ATP dependent DNA transport within cohesin: Scc2 clamps DNA on top of engaged heads while Scc3 promotes entrapment within the SMC-kleisin ring

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23 SUMMARY

24 In addition to extruding DNA loops, cohesin entraps within its SMC-kleisin ring (S-K)
 25 individual DNAs during G1 and sister DNAs during S-phase. All three activities require
 26 related hook-shaped proteins called Scc2 and Scc3. Using thiol-specific crosslinking we
 27 provide rigorous proof of entrapment activity in vitro. Scc2 alone promotes entrapment of
 28 DNAs in the E-S and E-K compartments, between ATP-bound engaged heads and the SMC
 29 hinge and associated kleisin, respectively. This does not require ATP hydrolysis nor is it
 30 accompanied by entrapment within S-K rings, which is a slower process requiring Scc3.
 31 Cryo-EM reveals that DNAs transported into E-S/E-K compartments are “clamped” in a sub-
 32 compartment created by Scc2’s association with engaged heads whose coiled coils are
 33 folded around their elbow. We suggest that clamping may be a recurrent feature of cohesin
 34 complexes active in loop extrusion and that this conformation precedes the S-K entrapment
 35 required for sister chromatid cohesion.

36 INTRODUCTION

37 Protein complexes containing SMC and kleisin subunits organise the spatial arrangement,
 38 or topology, of DNAs in most if not all living organisms (Nasmyth, 2001; Yatskevich et al.,
 39 2019). Best characterised are the eukaryotic cohesin and condensin complexes that are
 40 thought to organise chromosomal DNAs during interphase and mitosis, respectively, by a
 41 process of loop extrusion (LE) (Golfier et al., 2020). Cohesin in addition mediates the
 42 connections between sister DNAs that hold sister chromatids together during mitosis until
 43 their disjunction at the onset of anaphase (Oliveira et al., 2010; Uhlmann et al., 1999). Many
 44 clues as to their molecular mechanisms have emerged from structural studies. All contain a
 45 pair of rod-shaped SMC proteins with a dimerisation domain, known as the hinge, at one
 46 end and an ABC-like ATPase domain at the other, separated by a ~50 nm long anti-parallel
 47 intra-molecular coiled coil (Haering et al., 2002). Their association creates V-shaped dimers
 48 whose apical ATPase head domains are interconnected by a kleisin subunit (Scc1) whose
 49 N-terminal domain forms a three-helix bundle with the coiled coil emerging from Smc3's
 50 ATPase head (Gligoris et al., 2014), called its neck, and whose C-terminal winged helical
 51 domain binds to the base (or cap) of Smc1's ATPase head to complete the ring (Haering et
 52 al., 2004).

53
 54 Hinge dimerisation facilitates numerous other contacts between Smc1 and Smc3. First, their
 55 coiled coils interact with each other extensively, all the way from the hinge to the joint, a
 56 small break in the coiled coil roughly 5 nm above the heads, effectively zipping up the coiled
 57 coils (Bürmann et al., 2019; Chapard et al., 2019; Diebold-Durand et al., 2017; Soh et al.,
 58 2015). This process leads to the juxtaposition of the Smc1 and Smc3 heads, which are
 59 loosely associated under these conditions (Chapard et al., 2019; Diebold-Durand et al.,
 60 2017). Second, their coiled coils fold around an elbow, which results in an interaction
 61 between the hinge and a section of the coiled coils approximately 10 nm from the heads
 62 (Bürmann et al., 2019). Finally, the γ -phosphate of ATP bound to one ATPase head binds a
 63 signature motif on the other, resulting under appropriate conditions in engagement of the
 64 heads and a sandwiching of two molecules of ATP between them, a process that is a
 65 precondition for subsequent ATP hydrolysis (Arumugam et al., 2003; Lammens et al., 2004;
 66 Marcos-Alcalde et al., 2017). Head engagement has been proposed to disrupt coiled coil
 67 interactions, at least in the vicinity of the heads, yet the full extent of this disruption is not
 68 known.

69

70 The ATPase activities of SMC-kleisin complexes as well as all their biological functions in
 71 vivo depend on additional proteins that are recruited through their association with kleisin
 72 subunits and act by binding DNA and interacting with various SMC protein domains. In
 73 cohesin, this class of proteins consists of large hook-shaped proteins composed of HEAT
 74 repeats, known as Heat repeat containing proteins Associated With Kleisins (HAWKs) (Wells
 75 et al., 2017). Cohesin has three such HAWKs known as Scc2, Scc3, and Pds5. Scc3 is
 76 thought to be permanently bound to the complex (Tóth et al., 1999) while association of
 77 Scc2 and Pds5, whose occupancy is mutually exclusive, are more dynamic (Petela et al.,
 78 2018). Scc2 is essential for cohesin's ATPase activity (Petela et al., 2018), for its loading
 79 onto chromosomes (Ciosk et al., 2000), for maintaining cohesin's chromosomal association
 80 during G1 (Srinivasan et al., 2019), and for cohesin's ability to extrude loops in vitro
 81 (Davidson et al., 2019; Kim et al., 2019). However, Scc2 is not required to maintain cohesion
 82 during G2 or even establish cohesion during S phase from complexes previously associated
 83 with unreplicated DNAs (Srinivasan et al., 2019). Pds5 also has multiple functions. By
 84 recruiting Wapl, it promotes cohesin's dissociation from chromosomes, a process blocked
 85 by acetylation of two lysine residues on Smc3 during S phase (Beckouët et al., 2016; Chan
 86 et al., 2013, 2012). Pds5 also promotes acetylation during S phase and inhibits deacetylation
 87 during G2 and thereby protects sister chromatid cohesion which would otherwise be
 88 destroyed by Wapl-mediated release (Chan et al., 2013).

89
 90 Though the mechanism by which cohesin extrudes loops remains mysterious, there is a
 91 clear and simple hypothesis as to how cohesin holds sister DNAs together, namely by
 92 entrapping them both inside the S-K ring created through the binding of a kleisin subunit to
 93 the ATPase heads of an Smc1/Smc3 heterodimer (Gruber et al., 2003; Haering et al., 2002).
 94 This model explains the key observation that cleavage of cohesin's kleisin subunit by
 95 separase, or any other site-specific protease, is sufficient to trigger sister chromatid
 96 disjunction at anaphase (Oliveira et al., 2010; Uhlmann et al., 2000). To measure such
 97 entrapment in yeast, we have substituted residues within all three interfaces that make up
 98 S-K rings by pairs of cysteine residues that can be crosslinked by the thiol-specific reagent
 99 bis-maleimidoethane (BMOE). Around 20% of cohesin complexes can be crosslinked
 100 simultaneously at all three interfaces in vivo (Gligoris et al., 2014), and in post-replicative
 101 cells this is accompanied by formation of SDS-resistant structures that hold together the
 102 sister DNAs of circular minichromosomes, called catenated dimers or CDs (Chapard et al.,
 103 2019; Gligoris et al., 2014; Srinivasan et al., 2018). Because the two DNAs associated with

104 CDs are not otherwise intertwined (Haering et al., 2008), they must be held together by
 105 cohesin through a topological mechanism, either by co-entrapment within a chemically
 106 circularised S-K ring or conceivably in a three-way Borromean ring containing a pair of sister
 107 DNA rings and an SDS-resistant S-K ring. Importantly, the study of numerous mutants has
 108 revealed a perfect correlation between CD formation and cohesion establishment
 109 (Srinivasan et al., 2018), suggesting that these structures are actually responsible for sister
 110 chromatid cohesion or at the very least are produced by a highly related mechanism. Using
 111 cysteine pairs that crosslink heads that are not engaged, but are otherwise closely
 112 juxtaposed (J) (Chapard et al., 2019), it has been established that sister DNAs are at least
 113 some of the time entrapped between juxtaposed heads and the kleisin associated with them,
 114 namely within a J-K sub-compartment of the S-K ring (see Fig. 1A for an overview of the
 115 compartments).

116
 117 Loading of cohesin onto minichromosomes during G1 leads to a different topological
 118 interaction, namely catenation of individual circular DNAs by S-K rings (chemically
 119 circularised for detection), known as catenated monomers or CMs. Though loading of
 120 cohesin throughout the genome is normally accompanied by CM formation on
 121 minichromosomes, cohesin complexes containing a hinge with mutations within its lumen
 122 that neutralises its positive charge can load throughout the genome but cannot form either
 123 CMs or CDs (Srinivasan et al., 2018), implying that stable chromosomal association is not
 124 necessarily synonymous with entrapment of DNAs within S-K rings. That cohesin can
 125 associate with DNA in a functional manner without being topologically entrapped within S-K
 126 rings is supported by the finding that LE in vitro can be mediated by a version of human
 127 cohesin whose Smc1, Smc3, and kleisin subunits are expressed as a single polypeptide
 128 and whose hinge interface has been crosslinked by BMOE (Davidson et al., 2019).
 129 Importantly, neither of the above observations exclude the possibility that cohesin usually
 130 associates with chromosomal DNAs by entrapping a loop within its S-K ring, a type of
 131 association that has been termed pseudo-topological.

