked in ethidium bromide and scanned with a Typhoon 9400 imager (GE Healthcare) using a 532

nm laser and a 610 nm BP 30 emission filter. Images were processed with Adobe Photoshop.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Akhmedov AT, Frei C, Tsai-Pflugfelder M, Kemper B, Gasser SM, Jessberger R. Structural maintenance of chromosomes protein C-terminal domains bind preferentially to DNA with secondary structure. *J Biol Chem.* 1998; 273:24088–24094. [PubMed: 9727028]
- Akhmedov AT, Gross B, Jessberger R. Mammalian SMC3 C-terminal and coiled-coil protein domains specifically bind palindromic DNA, do not block DNA ends, and prevent DNA bending. *J Biol Chem.* 1999; 274:38216–38224. [PubMed: 10608896]
- Britton RA, Lin DC, Grossman AD. Characterization of a prokaryotic SMC protein involved in chromosome partitioning. *Genes Dev.* 1998; 12:1254–1259. [PubMed: 9573042]
- Chen JC, Viollier PH, Shapiro L. A membrane metalloprotease participates in the sequential degradation of a *Caulobacter* polarity determinant. *Mol Microbiol.* 2005; 55:1085–1103. [PubMed: 15686556]
- Christen B, Fero MJ, Hillson NJ, Bowman G, Hong SH, Shapiro L, McAdams HH. High-throughput identification of protein localization dependency networks. *Proc Natl Acad Sci U S A.* 2010; 107:4681–4686. [PubMed: 20176934]
- Collier J, Shapiro L. Feedback control of DnaA-mediated replication initiation by replisome-associated HdaA protein in *Caulobacter*. *J Bacteriol.* 2009; 191:5706–5716. [PubMed: 19633089]
- Danilova O, Reyes-Lamothe R, Pinskaya M, Sherratt D, Possoz C. MukB colocalizes with the *oriC* region and is required for organization of the two *Escherichia coli* chromosome arms into separate cell halves. *Mol Microbiol.* 2007; 65:1485–1492. [PubMed: 17824928]
- Ely B. Genetics of *Caulobacter crescentus*. *Methods Enzymol.* 1991; 204:372–384. [PubMed: 1658564]
- Georgescu RE, Yao NY, O'Donnell M. Single-molecule analysis of the *Escherichia coli* replisome and use of clamps to bypass replication barriers. *FEBS Lett.* 2010; 584:2596–2605. [PubMed: 20388515]
- Gruber S, Errington J. Recruitment of condensin to replication origin regions by ParB/SpoOJ promotes chromosome segregation in *B. subtilis*. *Cell.* 2009; 137:685–696. [PubMed: 19450516]
- Haering CH, Lowe J, Hochwagen A, Nasmyth K. Molecular architecture of SMC proteins and the yeast cohesin complex. *Mol Cell.* 2002; 9:773–788. [PubMed: 11983169]
- Haering CH, Schoffnegger D, Nishino T, Helmhart W, Nasmyth K, Lowe J. Structure and stability of cohesin's SMC1-kleisin interaction. *Mol Cell.* 2004; 15:951–964. [PubMed: 15383284]
- Hirano M, Hirano T. ATP-dependent aggregation of single-stranded DNA by a bacterial SMC homodimer. *EMBO J.* 1998; 17:7139–7148. [PubMed: 9843517]
- Hirano M, Anderson DE, Erickson HP, Hirano T. Bimodal activation of SMC ATPase by intra- and inter-molecular interactions. *EMBO J.* 2001; 20:3238–3250. [PubMed: 11406600]
- Hirano M, Hirano T. Hinge-mediated dimerization of SMC protein is essential for its dynamic interaction with DNA. *EMBO J.* 2002; 21:5733–5744. [PubMed: 12411491]
- Hirano M, Hirano T. Positive and negative regulation of SMC-DNA interactions by ATP and accessory proteins. *EMBO J.* 2004; 23:2664–2673. [PubMed: 15175656]