132
 133 Previous reports claiming entrapment of DNA within cohesin rings in vitro used salt
 134 resistance and sensitivity to cleavage as their criteria (Murayama and Uhlmann, 2015,
 135 2014). However, there are fundamental limitations to such experiments. Many types of
 136 association other than entrapment within a closed compartment could give rise to salt
 137 resistance and cleavage sensitivity. Equally serious, even if these criteria were indicative of

Fig 1

Figure 1. SMC-kleisin (S-K) rings entrap circular DNA in vitro

(A) Cohesin's different compartments and the position of cysteine pairs used in our crosslinking studies. **(B)** BMOE-induced crosslinking of S-K rings with cysteine pairs in the specified interfaces. CC = circular cohesin. **(C)** The entrapment assay scheme. **(D)** Entrapment of DNA in S-K rings in the presence or absence of 6C cohesin, or **(E)** the presence 5C cohesin lacking Scc1A547C, or 6C cohesin. **(F)** For DNA in the presence of Scc2, Scc3, and 6C cohesin and the presence or absence of BMOE, or **(G)** the presence or absence of ATP. Entrapment assays incubated for 40 min (* = damaged open circular DNA) (I = input DNA).

138 topological entrapment, they reveal little or nothing as to its nature, namely whether DNAs
 139 are entrapped in S-K rings or other closed compartments, for example the E-S and E-K
 140 compartments between ATP-bound engaged heads and the SMC hinge and associated
 141 kleisin, respectively, or indeed other types of compartment created by multiple contacts
 142 between HAWKs and SMC proteins. For these reasons, we describe here the use of thiol-
 143 specific crosslinking to measure bona fide topological entrapment of DNAs within S-K rings
 144 in vitro. Both Scc2 and Scc3 are essential for this process, as are their abilities to bind DNA.
 145 The process is dependent on ATP binding and stimulated by its hydrolysis, a feature largely
 146 absent from previous assays (Minamino et al., 2018).

147
 148 Remarkably, we find that Scc2 alone promotes the rapid entrapment of DNAs within E-S and
 149 E-K compartments in a process that is not accompanied by entrapment within S-K rings,
 150 and propose that E-S/E-K entrapment occurs simultaneously through a single mechanism.
 151 Because E-S/E-K entrapment is an order of magnitude more rapid than S-K entrapment, we
 152 suggest that creation of the former by Scc2 may be a precursor to the latter, a process
 153 contingent on the action of Scc3. Electron cryo-microscopy (cryo-EM) of complexes formed
 154 between cohesin's SMC-kleisin trimers and linear or circular DNAs in the presence of Scc2
 155 suggests that entrapment within E-S/E-K compartments involves transport of DNA between
 156 ATPase heads prior to their engagement, whereupon DNAs are "clamped" in a sub
 157 compartment formed by Scc2's association with engaged heads in a manner similar to that
 158 recently observed in a complex between DNA and both human and *Schizosaccharomyces*
 159 *pombe* cohesin associated with both Scc2^{NIPBL/Mis4} and Scc3^{SA2/Psc3} (Higashi et al., 2020; Shi
 160 et al., 2020). Our observations reveal key insights into the biochemical activities of Scc2 and
 161 Scc3 and suggest that the recurrent clamping of DNAs by Scc2^{NIPBL/Mis4} and engaged heads
 162 resulting in E-S/E-K entrapment, followed by their subsequent release, may be an integral
 163 aspect of cohesin's ability to load onto and translocate along DNA.

164

165 RESULTS

166 SMC-kleisin rings entrap circular DNA in vitro

167 We expressed cohesin trimers from *Saccharomyces cerevisiae* consisting of Smc1, Smc3,
 168 and Scc1 in insect cells using the baculovirus expression system (Fig. S1A). Scc3 was
 169 expressed separately because co-expression with trimers resulted in substoichiometric
 170 yields. We also expressed a version of Scc2 lacking its N-terminal domain (Scc2C, residues
 171 133-1493). Though this form no longer binds Scc4, it is fully capable of activating cohesin's
 172 ATPase activity (Petela et al., 2018), and for simplicity we will refer to this as Scc2 throughout
 173 most of the text. To measure entrapment of DNAs inside S-K rings (Fig. 1A i), we introduced
 174 cysteine pairs within all three ring interfaces (Smc1K639C-Smc3E570C, Smc1G22C-
 175 Scc1A547C, and Smc3S1043C-Scc1C56) that enables them to be crosslinked using
 176 BMOE. Individual interfaces were crosslinked with efficiencies varying from 30 - 70% and
 177 by comparing the migration of proteins following crosslinking cysteine pairs at single (2C),
 178 double (4C), and triple (6C) interfaces, we identified a covalently circular species only
 179 produced when all three interfaces were crosslinked (Fig. 1B).

180
 181 To measure DNA entrapment within cohesin's S-K compartment in vitro, 6C SMC-kleisin
 182 trimers were mixed with circular supercoiled DNAs, Scc2, and Scc3, and incubated for 40
 183 min at 24°C following addition of ATP. BMOE was then added and the mixture placed on ice
 184 for 6 min after which proteins were denatured by adding SDS to a final concentration of 1%
 185 and heating at 70°C for 20 min. The DNA was then fractionated by agarose gel
 186 electrophoresis and visualised by ethidium bromide staining (Fig. 1C & D). Addition of 6C
 187 trimers to Scc2/Scc3/DNA mixtures greatly reduced the amount of DNA co-migrating with
 188 supercoiled monomers and produced a ladder of retarded DNA species, most likely caused
 189 by successive entrapment by one, two, three and more S-K rings (Fig. 1D).

190
 191 We propose that the ladder corresponds to multiple cohesin rings entrapping individual
 192 DNAs and not entrapment of multiple DNAs by individual cohesin rings for two reasons.
 193 First, retardation caused by entrapment within E-S compartments (see below), which contain
 194 only Smc1 and Smc3, is less than that caused by entrapment within S-K or E-K
 195 compartments, which contain Scc1 as well as Smc1 and Smc3 (see Fig. 5B). Second,
 196 dimeric plasmid DNA, which is frequently present in plasmid preparations, although largely
 197 absent from these gels due to our purification protocol, runs roughly at the top of the gel with
 198 respect to our figures. Thus, if our ladders represented entrapment of multiple DNAs by

individual cohesin rings, the DNA retardation should be much greater. Those DNAs retarded by entrapment within a single ring correspond to the CMs previously observed in vivo (Gligoris et al., 2014). Ladder formation required cysteine pairs at all three interfaces. It was never observed with linear DNA (Fig. S1D) or when just a single cysteine (Fig. 1E) or BMOE (Fig. 1F) was omitted. Crucially, the ladders were strictly dependent on addition of ATP (Fig. 1G).

Entrapment of DNAs by S-K rings requires Scc3 and is stimulated by Scc2

To assess the roles of Scc2 and Scc3, we measured ladder formation at four successive 10 min intervals in the presence and absence of the two proteins. Ladders indicative of entrapment increased with time (up to 40 min), suggesting that formation is a slow process, were greatly reduced by omission of Scc2 (Fig. 2A), and almost completely abolished by omission of Scc3 (Fig. 2B). In the absence of both Scc2 and Scc3, the level of entrapment was comparable to that observed in the presence of Scc2 alone (data not shown).

Entrapment of DNAs by S-K rings depends on ATP binding to Smc3 and on ATP hydrolysis

To address the role of cohesin's ATPase, we mutated Smc3's Walker A site (Smc3K38I) to abolish ATP binding to Smc3. This almost completely abolished entrapment (Fig. 2C). We did not test the effect of mutating the equivalent residue in Smc1 as this has previously been shown to abolish association of Smc1/3 heterodimers with Scc1 (Arumugam et al., 2003). We next tested the effect of mutating both Walker B sites to residues that permit ATP binding but strongly inhibit hydrolysis (Smc1E1158Q Smc3E1155Q, 'EQEQ') (Fig. S1B), which caused a more modest, albeit still significant, reduction (Fig. 2D). These data suggest that cohesin's ability to complete the ATP hydrolysis cycle stimulates entrapment but is not strictly necessary. To address whether Smc3's K112 K113 are also important we analysed the effect of substituting them by glutamine (Smc3K112Q K113Q), mutations thought to mimic the acetylated state. This also reduced S-K entrapment (Fig. S1C), an effect that parallels its abrogation of cohesin loading in vivo (Hu et al., 2015).

DNA binding to Scc3 is required for its entrapment by S-K rings

During a search for cohesin domains that bind DNA, we discovered that Scc3's association with a fragment of Scc1 containing residues 269-451 greatly stimulates its association with double stranded DNA, as measured using an electrophoretic mobility shift assay (EMSA)

Fig 2

Figure 2. Entrapment within S-K rings requires both Scc2 and Scc3, ATP binding to Smc3, and is stimulated by ATP hydrolysis

(A) Entrapment of DNA in S-K rings in the presence of Scc3, and the presence or absence of Scc2, or (B) the presence of Scc2, and the presence or absence of Scc3 (* = damaged open circular DNA). (C) DNA entrapment in the presence of Scc2 and Scc3, comparing WT cohesin to Smc3K38I (K38I), or (D) the effect of Smc1E1158Q Smc3E1155Q (EQEQ) in the presence of both Scc2 and Scc3. Entrapment assays incubated for 40 min with time points taken every 10 min (I = input DNA).

(Fig. S2A). Reasoning that Scc3/Scc1 complexes might bind DNA in a similar manner to that recently observed in a co-crystal of DNA bound to condensin's Ycg1 HAWK bound to its kleisin partner Brn1 (Kschonsak et al., 2017), we mutated two clusters of positively charged residues (Scc3K224E K225E R226E and Scc3 K423E K513E K520E) on opposite sides of the groove within Scc3 that is equivalent to Ycg1's DNA binding groove (Fig. 3A). Neither triple (3E) mutant eliminated DNA binding (Fig. S2B) nor caused lethality (Fig. S2E). Despite this, both reduced cohesin's association with all genomic sequences except point centromeres (*CENs*) (Fig. S2C & D). In contrast, combining the two triple mutations (to create 6E) was lethal (Fig. S2E), abolished binding of Scc3 to DNA in the presence of Scc1 (Fig. 3B), and with the exception of *CENs* eliminated cohesin's association with the genome (Fig. 3C & D).

Remarkably, cohesin containing Scc3-6E accumulated to exceptionally high levels at *CENs* (Fig. 3C & D), which are the loading sites for most peri-centric cohesin (50 kb intervals surrounding *CENs*). This distribution resembles that of Scc2 in wild type cells and indeed, *scc3-6E* had little or no effect on Scc2's accumulation with *CENs* (Fig. 3E). This implies that cohesin containing Scc3-6E forms complexes with Scc2 at *CENs* but subsequently fails to form a stable association with chromatin or translocate into neighbouring sequences. Our ability to detect such complexes at *CENs* but not at other loading sites along chromosome arms can be attributed to the fact that Scc2's partner Scc4 binds to the kinetochore protein Ctf19 and this association transiently tethers complexes at *CENs* while they are attempting to load (Hinshaw et al., 2017). Though accumulation of cohesin bound by Scc2 at *CENs* does not depend on Scc3's ability to bind DNA, it does still require Scc3 (Fig. S2F). Crucially, cohesin containing Scc3-6E failed to support entrapment of DNAs inside S-K rings in vitro (Fig. 3F). During the course of our work, a crystal structure of DNA bound to a Scc3/Scc1 complex confirmed that it does indeed bind DNA (Li et al., 2018 PDB 6H8Q) in a manner resembling that of Ycg1. Moreover, K224, K225 R226, K423, K513, and K520 are all predicted to contribute to the association. These data imply that Scc3's ability to bind DNA has an important role in cohesin's ability to load onto and translocate along chromosomal DNA in vivo, as well as entrap in S-K rings in vitro.

DNA binding to Scc2 facilitates entrapment by S-K rings

The *S. pombe* Scc2/4 complex has previously been shown to bind DNA in vitro (Murayama and Uhlmann, 2014) but the physiological significance of this activity has never been

Fig 3

Figure 3. DNA binding to Scc3 is required for its entrapment by S-K rings

(A) Structure of *S. cerevisiae* Scc3 (orange) protein in complex with a fragment of Scc1 (green) (PDB 6H8Q). Labelled are the six residues within the DNA binding groove of Scc3 that were mutated to glutamate (Scc3-6E). (B) EMSA comparing the ability of WT Scc3-Scc1²⁶⁹⁻⁴⁵¹ and Scc3-6E-Scc1²⁶⁹⁻⁴⁵¹ complexes to bind dsDNA. (C) Average calibrated ChIP-seq profiles of Scc1-PK6 60 kb either side of *CENs* in the presence of ectopic WT Scc3 (KN27821) or Scc3-6E (KN27804). Cells were arrested in G1 with α -factor prior to release into auxin and nocodazole containing media at 25°C to deplete the endogenous Scc3. ChIP-seq samples were taken 60 min after release. (D) Average calibrated ChIP-seq profile of ectopic WT (KN27796) or mutant (KN27802) Scc3-HA performed as in C. (E) Average calibrated ChIP-seq profile of Scc2-PK6 in the presence of ectopic WT Scc3 (KN27821) or Scc3-6E (KN27804). Experiment was performed as in C. (F) Entrapment of DNA within S-K rings in the presence Scc2 and either WT Scc3 or Scc3-6E. Entrapment assay incubated for 40 min with time points taken every 10 min (I = input DNA).

investigated. EMSA revealed that *S. cerevisiae* Scc2 also binds DNA (Fig. 4A), as do Scc2/4 complexes with slightly higher affinity (Fig. S3A). Unlike Scc3, whose DNA binding was greatly enhanced by Scc1, DNA binding by Scc2 was reduced by addition of a Scc1 fragment (Scc1¹⁵⁰⁻²⁹⁸) that contains sequences necessary for Scc2-dependent loading in vivo (Fig. 4A) (Petela et al., 2018). Interestingly, the inhibitory effect of Scc1¹⁵⁰⁻²⁹⁸ was not observed in the binding of DNA to full length Scc2/4 (Fig. S3A), suggesting that DNA binding sites also exist in Scc4, or in sequences N-terminal of the deletion in our Scc2C construct.

An alignment of the crystal structure of *E. gossypii* Scc2 (Chao et al., 2017) with that of Ycg1/Brn1 bound to DNA (Kschonsak et al., 2017) revealed not only a remarkable similarity in the overall shape of their hook-shaped HEAT repeats but also a set of potential DNA binding residues on the surface of the shallow concave groove corresponding to Ycg1's DNA binding pocket (Petela et al., 2018) (Fig. 4B). Four of these are particularly conserved and correspond to S717, K721, K788, and H789 in *S. cerevisiae*. Both *scc2S717L K721E* and *scc2K788E H789E* double mutants are lethal and abolish loading of cohesin throughout most of the genome (Petela et al., 2018) (Fig S3C). To test whether these residues participate in binding DNA, we used EMSAs to measure the effect on DNA binding of mutating the above residues to glutamate. Both *Scc2S717E K721E* and *Scc2K788E H789E* double mutants reduced binding (Fig. S3B) but caused only a modest reduction in S-K entrapment (data not shown). In contrast, the quadruple mutant *Scc2S717E K721E K788E H789E* (Scc2-4E) not only greatly reduced DNA binding (Fig. 4C) but also S-K entrapment (Fig. 4D). These results suggest that Scc2's ability to bind DNA has a crucial role in entrapping DNA within S-K rings in vitro, an activity also required for loading cohesin onto chromosomes in vivo (Petela et al., 2018) (Fig. S3C). They also demonstrate that the stimulation of DNA entrapment within S-K rings by Scc2 is not merely an adventitious property of Scc2 but an activity dependent on conserved surface residues that have unambiguous physiological functions.

DNA is never entrapped in J-S and only rarely in J-K compartments

Using the Smc1S161C-Smc3K160C cysteine pair, cohesin's ATPase heads can also be efficiently crosslinked in the J-state (Chapard et al., 2019). Moreover, this crosslinking can be combined with simultaneous crosslinking of N- and C-terminal kleisin domains to Smc3 and Smc1 ATPase heads respectively, to measure entrapment within J-K compartments

Fig 4

Figure 4. DNA binding to Scc2 facilitates entrapment by S-K rings

(A) EMSA comparing the ability of Scc2 and Scc2-Scc1¹⁵⁰⁻²⁹⁸ complexes to bind dsDNA. (B) *S. cerevisiae* Scc2 from the cryo-EM structure (Fig. 8) with the four residues within the putative DNA binding surface labelled that were mutated to glutamate (Scc2-4E). (C) EMSA comparing the ability of Scc2 and Scc2-4E complexes to bind dsDNA. (D) Entrapment of DNA in S-K rings in the presence of Scc3 and either Scc2 or Scc2-4E. Entrapment assay incubated for 40 min with time points taken every 10 min (* = damaged open circular DNA; I = input DNA).

(Fig. S4A), or with simultaneous crosslinking of the hinge (Fig. S4B), to measure entrapment of DNAs in J-S compartments (Fig. 1A ii). J crosslinking alone or in combination with hinge (J-S) or kleisin (J-K) was efficient even in the presence of ATP, DNA, Scc2, and Scc3 (Fig. S4C). In other words, both J-S and J-K circularisation occurred efficiently under conditions that promote efficient entrapment of DNAs inside S-K rings. However, DNAs were never entrapped within J-S compartments and only rarely by J-K ones (Fig. S4D). J-K entrapment was not only much less frequent than S-K entrapment but also independent of Scc2. The fact that J-K entrapment was comparable to S-K entrapment in the absence of Scc2 (compare Fig. 2A and S4D) suggests that the low-level entrapment of DNAs in S-K rings induced by Scc3 alone may in fact correspond to DNAs entrapped in J-K compartments. Though J-K circularisation by BMOE is modestly lower than that of S-K, this cannot account for its far lower DNA entrapment. We therefore suggest that most ATPase heads associated with DNA entrapped within S-K rings in vitro are not juxtaposed. They are either fully disengaged, in the E-state, or in some other conformation.