- Hirano M, Hirano T. Opening closed arms: long-distance activation of SMC ATPase by hinge-DNA interactions. *Mol Cell*. 2006; 21:175–186. [PubMed: 16427008]
- Holland IB, Blight MA. ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. *J Mol Biol*. 1999; 293:381–399. [PubMed: 10529352]
- Hopfner KP, Karcher A, Shin DS, Craig L, Arthur LM, Carney JP, Tainer JA. Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. *Cell*. 2000; 101:789–800. [PubMed: 10892749]
- Hopfner KP, Tainer JA. Rad50/SMC proteins and ABC transporters: unifying concepts from high-resolution structures. *Curr Opin Struct Biol*. 2003; 13:249–255. [PubMed: 12727520]
- Jensen RB, Shapiro L. The *Caulobacter crescentus smc* gene is required for cell cycle progression and chromosome segregation. *Proc Natl Acad Sci U S A*. 1999; 96:10661–10666. [PubMed: 10485882]
- Jensen RB, Wang SC, Shapiro L. A moving DNA replication factory in *Caulobacter crescentus*. *EMBO J*. 2001; 20:4952–4963. [PubMed: 11532959]
- Jensen RB, Shapiro L. Cell-cycle-regulated expression and subcellular localization of the *Caulobacter crescentus* SMC chromosome structural protein. *J Bacteriol*. 2003; 185:3068–3075. [PubMed: 12730166]
- Lammens A, Schele A, Hopfner KP. Structural biochemistry of ATP-driven dimerization and DNA-stimulated activation of SMC ATPases. *Curr Biol*. 2004; 14:1778–1782. [PubMed: 15458651]
- Leonard TA, Butler PJ, Lowe J. Bacterial chromosome segregation: structure and DNA binding of the Soj dimer—a conserved biological switch. *Embo J*. 2005; 24:270–282. [PubMed: 15635448]
- Lesley JA, Shapiro L. SpoT regulates DnaA stability and initiation of DNA replication in carbon-starved *Caulobacter crescentus*. *J Bacteriol*. 2008; 190:6867–6880. [PubMed: 18723629]
- Löwe J, Cordell SC, van den Ent F. Crystal structure of the SMC head domain: an ABC ATPase with 900 residues antiparallel coiled-coil inserted. *J Mol Biol*. 2001; 306:25–35. [PubMed: 11178891]
- Margolin W. FtsZ and the division of prokaryotic cells and organelles. *Nat Rev Mol Cell Biol*. 2005; 6:862–871. [PubMed: 16227976]
- Mascarenhas J, Soppa J, Strunnikov AV, Graumann PL. Cell cycle-dependent localization of two novel prokaryotic chromosome segregation and condensation proteins in *Bacillus subtilis* that interact with SMC protein. *EMBO J*. 2002; 21:3108–3118. [PubMed: 12065423]
- Melby TE, Ciampaglio CN, Briscoe G, Erickson HP. The symmetrical structure of structural maintenance of chromosomes (SMC) and MukB proteins: long, antiparallel coiled coils, folded at a flexible hinge. *J Cell Biol*. 1998; 142:1595–1604. [PubMed: 9744887]
- Mohl DA, Gober JW. Cell cycle-dependent polar localization of chromosome partitioning proteins in *Caulobacter crescentus*. *Cell*. 1997; 88:675–684. [PubMed: 9054507]
- Moody JE, Millen L, Binns D, Hunt JF, Thomas PJ. Cooperative, ATP-dependent association of the nucleotide binding cassettes during the catalytic cycle of ATP-binding cassette transporters. *J Biol Chem*. 2002; 277:21111–21114. [PubMed: 11964392]
- Nasmyth K, Haering CH. The structure and function of SMC and kleisin complexes. *Annu Rev Biochem*. 2005; 74:595–648. [PubMed: 15952899]
- Nielsen HJ, Ottesen JR, Youngren B, Austin SJ, Hansen FG. The *Escherichia coli* chromosome is organized with the left and right chromosome arms in separate cell halves. *Mol Microbiol*. 2006; 62:331–338. [PubMed: 17020576]
- Niki H, Jaffe A, Imamura R, Ogura T, Hiraga S. The new gene *mukB* codes for a 177 kd protein with coiled-coil domains involved in chromosome partitioning of *E. coli*. *EMBO J*. 1991; 10:183–193. [PubMed: 1989883]
- Niki H, Imamura R, Kitaoka M, Yamanaka K, Ogura T, Hiraga S. *E. coli* MukB protein involved in chromosome partition forms a homodimer with a rod-and-hinge structure having DNA binding and ATP/GTP binding activities. *EMBO J*. 1992; 11:5101–5109. [PubMed: 1464330]
- Ptacin JL, Lee SF, Garner EC, Toro E, Eckart M, Comolli LR, Moerner WE, Shapiro L. A spindle-like apparatus guides bacterial chromosome segregation. *Nat Cell Biol*. 2010; 12:791–798. [PubMed: 20657594]

- Rao R, Pagan J, Senior AE. Directed mutagenesis of the strongly conserved lysine 175 in the proposed nucleotide-binding domain of alpha-subunit from *Escherichia coli* F1-ATPase. *J Biol Chem.* 1988; 263:15957–15963. [PubMed: 2903146]
- Schleiffer A, Kaitna S, Maurer-Stroh S, Glotzer M, Nasmyth K, Eisenhaber F. Kleisins: a superfamily of bacterial and eukaryotic SMC protein partners. *Mol Cell.* 2003; 11:571–575. [PubMed: 12667442]
- Smith PC, Karpowich N, Millen L, Moody JE, Rosen J, Thomas PJ, Hunt JF. ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimer. *Mol Cell.* 2002; 10:139–149. [PubMed: 12150914]
- Soppa J, Kobayashi K, Noirot-Gros MF, Oesterhelt D, Ehrlich SD, Dervyn E, Ogasawara N, Moriya S. Discovery of two novel families of proteins that are proposed to interact with prokaryotic SMC proteins, and characterization of the *Bacillus subtilis* family members ScpA and ScpB. *Mol Microbiol.* 2002; 45:59–71. [PubMed: 12100548]
- Sullivan NL, Marquis KA, Rudner DZ. Recruitment of SMC by ParB-parS organizes the origin region and promotes efficient chromosome segregation. *Cell.* 2009; 137:697–707. [PubMed: 19450517]
- Thanbichler M, Shapiro L. MipZ, a spatial regulator coordinating chromosome segregation with cell division in *Caulobacter*. *Cell.* 2006; 126:147–162. [PubMed: 16839883]
- Toro E, Hong SH, McAdams HH, Shapiro L. *Caulobacter* requires a dedicated mechanism to initiate chromosome segregation. *Proc Natl Acad Sci U S A.* 2008; 105:15435–15440. [PubMed: 18824683]
- Viollier PH, Thanbichler M, McGrath PT, West L, Meewan M, McAdams HH, Shapiro L. Rapid and sequential movement of individual chromosomal loci to specific subcellular locations during bacterial DNA replication. *Proc Natl Acad Sci U S A.* 2004; 101:9257–9262. [PubMed: 15178755]
- Woo JS, Lim JH, Shin HC, Suh MK, Ku B, Lee KH, Joo K, Robinson H, Lee J, Park SY, Ha NC, Oh BH. Structural studies of a bacterial condensin complex reveal ATP-dependent disruption of intersubunit interactions. *Cell.* 2009; 136:85–96. [PubMed: 19135891]
- Yasuoka K, Kawakita M, Kaziyo Y. Interaction of adenosine-5'-O-(3-thiotriphosphate) with Ca^{2+} , Mg^{2+} -adenosine triphosphatase of sarcoplasmic reticulum. *J Biochem.* 1982; 91:1629–1637. [PubMed: 6124538]
- Yeh YC, Comolli LR, Downing KH, Shapiro L, McAdams HH. The *caulobacter* Tol-Pal complex is essential for outer membrane integrity and the positioning of a polar localization factor. *J Bacteriol.* 2010; 192:4847–4858. [PubMed: 20693330]

Figure 1. Expression of SMC-E1076Q causes a dominant-negative cell division phenotype

(A) Left: Diagram of the SMC protein black: bi-partite ATPase domain, gray: coiled-coil domain, white: dimerization domain. Amino acid substitutions at sites known to disrupt ATPase function are marked. Right: Schematic of open SMC dimer.

(B) Phase contrast images of *Caulobacter* following xylose induction of *smc* (MS496) or *smc-E1076Q* (MS487). Time after induction is indicated in hours. Arrows indicate examples of incomplete cell division. Scale bar represents 2 μ m.

(C) Colony forming units of SMC (MS496) and SMC-E1076Q (MS487) merodiploids in minimal M2G medium. Expression of SMC and SMC-E1076Q was induced for the indicated amount of time in hours.