314

315 **Rapid DNA entrapment in E-S and E-K compartments**

316 We used the same approach to measure entrapment in E-S or E-K compartments (Fig. 1A
317 iii), in this case replacing J-specific cysteines by a pair specific for the E-state
318 (Smc1N1192C-Smc3R1222C). Unlike J crosslinking, which was readily detected in cohesin
319 trimers, efficient E-state crosslinking was dependent on the presence of ATP (Fig. S5A). As
320 with J-, E-state crosslinking can be combined with simultaneous crosslinking of N- and C-
321 terminal kleisin domains to Smc3 and Smc1 ATPase heads respectively, to measure
322 entrapment within E-K compartments (Fig. S5B), or with simultaneous crosslinking of the
323 hinge (Fig. S5C), to measure entrapment of DNAs in E-S compartments. As previously
324 reported (Chapard et al., 2019), Smc1/3 dimers crosslinked simultaneously at the hinge and
325 engaged heads co-migrate with those crosslinked at the hinge alone, which hinders
326 detection of E-S circularisation directly. Given that double crosslinking has been detected in
327 vivo using differently tagged proteins (Chapard et al., 2019) and that DNAs are readily
328 entrapped by Smc1/3 dimers containing hinge and E-specific cysteine pairs treated with
329 BMOE, we can reason that efficient double crosslinking does indeed occur. DNAs were
330 entrapped in an ATP-dependent fashion in both E-S and E-K compartments in the presence
331 of Scc2 and Scc3 (Fig. 5A). Notably, both processes occurred much more rapidly than S-K
332 entrapment, with significant amounts of DNA entrapped by multiple rings within 2 min (Fig.
333 5B). Because S-K entrapment occurs much more slowly, the efficient entrapment of DNAs

Fig 5

Figure 5. Rapid DNA entrapment in E-S and E-K compartments

(A) Entrapment of DNA in E-S/E-K compartments in the presence of Scc2 and Scc3, and the presence or absence of ATP, incubated for 40 min (* = damaged open circular DNA). (B) DNA entrapment in S-K rings, or E-S/E-K compartments in the presence of Scc2 and Scc3, incubated for either 2 or 40 min. (C) DNA entrapment in E-S/E-K compartments in the presence of Scc2, Scc3, Scc2 and Scc3, or absence of both, incubated for 2 min. (D) DNA entrapment in E-S/E-K compartments in the presence of Scc2, and either the presence or absence of Scc3, incubated for either 2 min or 40 min. (E) DNA entrapment in E-S/E-K compartments in the presence of either Scc2 or Scc2-4E, or (F) Entrapment in the presence of Scc2 alone, comparing WT and Smc1E1158Q Smc3E1155Q (EQEQ) cohesin, incubated for 2 min. (G) DNA entrapment in E-S compartments in the presence of Scc2 comparing WT and Smc3K38I (K38I) cohesin, or (H) Entrapment of DNAs in S-K rings, or E-S/E-K compartments in the presence of Scc2, incubated for 2 min (I = input DNA).

inside E-S/E-K compartments within a few minutes is presumably not accompanied by S-K entrapment. Though it occurs efficiently in vitro, entrapment of circular DNAs by cohesin in E-S compartments has not so far been detected in vivo, although *Bacillus subtilis* SMC possessing Walker B mutations have been shown to have such an activity inside cells (Vazquez Nunez et al., 2019).

339

340 **Entrapment within the E-S and E-K compartments depends on Scc2 but not Scc3**

341 In contrast to entrapment within S-K rings, which depends on Scc3, the rapid entrapment of
342 DNA in the E-S/E-K compartments was Scc3 independent (Fig. 5C). However, it was highly
343 dependent on Scc2, both in the presence or absence of Scc3 (Fig. 5C). Levels of E-S
344 entrapment increased between 2 and 40 min in the presence of Scc2, as well as in the
345 presence of Scc2 and Scc3 (Fig. 5D). However, while a similar result was seen for E-K
346 entrapment in the presence of Scc2 alone, this increase was not observed in the presence
347 of both Scc2 and Scc3 which instead showed no increase by the longer time point and
348 possibly even a small reduction.

349

350 The rapid entrapment of DNAs within E-S/E-K compartments in the presence of Scc2 was
351 reduced by Scc2-4E (Fig. 5E), suggesting that the reaction at least partly depends on Scc2's
352 ability to bind DNA. Strikingly, both types of entrapment were unaffected by Smc1E1158Q
353 Smc3E1155Q mutations (EQEQ), implying that neither form of entrapment requires ATP
354 hydrolysis (Fig. 5F). In contrast to S-K entrapment in the presence of both Scc2 and Scc3
355 (Fig. 2C), E-S entrapment in the presence of Scc2 alone was only modestly reduced by
356 Smc3K38I, implying that ATP bound merely to Smc1's ATPase head is sufficient (Fig. 5G).
357 Indeed, Smc3K38I does not prevent E-specific crosslinking under these reaction conditions,
358 namely in the presence of ATP, DNA, and Scc2 (Fig. S5D) and its modest reduction of E-S
359 entrapment is in line with its effect on E-state crosslinking (Fig. 5G & S5D). Though it does
360 not abolish head engagement, Smc3K38I clearly compromises the process. As long as ATP
361 is present, neither DNA nor Scc2 are required for efficient E-state crosslinking of wild type
362 complexes but both are important for Smc3K38I complexes (Fig. S5D). Smc3K38I
363 presumably destabilises head engagement in a manner that can be overcome by the
364 presence of Scc2 and DNA. To explore whether Scc2 and DNA also promote head
365 engagement of otherwise wild type complexes, we tested their effect when ATP's ability to
366 promote head engagement is compromised by omission of Mg²⁺ (Fig. S5E). Under these
367 circumstances, addition of both Scc2 and DNA restored efficient head engagement and both

368 factors were required for this effect. Our finding that Scc2 and DNA collaborate to promote
369 ATP-dependent head engagement suggests that DNA binds to a site created by head
370 engagement as well as to Scc2.

371

372 **Scc2 causes DNAs to be entrapped in E-S and E-K compartments without entering S-** 373 **K rings**

374 Because Scc3 is crucial for S-K entrapment, the rapid entrapment of DNAs within E-S and
375 E-K compartments in the presence of Scc2 alone should be unaccompanied by S-K
376 entrapment. This is indeed the case. In contrast with E-S or E-K entrapment, which is very
377 efficient, few if any DNAs are entrapped in S-K compartments by 2 min (Fig. 5H). Though
378 paradoxical, this striking observation has a very simple explanation. The similarity in kinetics
379 suggests that E-S and E-K entrapments are created simultaneously as part of the same
380 reaction. In other words, a single type of DNA passage followed by head engagement gives
381 rise to both types. We envisage two types of mechanism to explain how this occurs without
382 S-K entrapment. According to the first (and simplest), DNA moves “upwards” between
383 disengaged ATPase heads, and is subsequently trapped in the E-S compartment following
384 ATP-driven head engagement (Fig. 9A). An alternative is that a loop of DNA is inserted into
385 an open S-K ring. If one of the loop’s segments were located above the ATPase domains
386 while the other below, then subsequent head engagement would lead to simultaneous
387 entrapment in both E-S/E-K compartments (Fig. 9B). Neither type of DNA movement
388 involves passage through a gate created by opening the S-K ring, hence explaining the lack
389 of S-K entrapment. Entrapment within E-S/E-K, but not S-K compartments, in the presence
390 of Scc2 alone was also observed with relaxed (nicked) DNAs (data not shown).

391

392 **Entrapment of DNA within E-S compartments disrupts coiled coil interactions** 393 **proximal to the heads**

394 To address whether the coiled coils are associated when Smc1/3 heads engage in vitro in
395 the presence of Scc2, we combined the E-specific cysteine pair with one specific for the
396 coiled coils (Smc1K201C-Smc3K198C), in close proximity to the joint (Fig. 6A). This
397 revealed that double crosslinking can indeed occur in the presence of ATP (Fig. 6B). As
398 expected, double crosslinking also occurred efficiently when the coiled coil pair was
399 combined with one within the hinge interface (Smc1K639C Smc3E570C). These cysteine
400 pair combinations enabled us to measure entrapment within two sub-compartments within
401 the E-S compartment (Fig. 6A iii): one created by simultaneous crosslinking of the hinge and

A **B** **C** **Fig 6**

Figure 6. E-S/E-K entrapment leads to dissociation of the coiled coil around the joint

(A) Scheme showing the location of the joint cysteine pair and how head engagement could lead to different degrees of coiled coil dissociation and sub-compartment formation. (B) BMOE crosslinking of cohesin containing cysteine pairs at the specified interfaces in the presence of ATP. CC = coiled coils. (C) DNA entrapment in E-S compartments, or either E-C or C-H sub-compartments, in the presence of Scc2, incubated for 2 min (I = input DNA).

coiled coils (C-H compartment) (Fig. 6A ii) and a complementary one made by the simultaneous crosslinking of coiled coils and engaged heads (E-C compartment) (Fig. 6A i). If DNAs entrapped in E-S compartments are in molecules whose coiled coils are associated, at least in the vicinity of their joint regions, then they must be entrapped either in the E-C or the C-H sub compartments. On the other hand, if the entrapment of DNAs within E-S compartments is accompanied by (or indeed causes), dissociation of the coiled coils in the vicinity of the Smc1K201C-Smc3K198C cysteine pair, then DNA should not be trapped in either of these sub-compartments.

Despite efficient crosslinking at both cysteine pairs (Fig. 6B), few if any DNAs were entrapped in the presence of Scc2 and ATP following BMOE treatment of cohesin trimers containing hinge and coiled coil cysteine pairs (Fig. 6C). Likewise, few if any DNAs were entrapped by cohesin trimers containing both E-state and coiled coil cysteine pairs. We deduce from this result that entrapment of DNAs within E-S compartments in the presence of Scc2 is accompanied by dissociation of their coiled coils in a manner that precludes crosslinking between Smc1K201C and Smc3K198C. It is important to point out that this feature was not apparent when analysing the crosslinking efficiency between Smc1K201C and Smc3K198C under the same conditions (data not shown), namely in the presence of Scc2, ATP and DNA. To explain this, we suggest that despite the addition of DNA, complexes exist, at least transiently, that have engaged their heads and have zipped up their coiled coils but have not in fact trapped DNA within their E-S compartments. Consistent with this notion is our finding that simultaneous crosslinking of coiled coils and engaged heads occurs efficiently even in the absence of DNA (Fig. 6B).