Figure 2. SMC-E1076Q is ATPase deficient *in vitro*, and can interact with native SMC *in vivo*
 (A) Purified SMC and SMC-E1076Q visualized on 10% acrylamide gel by Coomassie Blue staining
 (B) Rate of ATP hydrolysis. ATPase rate was measured over 180 min with 1 μ M SMC, 1 μ M SMC-E1076Q or no protein. The reaction was carried out in the presence of 2 mM ATP, 5 mM $MgCl_2$ and in the presence or absence of single (ss) or double stranded (ds) DNA (31.2 μ M nucleotides). Depicted are the average and standard deviation of two independent experiments with independent protein preparations.
 (C) Co-Immunoprecipitation of native and induced SMC or SMC-E1076Q in *Caulobacter* cells. Native SMC-FLAG was immunoprecipitated with FLAG-M2 beads. Co-

immunoprecipitation of induced SMC-His6 or SMC-E1076Q-His6 (xylose induced for 6 hours) was determined by Western blotting with α -His antibody (MS597, MS598, MS627, MS628).

Figure 3. In the presence of SMC-E1076Q, centromeres localize in clusters at one pole, but the DNA is deployed throughout the cell

(A) Diagram of chromosomal replication in wild type *Caulobacter*. In non-replicating swarmer cells, the chromosome is positioned with the ParB-bound *parS* centromere (green) at the flagellated pole and the terminus at the opposite pole (left). The positions of the intermediate loci reflect their position on the genetic map (for example *pilA*: red, *podJ*: yellow). Once replication initiates and the *parS* centromere is duplicated (chromosomes in black and gray), one centromere is rapidly segregated to the opposite pole, resulting in one centromere at each pole. As replication proceeds, further loci (*pilA*) are replicated and segregated, maintaining the organization of the chromosome.

(B) Model of chromosome organization in SMC-E1076Q expressing cells. We propose that several chromosomes (shades of gray) are present and line up along the length of the cell. Multiple foci of labeled loci (for example *parS*: green, *pilA*: red and *podJ*: yellow) are localized to a specific region of the cell, thereby forming a cluster.

(C) The position of the *parS* centromere was followed by expressing an mCherry fusion of its binding protein ParB. The chromosomal DNA was visualized by 4',6-diamidino-2-phenylindole (DAPI) staining. Images are of cells induced for 0 hours (uninduced) and cells expressing SMC (MS546) or SMC-E1076Q (MS545) for 6 hours (induced). Inset contains DNA free mini-cell. Scale bar represents 2 μ m.

Figure 4. SMC-E1076Q expression leads to clustering of marked chromosomal loci, but overall organization of the chromosome is maintained

(A) Top: Map of *Caulobacter* chromosome showing the chromosomal position of *parS* and insertions of *parS(pMT1)* at the *MPO7*, *pilA*, *pleC* and *podJ* loci (approximate distances from *parS* - *MPO7*: 100 kb, *pilA*: 850 kb, *pleC*: 1.3 Mb, *podJ*: 1.8 Mb). Bottom: Visualization of locus position by labeling of the cognate binding proteins eCFP-ParB and mCherry-ParB(pMT1) in overlay of phase contrast image, eCFP and mCherry signals. Green: *parS*/eCFP-ParB, red: *parS(pMT1)*/mCherry-ParB(pMT1), insertion site of *parS(pMT1)* is indicated above images (MS522–MS529). Expression of SMC or SMC-

E1076Q was induced for 0 (uninduced) or 6 (induced) hours. Arrow indicates DNA free mini-cell. Scale bar represents 2 μm .

(B) FACS analysis of chromosome content. Expression of SMC (MS496) or SMC-E1076Q (MS487) was not induced (uninduced) or induced with xylose for 6 hours (induced).

Figure 5. SMC and SMC-E1076Q do not interact with ParB

(A) Visualization of SMC-eYFP and eCFP-ParB (MS333). Double arrow indicates an eCFP-ParB focus (green) that does not colocalize with SMC-eYFP (red). Scale bar represents 2 μ m.

(B) Co-Immunoprecipitation of native SMC and ParB in *Caulobacter* cells. FLAG-M2 tagged SMC was pulled down and co-immunoprecipitation of ParB, ScpA and ScpB was determined by Western blotting. Western blots of whole-cell extracts (lysate) and eluted samples were probed with α -FLAG-M2 (SMC-FLAG), α -GFP (ScpA-eCFP, ScpB-eCFP), α -ParB (ParB). (MS455, MS457, MS550, MS551)

(C) Visualization of SMC-eCFP or SMC-E1076Q-eCFP and mCherry-ParB (MS651, MS652). Single arrow indicates co-localization of mCherry-ParB (green) and SMC-eCFP or SMC-E1076Q-eCFP (red); Double arrow indicates an mCherry-ParB focus (green) that does not colocalize with SMC-eCFP or SMC-E1076Q-eCFP (red). Expression of SMC or SMC-E1076Q was induced with xylose for 6 hours. Scale bar represents 2 μ m.

(D) Co-Immunoprecipitation of induced SMC or SMC-E1076Q and ParB in *Caulobacter* cells. FLAG-M2 tagged SMC or SMC-E1076Q was pulled down and co-immunoprecipitation ParB was determined by Western blotting. As a positive control, the co-immunoprecipitation of the SMC complex protein ScpA was determined. Western blots of whole-cell extracts (lysate) and eluted samples were probed with α -FLAG-M2 (SMC-FLAG), α -GFP (ScpA-eCFP), α -ParB (ParB). (MS608, MS610, MS631, MS632)

Figure 6. Aberrant localization of proteins involved in replication, segregation and cell division in SMC-E1076Q expressing cells

The centromere *parS* is visualized through its binding partner eCFP-ParB (green) in all experiments. Expression of SMC or SMC-E1076Q was induced with xylose for 0 (uninduced) or 6 (induced) hours. All images are overlays of phase contrast image, eCFP and eYFP or mCherry signal. Scale bar represents 2 μ m.

(A) Localization of replisome component DnaN-mCherry (red) expressed from the native promoter (MS648, MS649).

(B) Localization of segregation protein ParA-eYFP (red) expressed from the vanillate promoter (MS614, MS615). Arrow indicates ParA-eYFP extending from the pole opposite the ParB focus; double arrow indicates segregating ParB focus on the trailing edge of ParA-eYFP.

(C) Localization of divisome component FtsZ-eYFP (red) expressed from the vanillate promoter (MS519, MS520). Arrow indicates tight FtsZ-eYFP ring at midcell; double arrow indicates polar focus of FtsZ-eYFP; black arrowhead indicates FtsZ-eYFP ring found separated from constriction; white arrowhead indicates faint bands or foci of FtsZ-eYFP.

Figure 7. ATP hydrolysis modulated the stability of SMC-DNA binding interactions

(A) Gel shift assay with purified SMC and SMC-E1076Q in the presence or absence of 1 μ M ATP. No protein (lanes 1, 6, 11, 16) or increasing amounts of protein (125 nM, 250 nM, 500 nM, 1 μ M) were added to pBluescript KS+ (15.6 μ M nucleotides) and incubated for 60 minutes. Samples were run on a 0.7% agarose gel at 5.5 V/cm for 4 hours at 4°C. Arrowhead indicates SMC-DNA complex.

(B) Gel shift assay with purified SMC and SMC-E1076Q in the presence of 1 μ M ATP or 1 μ M ATP γ S (γ S). No protein, 125 nM or 1 μ M protein were added to pBluescript KS+ (15.6 μ M nucleotides) and incubated for 60 minutes. Samples were run on a 0.7% agarose gel at 5.5 V/cm for 4 hours at 4°C.

A

B

Figure 8. Model of the role of SMC ATP hydrolysis in chromosome segregation

(A) Top: Diagram of the *Caulobacter* chromosome soon after replication initiation. Duplicated DNA (black and gray) containing the *parS* centromere (green) is emerging from the replisome (blue). The boxed area is magnified in the model below. Bottom: SMC cannot distinguish between the two newly replicated chromosomes (black and gray) and so can spuriously embrace both chromosomes. However, the SMC-E1076Q dimer remains stably bound to the DNA because it lacks the ability to hydrolyze ATP, and the links between the two chromosomes inhibit segregation. Once the two chromosomes are held together, further binding of mutant dimers elsewhere on the adjacent chromosomes can reinforce the linkage

and the chromosomes stack up on each other (Fig. 3B), while maintaining the organization of the chromosome.

(B) Upon ATP hydrolysis, the arms of the wild type SMC dimer spuriously bound to both newly replicated chromosomes open and release the DNA. The temporary loss of cohesion allows the segregation machinery to move one of the chromosomes to the opposite pole. As the chromosomes are moved further apart, the SMC dimer is more likely to interact with only one chromosome.