The DNA is ‘clamped’ between Scc2 and the engaged heads during entrapment within E-S/E-K compartments, as revealed by cryo-EM

The dissociation of Smc1 and Smc3 coiled coils in the vicinity of their joint regions would create space for DNA to bind to engaged heads, as observed in Mre11/Rad50 complexes (Liu et al., 2016) and more recently in both human and *S. pombe* cohesin containing Scc2^{NIPBL/Mis4} and Scc3^{SA2/Psc3} (Higashi et al., 2020; Shi et al., 2020). In the latter structures, the coiled coils of Smc1 and Smc3 diverge from each other at 70° as they emerge from the engaged heads, thereby creating a site for DNA to bind a surface on top of the heads. The DNA is also bound by Scc2, whose simultaneous association with Smc1’s ATPase head

435 and the coiled coil emerging from Smc3's head creates a new type of sub compartment
436 within which DNA bound to the engaged heads is entrapped or 'clamped'.

437

438 Our findings that Scc2 and DNA together promote head engagement (Fig. S5D & E) and
439 that Scc2-4E reduces E-S/E-K entrapment (Fig. 5E) raise the possibility that DNAs en-
440 trapped within E-S/E-K compartments through the action of Scc2 are bound in a similar
441 manner. However, since the human (PDB 6WG3) and *S. pombe* cryo-EM complexes
442 (Higashi et al., 2020; Shi et al., 2020) were formed by cohesin containing Scc3^{SA2/Psc3}, the
443 complexes described here, which are formed in the absence of Scc3, could in fact have a
444 very different conformation. Furthermore, because it is not possible to trace the entire kleisin
445 chain, and because the complexes contain only short linear DNA molecules, the topology of
446 DNA's association with the SMC-kleisin trimers in the existing structures cannot be inferred
447 definitively. In other words, it is not possible to determine whether the DNAs in the structures
448 are entrapped within E-S and E-K and/or S-K compartments. As it happens, the kleisin path
449 deduced for PDB 6WG3 (Shi et al., 2020) suggests that DNA, if it were circular, would be
450 trapped in E-S and S-K but not E-K compartments. Thus, the presence of Scc3^{SA2} in this
451 complex may have had an important influence on the topology of cohesin's association with
452 DNA.

453

454 To elucidate how DNA actually associates with EQEQ cohesin trimers in the presence of
455 ATP and Scc2, but lacking Scc3, namely under conditions in which DNAs are clearly en-
456 trapped within E-S and E-K but not S-K compartments, we used cryo-EM to solve the struc-
457 ture of EQEQ cohesin trimer (Smc1, Smc3 and Scc1) bound to ATP, linear DNA (40 bp),
458 and Scc2C2 (residues 151-1493) to a resolution of 3.4 Å (Fig. 7A). Processing followed
459 standard cryo-EM single particle procedures as implemented in RELION 3.1 pipelines
460 (Scheres, 2012), but significant preferred orientation required the use of tilted data acquisi-
461 tion (Methods). With the help of previous crystal structures (PDBs 5ME3, 1W1W and 4UX3)
462 (Chao et al., 2017; Gligoris et al., 2014; Haering et al., 2004) the electron density map ena-
463 bled us to build and refine a reliable atomic model, with DNA rigidly clamped between the
464 head domains and the HAWK subunit Scc2 (Table 1, Fig. 8A).

465

466 A key question arising from our crosslinking studies concerns the mechanism by which cir-
467 cular DNA is entrapped within E-S/E-K compartments without being entrapped within the S-
468 K ring, namely whether a single segment of DNA is passed between the heads prior to their

Fig 7

Figure 7. Cryo-EM of cohesin clamping DNA in the E-S/E-K state

(A) Cryo-EM map of 40 bp DNA clamped by Scc2- and ATP-bound cohesin EQEQ trimer at 3.4 Å resolution. Both front and side views are coloured by subunit. **(B)** Same complex as shown in A but bound to ~1.8 Kbp relaxed circular DNA as a cryo-EM field view (using Volta phase plate, left) and a selection of 2D class averages (right) clearly showing DNA emanating from cohesin/Scc2 complexes. **(C)** 7.3 Å resolution cryo-EM map of the complex shown in B, coloured by subunit, demonstrating that the same conformation of the complex has been obtained as with linear DNA (panel A). Same orientations and colours as in A. **(D)** 2D class averages obtained by reprocessing of the same data set as used for A with an enlarged box size show the position of the coiled coils and the hinge. **(E)** ~10 Å resolution cryo-EM map of the entire tetramer complex as shown in D. Since we used the same complex as used in the in vitro entrapment reactions, we can deduce that the DNA within the clamped structure depicted in A, C and E must be entrapped in both the E-S and E-K compartments.

engagement or whether a loop of DNA is first passed through the S-K ring before head engagement traps one segment of the loop above and another below the heads. Visualising how DNA is actually grasped by cohesin and Scc2 under these conditions should in principle be revealing. However, the linearity of the 40 bp oligonucleotide used for our high-resolution structure (Fig. 7A) precludes any conclusions as to the topology of its association with cohesin. In other words, we cannot say whether it corresponds to E-S/E-K entrapment. It also precludes any insight as to how DNA actually enters the Scc2-SMC clamp because the DNA could either have been passed through the heads prior to their engagement (or any other gate), or it could simply have been threaded through the clamp after head engagement. For this reason, we also solved to a resolution of ~ 7 Å the structure of the same clamped state associated with circular relaxed DNA (Table 1, Fig. 7B & C). Crucially, the structure associated with circular DNA is virtually identical to that associated with the linear oligonucleotide and since the former is known to involve E-S/E-K but not S-K entrapment, we can with some certainty infer the path of the kleisin chain with respect to the DNA (Fig. 8F). Two important conclusions can therefore be drawn. First and foremost, E-S/E-K entrapment does indeed arise from the clamping of DNA between Scc2 and engaged heads in the manner revealed by both high (Fig. 7A) and medium (Fig. 7B) resolution cryo-EM structures. Second, because the circular DNA is demonstrably not highly-bent when clamped (Fig. 7B, right), the DNA must have entered the clamped state and thereby been entrapped in E-S/E-K compartments without formation of a loop (Fig. 9B). In other words, a single segment of DNA must have been passed between the heads prior to their engagement (Fig. 9A).

Processing of the linear DNA dataset using boxes large enough to cover entire cohesin complexes (Table 1, Methods) revealed a significant subset of particles ($\sim 30\%$) that contained lower-resolution information about the conformation of the coiled coils and placement of the hinge (Fig. 7D & E). Despite partial head-proximal unzipping of the coiled coils in this state, the reconstructed map clearly revealed a folded conformation reminiscent of *apo* condensin, a fraction of ATP-bound condensin (Lee et al., 2020) and *apo* cohesin (Bürmann et al., 2019). A folded state was also presumed to exist in the human cohesin structure where the HAWKS and the hinge interacted directly, but the coiled coils could not be resolved (Shi et al., 2020). Our map did not have sufficient resolution to determine whether the hinge was partially open or closed (Fig. 7E).

Binding of DNA to Scc2 is consistent with Scc2-4E effects

Binding of DNA by Scc2 in *S. cerevisiae* cohesin occurs in a similar manner to that recently described for human Scc2^{NIPBL} (Shi et al., 2020) (Fig. 8A). Scc2 holds DNA by way of a curved basic and polar surface located around the transition between its neck and head regions (Fig. 8B). The surface, which causes the DNA to bend slightly (~9°), is created by the spatial arrangement into a semicircle of a series of residues (e.g. S508, N557, K714, S753, S783, and K1324/25) from the ends of six α-helices and one loop (containing K427) that together engulf the phosphate backbone. This region includes all four positions mutated in Scc2-4E (S717E K721E K788E H789E, Fig. 4), thus neatly explaining why the charge-reversing mutations lowered the binding affinity for DNA and inhibited entrapment of DNAs in vitro.

Scc2 binds both Smc1 and Smc3

Clamping involves not only the binding of DNA to Scc2 and to engaged heads (see below) but also entrapment in a novel compartment created by Scc2's association with both Smc1 and Smc3. The latter involves multiple binding sites (Fig. 8C) and is therefore much more delocalized than Scc2's DNA binding. Highly prominent is the binding of Scc2 to Smc1 through the docking of HEAT repeats 18-24 (residues 1127-1493) onto the F-loop (residues 1095-1118) on Smc1 head's C-lobe and the coiled coils that emerge above (Fig. 8C i). This mode of association is highly analogous to the binding of *S. cerevisiae* condensin Smc4 by the HAWK Ycs4 (Lee et al., 2020) (Fig. S6). Scc2 simultaneously interacts with Smc3's N-lobe - thereby providing a mechanism by which it promotes head engagement and subsequent ATP hydrolysis. One key contact in this regard involves residues in an otherwise disordered loop (1178-1203) that form a β-strand which docks onto the end of the central β-sheet of the Smc3 head (Fig. 8C iii). More conserved is a major contact mediated by salt bridges between a collection of highly conserved aspartate and glutamate residues located within two loops of Scc2 (819-EDEED-823 and 781-DD-782) and two key lysine residues (K112 K113) in Smc3 (Fig. 8C ii). Entrapment of DNA between Scc2 and engaged heads arises because in addition to the above contacts, Scc2 contacts Smc3 through HEAT repeats 1-4 (residues 151-409), which bind to the start of the joint module (coiled coil arm, residues 999-1004) in a manner that — when compared to the unbound crystal structure (Kikuchi et al., 2016) — causes a conformational rearrangement of Scc2's head segment. This movement is necessary to accommodate the simultaneous binding of DNA by both Scc2 and the heads while presumably playing a role in stabilising the unzipped conformation of the coiled coils (Fig. 8C iv). Several residues within this interface (for example, Smc3

Fig 8

Figure 8. Molecular interactions in the E-S/E-K state

(A) Cartoon representation of the refined atomic model of cohesin's clamped (E-S/E-K) state based on the 3.4 Å resolution cryo-EM map (Fig. 7A, same orientation and colours, Table 1). (B) Basic and polar residues of Scc2 involved in the interaction with DNA. Scc2 interacts only with the backbone. Residues in its vicinity are labelled in black while those mutated in Scc2-4E (Fig. 4) in purple. (C) Scc2 makes extensive contacts with both Smc1 and Smc3 heads: i) Scc2 binds Smc1 through its HEAT repeats 18-24 (residues 1127-1493) that dock onto the F-loop on Smc1 (residues 1095-1118) and the emerging coiled coils above it. ii) Smc3's K112 K113, whose acetylation reduces loading efficiency, are in the vicinity of a negatively charged patch on Scc2 (819-EDEED-823 and 781-DD-782). iii) Scc2 binds to Smc3 through a β -strand (part of the otherwise disordered loop 1178-1203) that complements the central β -sheet of Smc3. iv) The N-terminal section of Scc2 contacts parts of Smc3's coiled coil arm/neck, close to where the last ordered region of Scc1's N-terminal domain is bound to the Smc3 coiled coil. (D) DNA binding to the SMC head domains is pseudo-symmetrical. Top: the pseudo two-fold axis of the DNA neatly aligns with that of the head domains underneath. Bottom: The head domains interact with the DNA almost exactly two full DNA turns apart, utilising pseudo symmetry-related surfaces (Smc1: K63, S112, R113, & K124; Smc3: K57, K112, K113, & K125). (E) The two lysines K112 K113 are in contact with a negatively charged patch on Scc2 (see panel C iii), but are also in the vicinity of the DNA backbone. (F) The N-and C-terminal domains of the kleisin Scc1 bind canonically to Smc3 and Smc1, linking the heads and topologically closing the tripartite Smc1/Smc3/Scc1 (S-K) cohesin ring. A tentative path of the disordered regions of Scc1, not visible in our cryo-EM map is shown to demonstrate the topology as deduced from the loading reactions and subsequent crosslinking that show that the DNA must be outside the tripartite S-K ring.

537 R225 K228 and Scc2 E304) are highly conserved, suggesting that it has an important func-
538 tion.

539

540 **Pseudo-symmetric binding of DNA to the engaged Smc1/3 ATPase heads**

541 While Scc2 holds the upper half of the DNA's backbone through a spiral of basic and polar
542 residues (Fig. 8B), the engaged heads of Smc1 and Smc3 produce a 2-fold pseudo-sym-
543 metrical ABC ATPase heterodimer that binds DNA through two sites that are exactly two
544 turns of the DNA apart and coincide with the major groove in the DNA (Fig. 8D). As expected
545 for DNA-binding proteins that are not sequence specific, neither protein inserts residues into
546 the major or minor grooves and both rely solely on interactions with the DNA backbone. A
547 consequence of this binding mode is that the two-fold symmetry of DNA is matched almost
548 perfectly by the Smc1/3 heterodimer (Fig. 8D top). The two pseudo-symmetrical DNA bind-
549 ing sites close to the major groove are formed through basic and polar amino acids in Smc1
550 (e.g. K63, S112, R113, & K124) and Smc3 (e.g. K57, K112, K113, & K125) (Fig. 8D bottom).
551 The DNA is bent slightly ($\sim 9^\circ$) and it seems likely that without bending the DNA binding sites
552 on Smc1 and Smc3 would be too close together. Overall, DNA binding is linked to head
553 engagement and the ATPase cycle, as the complete binding path for DNA along the heads
554 only arises when both heads come together in the E-state.

555

556 **How do Smc3 K112 and K113 affect loading?**

557 In yeast, Smc3 K112 and K113 have important roles in loading of cohesin onto chromo-
558 somes. We show here that changing KK to QQ reduces S-K entrapment in the presence of
559 Scc2 and Scc3 in vitro (Fig. S1C), recapitulating the adverse effect on genome wide asso-
560 ciation in vivo (Hu et al., 2015). The QQ double mutation is thought to mimic acetylation of
561 K112 K113, which takes place as cells undergo S phase and may have a role in altering
562 how cohesin interacts with DNA, principally whether it can associate (de novo) with and
563 translocate along chromosomes. Our high resolution cryo-EM structure (Figs. 8A and C)
564 reveals that K112 K113 belong to the array of residues that create a basic environment for
565 charge-mediated binding to the DNA backbone (Fig. 8E). This raises the possibility that
566 K112 K113 participate directly in the binding of DNA to engaged heads. However, close
567 inspection of their side chains shows that they in fact face towards the two aspartate and
568 glutamate rich loops in Scc2 (819-EDEED-823 and 781-DD-782) (Fig. 8C ii), implying that
569 they engage in ionic interactions between Smc3 and Scc2 as well as or instead of DNA. If
570 so, one consequence of acetylation or replacement by QQ may be disruption of this mode

571 of Scc2-Smc3 binding, a notion consistent with our previous finding that QQ greatly reduces
572 stimulation of cohesin's ATPase activity by Scc2 in the absence of DNA, at least when Scc3
573 is present (Petela et al., 2018).

574

575 Remarkably, the charge reversal substitution Scc2E822K was isolated as a spontaneous
576 mutation that suppresses the lethality of *scc4* mutants whose Scc2's activity is greatly com-
577 promised (Petela et al., 2018). Because Scc2E822K would be predicted to reduce binding
578 to Smc3 K112 K113, which might have been expected to further reduce not improve the
579 compromised Scc2 activity in of *scc4* mutants, we suggest that E822K might loosen but not
580 eliminate the ionic interactions between Scc2 and K112 K113, permitting the latter to make
581 a greater contribution to DNA binding and thereby increase the affinity between engaged
582 heads and DNA. Given the extreme conservation of residues equivalent to Smc3 K112 K113
583 and Scc2 E822 D823, it seems likely that the interface has a similar function in most eukar-
584 yotes and yet mutations equivalent to *smc3 K112Q K113Q* in *S. pombe* and in human tissue
585 culture cells (where they are not lethal) (Feytout et al., 2011; Ladurner et al., 2016) do not
586 eliminate cohesin loading in the manner observed in yeast (Davidson et al., 2016). Cohesin's
587 ATPase is necessary for LE as well as for loading and we therefore suggest that QQ muta-
588 tions may turn out to compromise LE.

589

590 Smc3 K112 K113 are required for Wapl-dependent release of cohesin from chromosomes
591 as well as for optimal ATPase activity (Ladurner et al., 2014; Petela et al., 2018). Because,
592 release only occurs when Scc2 is replaced by Pds5, our cryo-EM structures provide little
593 direct insight as to their role during release. A key question is whether K112 K113 interact
594 with Pds5 in a similar manner to Scc2 or whether their primary role during release is to bind
595 DNA. Acetylation during S phase blocks release and helps to stabilize Pds5's association
596 with chromosomal cohesin.

597

598 **Scc1 is bound to both heads and does not engage in DNA binding**

599 Though our map (Fig. 7A) shows little to no density for residues of Scc1 known to bind the
600 central cleft of Scc2 (Scc1 residues ~190-290), it shows very clearly that Scc1's two struc-
601 tured domains, Scc1-N (residues 67-103) and Scc1-C (residues 502-555), are bound in a
602 canonical manner to Smc3's neck and the base of Smc1's ATPase respectively (Gligoris et
603 al., 2014; Haering et al., 2004) thereby bridging the two heads to form the S-K ring (Fig. 8F).
604 We can therefore exclude the possibility that head engagement, at least in the presence of

605 Scc2 and DNA, causes the sort of rearrangement of Smc3's coiled coil thought to induce
 606 Scc1-N's release from Smc3, as suggested by recent structural studies of an ATP γ S-bound
 607 cohesin trimer (Muir et al., 2020). Similarly, even though it is evident that there is a high level
 608 of conservation between the structures of human (Shi et al., 2020), *S. pombe* (Higashi et
 609 al., 2020) and *S. cerevisiae* cohesin reported here, the latter shows no sign of any structured
 610 part of Scc1 participating in the binding of DNA. Indeed, despite its conservation among
 611 most eukaryotes, the positively charged loop within Scc1-N that binds DNA in the human
 612 and *S. pombe* complexes (residues 23 - 28 in both) is not present in yeast.

613

614 Scc2 and DNA disrupt the J-state in the absence of ATP

615 Passage of DNA between disengaged heads prior to ATP-driven head engagement would
 616 be essential for entrapment simultaneously in E-S and E-K compartments (see Discussion).
 617 There must therefore exist a mechanism by which the heads are moved sufficiently far apart
 618 to permit DNA passage. To investigate this, we tested the effect of ATP, Scc2, and DNA on
 619 crosslinking between the J-state cysteine pair. Conditions that promote efficient E-state
 620 crosslinking, namely addition of ATP, Scc2, and DNA, caused a modest ~20% reduction in
 621 J-state crosslinking (Fig. S5F), confirming that the E-state is formed at the expense of J. As
 622 expected, other combinations of these three factors had less effect. Surprisingly, addition of
 623 Scc2 and DNA in the absence of ATP had the greatest effect, causing a ~50% reduction in
 624 J crosslinking, an effect that was highly reproducible. Such a marked reduction is
 625 presumably caused by the heads adopting a different conformation. Importantly, this does
 626 not correspond to the E-state as very little crosslinking takes place between Smc1N1192C
 627 and Smc3R1222C under these conditions (Fig. S5A). We therefore suggest that in the
 628 absence of ATP, both Scc2 and DNA reduce J-specific crosslinking by driving or indeed
 629 holding the ATPase heads apart, a process that could facilitate passage of DNA between
 630 them and thereby facilitate its entrapment in E-S compartments when heads engage in the
 631 presence of ATP.

632

633 **DISCUSSION**

634 **In vitro reproduction of DNA entrapment within cohesin SMC-kleisin rings**

635 In vivo studies have shown that cohesin entraps circular minichromosomes within its S-K
 636 ring (Gligoris et al., 2014; Srinivasan et al., 2018). We demonstrate here that purified cohesin
 637 possesses such an activity also in vitro. Unlike previous assays that have merely measured
 638 the physical association between cohesin and DNA and investigated its resistance to salt or
 639 sensitivity to kleisin cleavage (Murayama and Uhlmann, 2015, 2014), our method measures
 640 topological association directly. By covalently circularising the cohesin ring and its
 641 component compartments we can make unambiguous deductions about the topology
 642 between DNA and cohesin. The entrapment of DNAs within S-K rings measured by this
 643 method depends on Scc2, Scc3, and ATP. Importantly, it is also stimulated by ATP
 644 hydrolysis, a feature that has been lacking in previous assays but is of paramount
 645 importance for entrapment in vivo (Srinivasan et al., 2018). Four other key properties of the
 646 in vitro S-K entrapment activity reflect cohesin's behaviour in vivo, namely it depends on the
 647 ability of Scc2 and Scc3 to bind DNA, on the ability of ATP to bind Smc3 heads, and on
 648 Smc3's K112 K113 residues, whose lack of acetylation is necessary for loading in yeast (Hu
 649 et al., 2015). We therefore suggest that the in vitro DNA S-K entrapment described here
 650 involves mechanisms similar or identical to those of cohesin operating within cells. If we
 651 assume that the efficiency of BMOE induced S-K circulation is around 20%, we estimate
 652 that many DNAs are entrapped by 15 or more cohesin rings in our assay after a 40 min
 653 incubation.

654

655 **Potential mechanisms for Scc2-driven E-S/E-K entrapment**

656 An obvious question concerns the state of the Smc1 and Smc3 ATPase heads when DNA
 657 is entrapped. They could either be engaged in the presence of ATP, juxtaposed together in
 658 the absence of ATP (a state facilitated by extensive association of the Smc1 and Smc3 coiled
 659 coils), or fully disengaged. To address this, we used a Smc1-Smc3 cysteine pair specific for
 660 engaged heads (Smc1N1192C Smc3 R1222C), which revealed that DNAs are also
 661 entrapped efficiently between the hinge and engaged heads (the E-S compartment),
 662 between engaged heads and the kleisin subunit associated with them (the E-K
 663 compartment), but only rarely between juxtaposed heads and their associated kleisin (the
 664 J-K compartment). For obvious reasons, we were not able to address using cysteine-specific
 665 crosslinking whether DNAs are also entrapped within S-K rings with fully disengaged heads.
 666 However, given that cohesin's ability to hydrolyse ATP is important for S-K entrapment, it is

likely that at least some S-K rings that have entrapped DNA in vitro are in this state. Our failure to observe efficient entrapment within J-K compartments was unexpected given that this state has been documented in vivo (Chapard et al., 2019).

We were surprised to find that unlike entrapment within S-K rings, which requires Scc3, entrapment within E-S/E-K compartments was entirely Scc3 independent. In other words, entrapment within E-S/E-K compartments in the presence of Scc2 alone is not accompanied by entrapment within S-K rings. The similarity in kinetics suggests that entrapment within E-S and E-K compartments driven solely by Scc2 occurs simultaneously as part of the same reaction. The simplest explanation for this is that DNA is transported (upwards) between disengaged ATPase heads and then subsequently trapped in the E-S compartment due to ATP-driven head engagement (Fig. 9A). The simultaneous entrapment within E-K compartments arises naturally from this, as the kleisin polypeptide must be looped “upwards” to accommodate DNA entry in this manner. Crucially, this process would not require opening of the hinge or either SMC-kleisin interface, processes that would be necessary for entrapment within S-K rings. Our cryo-EM structures reveal that entrapment within E-S/E-K compartments is accompanied and probably driven by the binding of DNA to Scc2 and DNA binding sites on the upper surface of Smc1 and Smc3 ATPase heads created upon head engagement. In other words, entrapment within E-S/E-K compartments (in the absence of S-K entrapment) arises from the clamping of DNA between Scc2 and engaged heads. The remarkable similarity between this structure and one formed between DNA, Scc2^{NIPBL}, and tetrameric human cohesin (PDB 6WG3) (Shi et al., 2020) shows that the clamping of DNA between Scc2^{NIPBL} and engaged heads not only does not involve Scc3^{SA2} (whose ortholog SA2 was present in the human structure) but more importantly the same clamping happens even when Scc3 is absent (Fig. S6).

An alternative is that simultaneous E-S/E-K entrapment arises from insertion of a loop of DNA into an open S-K ring. If one segment of this loop were located above the heads while the other located below them, head engagement would also lead to simultaneous entrapment in E-S and E-K compartments (Fig. 9B). This scenario, which could also involve the clamping of DNA between Scc2 and engaged heads, is not only more complex, but it must somehow explain how DNA is bent prior to insertion, a process which would carry a clear entropic penalty and more importantly is not apparent from cryo-EM images of EQEQ cohesin associated with circular DNAs (Fig. 7B right). Importantly, the states created by the

Fig 9

Figure 9. Potential mechanisms for Scc2-driven E-S and E-K entrapment and subsequent S-K ring entrapment

(A) ES/EK entrapment by DNA passing through open heads, or (B) through a DNA loop being inserted. (C) Topological isomerism between A and B. (D) ES/EK entrapment due to two distinct populations. (E) Models for converting E-S/E-K entrapment to S-K entrapment.

two mechanisms are topologically isomeric. In other words, it is possible to transform the state described in Fig. 9A to that of Fig. 9B merely by moving Scc1's central domain "downwards" to below the heads and simultaneously bending the DNA through 180°.

It is nevertheless important to point out that because our assays measuring E-S/E-K entrapment driven by Scc2 alone use complexes with different sets of cysteine pairs, they do not per se prove simultaneous entrapment of DNAs within both compartments. Thus, DNAs trapped in E-S/E-K compartments could in principle belong to separate populations (Fig. 9D). According to this scenario, and because E-S/E-K entrapment is not accompanied by S-K entrapment, DNAs entrapped solely within E-S compartments would have to be held by complexes whose kleisin subunit had dissociated from one or both ATPase heads while DNAs entrapped solely within E-K compartments would have to be trapped by complexes whose hinge had opened. There are two arguments against this interpretation. First, it is very unclear why head engagement in the presence of ATP, Scc2, and DNA should be associated with two such different events. An even more compelling argument stems from our cryo-EM structures of DNA clamped by Scc2 and engaged heads. A low resolution structure reveals coiled coils folded around their elbow and a dimerised hinge associated with Smc3's coiled coil (Fig. 7E), while a high resolution structure shows that both N- and C-terminal kleisin domains (Scc1-N and -C) are bound to Smc3's neck and the base of Smc1's ATPase respectively (Fig. 8A). In other words, association of DNA with engaged heads in the presence of Scc2 does not appear to be accompanied by opening of any of the S-K ring's three interfaces, ruling out the possibility that E-S and E-K entrapment are independent processes.

The notion that DNA can be engaged by cohesin rings in a manner that does not require opening of the hinge or either SMC-kleisin interface (Fig. 9A & B) is consistent with the recent observation that sealing all three interfaces does not adversely affect DNA loop extrusion by human cohesin complexes (Davidson et al., 2019) as well as the finding that the association between chromosomes and cohesin complexes with certain hinge mutations is not accompanied by S-K entrapment in vivo (Srinivasan et al., 2018).

Though the path of the kleisin chain connecting the ATPase heads is not discernible in any of the cryo-EM structures (this work) (Higashi et al., 2020; Shi et al., 2020), our knowledge that circular DNAs are entrapped within E-S/E-K but not S-K compartments under identical

conditions makes clear that the kleisin chain, whose Scc1-N domain is bound to Smc3's neck, must pass over the DNA bound to the engaged heads before its Scc1-C domain binds to the base of Smc1's ATPase (Fig. 9A and molecular equivalent Fig. 8F). This topology is not merely of academic interest as it provides crucial insight into the pathway by which DNAs are clamped by Scc2 and engaged ATPase heads. Because EQEQ cohesin's association with Scc2 and circular DNA does not appear to cause much DNA bending (Fig. 7B right), we favour the notion that E-S/E-K entrapment arises when DNAs pass (in an upwards direction) between heads prior to their engagement in the presence of ATP and not by insertion of a loop into the S-K ring.

Given that the ATPase heads are frequently associated either in the E- or J-state, there must exist a mechanism to create an opening between them, if only transiently, in order for DNA to pass through before being clamped by their subsequent engagement. Our observation that Scc2 and DNA disrupts the J-state, albeit only in the absence of ATP, may be relevant in this regard (Fig. S5F). This J-state disruption was not caused by adoption of the E-state (Fig. S5A), which requires ATP, and it must therefore involve transition to a state in which Smc1 and Smc3 ATPase heads adopt yet another conformation. This could be a state in which Scc2 and DNA together drive apart the ATPase head domains, thereby enabling DNA to pass between them. Subsequent ATP binding would then cause head engagement and E-state formation, trapping DNA inside both the E-S and E-K compartments. Interestingly, a conformation of this nature has recently been observed in condensin bound to Ysc4 (Lee et al., 2020), where the latter bridges the Smc2 and Smc4 heads, holding them apart by some distance. Although DNA was absent from this structure, the separation of the heads would be sufficient for DNA to pass between them and exposes the HAWK's DNA-binding surface without any impediment. If we assume that Scc2 bridges Smc1 and Smc3 heads in a similar fashion prior to their engagement, then one merely has to propose that DNA initially binds to Scc2 while in the bridged state and remains associated as the Smc3 head pivots around and the Smc3 ATPase head engages with that of Smc1 (Movie 1). We envisage that Scc2's association with Smc1 heads (which strongly resembles that between Ycs4 and Smc4, Fig. S6) remains unaltered during this transition, as it did between Smc4 and Ycs4 in condensin (Lee et al., 2020).

Our demonstration that DNAs transported into the sub-compartment created by Scc2's association with engaged ATPase heads results in entrapment in E-S/E-K but not S-K

compartments is difficult to reconcile with the proposal that DNAs must first pass through a transiently opened Scc1-Smc3 interface before they enter the clamped state created by head engagement (Higashi et al., 2020), a process that has been termed DNA “gripping”. Passage through a gate created by opening the Scc1-Smc3 interface before being clamped by engaged heads and Scc2 would be accompanied by E-K and S-K entrapment but not by E-S entrapment, which is contrary to what we observe.

It is also worth pointing out that E-K entrapment would not be possible if Scc1’s NTD were dissociated from Smc3’s neck upon head engagement, as has been suggested by a cryo-EM structure of heads engaged in the absence of both DNA and Scc2 (Muir et al., 2020). The fact that E-K entrapment accompanies E-S entrapment during our Scc2-only reaction implies that Scc1’s NTD does not in fact dissociate from Smc3’s neck upon head engagement when DNA and Scc2 are present, a feature also revealed by cryo-EM of our yeast (Fig. 8A), human (Shi et al., 2020) and *S. pombe* (Higashi et al., 2020) structures of cohesin heads in complex with DNA and Scc2^{NIPBL/Mis4}. Because Scc2 is necessary to prevent cohesin’s release from chromosomes during G1, and because release is accompanied by disengagement of Scc1’s NTD from Smc3’s neck (Srinivasan et al., 2019), we suggest that head engagement may indeed promote Scc1’s dissociation from Smc3 but that this process is actively inhibited by Scc2. One of the functions of Pds5 and Wapl in mediating release during G1 when Smc3 is not acetylated may be to replace Scc2 and thereby abrogate this protection mechanism. In this regard, it is interesting that Scc2 contacts the joint region within Smc3’s coiled coil adjacent to where Scc1’s NTD binds to Smc3’s neck (Fig. 8C iv), an interaction also observed in the human structure (Shi et al., 2020) and could have a role in hindering Scc1’s dissociation from Smc3 upon head engagement.

The remarkable similarity in the structures by which yeast and human cohesin clamp DNA between Scc2^{NIPBL} and engaged ATPase heads (Fig. S6) suggests that this highly conserved conformation must have crucial physiological functions. We propose two possibilities. The first is as follows. Because Scc2 is required for S-K entrapment in the presence of Scc3, as well as for E-S/E-K entrapment in its absence, we suggest that entrapment driven by Scc2 and the binding of DNA to engaged heads is necessary for subsequent S-K entrapment. However, this does not exclude the possibility that Scc2 has roles in S-K entrapment additional to formation of an E-S/E-K intermediate. In other words,

803 the clamping of DNAs between Scc2^{NIPBL} and engaged heads may be a key intermediate
804 during the process of S-K entrapment and hence crucial for the establishment of sister
805 chromatid cohesion.

806

807 The observation that S-K entrapment is clearly not necessary for DNA translocation or loop
808 extrusion and may in fact be a rare event in the life of chromosomal cohesin suggests
809 another possibility. The clamping of DNA between Scc2^{NIPBL} and engaged heads and
810 subsequent release upon ATP hydrolysis, all in the absence of S-K entrapment, may be the
811 driving force for cohesin's translocation along DNA, a notion fully consistent with Scc2's key
812 role in stimulating DNA-dependent ATP hydrolysis (Petela et al., 2018) and loop extrusion
813 (Davidson et al., 2019). If so, a crucial question for the future is how transport of DNA into
814 the sub-compartment created by Scc2 and engaged heads is harnessed to mediate
815 translocation along DNA. We presume that DNA translocation is accompanied (and indeed
816 driven) by recurrent cycles of DNA uptake into the clamped state, with each cycle involving
817 segments of DNA further along the chromosome fibre. However, functional translocation
818 would not be possible without a second (reciprocal) mechanism by which DNAs are
819 recurrently bound and released. Scc3's ability to bind DNA may be crucial in this regard.
820 Another idea is that cohesin's hinge provides the second site and that the clamp/release
821 transport cycle is accompanied by changes in the folding of Smc1/3 coiled coils around their
822 elbow region, which could be the key to walking along the DNA. However, this notion is
823 difficult to reconcile with the observation that cohesin's coiled coils can be folded whether its
824 ATPase heads are engaged (Fig. 7E) or disengaged (Bürmann et al., 2019).

825

826 **Scc3 catalyses entry of DNA inside the SMC-kleisin ring**

827 Our finding that Scc3 is essential for S-K but not E-S/E-K entrapment reveals that Scc3 has
828 a unique role in promoting entry of DNA inside the SMC-kleisin ring as well as being
829 necessary for loop extrusion (Davidson et al., 2019). In principle, Scc3 could catalyse DNA
830 entry either via a gate created by transient hinge opening or through one produced by
831 transient dissociation of one or both SMC-kleisin interfaces (Fig. 9E). Crucially, S-K
832 entrapment in vivo is not abolished by fusing Scc1's NTD to Smc3 or by fusing its CTD to
833 Smc1, implying that DNA must enter either through the hinge or through dissociation of
834 either one of the two SMC-kleisin interfaces (possibly through simultaneous dissociation of
835 both) (Srinivasan et al., 2018). There is little or no direct evidence regarding which
836 mechanism is correct. SMC-kleisin dissociation has been strongly implicated in release and

837 is therefore also a plausible mechanism for entry (Beckouët et al., 2016). Nevertheless,
838 hinge opening is equally plausible, especially in the light of recent findings that folding of
839 cohesin's coiled coils around an elbow brings its hinge domain into close proximity to DNA
840 bound to the heads, and that Scc3^{SA2} interacts with a half opened hinge when DNA is bound
841 to human cohesin-Scc2^{NIPBL} complexes (Bürmann et al., 2019; Shi et al., 2020). Ascertaining
842 which mechanism is at play will require a method to measure the effect on S-K entrapment
843 of chemically linking interfaces together in a manner that is orthogonal to the BMOE-induced
844 crosslinking. For example, prior crosslinking of both SMC-kleisin interfaces would abolish
845 entrapment via a kleisin gate (Fig. 9E bottom pathway) but not via a hinge gate (Fig. 9E top
846 pathway).

847
848 This feature of Scc3's activity depends on its ability to bind DNA in a manner similar to that
849 employed by Scc2 (this work) (Li et al., 2018, Shi et al., 2020) and condensin's Ycg1 HAWK
850 (Kschonsak et al., 2017). Two sets of residues are implicated in DNA binding (K224 K225
851 R226 and K423 K513 K520). Mutation of one or other set does not abrogate DNA binding
852 or cause lethality but does reduce cohesin's association with chromosomes while mutation
853 of both sets (Scc3-6E) abolishes not only DNA binding and S-K entrapment in vitro but is
854 lethal and abolishes all loading throughout the genome in vivo. Though it abrogates
855 entrapment of DNA within S-K compartments (Fig. 3F), Scc3-6E has no effect on Scc2
856 driven E-S/E-K entrapment (data not shown). Thus, if S-K entrapment in vivo involved prior
857 formation of an E-S/E-K intermediate, which is consistent with the latter's more rapid kinetics
858 in vitro, then cohesin containing Scc3-6E should form this intermediate and accumulate in
859 this state, possibly at loading sites. Our observation that, despite failing to associate with
860 the vast majority of the genome, Scc3-6E cohesin accumulates at especially high levels at
861 *CEN* sequences, which are highly efficient loading sites, suggests that this may indeed be
862 the case. Unlike Scc3-6E, complete depletion of Scc3 abrogates cohesin's association at
863 *CENs* as well as along chromosomes arms, which implies that Scc3 has additional functions
864 that do not involve or require its ability to bind DNA.

865
866 The notion that entrapment of DNA within S-K rings is preceded by its prior entrapment
867 within E-K/E-S compartments to Scc2 and SMC heads in a manner observed in our cryo-
868 EM structure raises the interesting possibility that DNA is eventually entrapped within the S-
869 K ring, not by passing from outside to inside, but instead by being allowed to exit from either
870 the E-S or the E-K compartment by transiently opening one of the S-K ring's three interfaces.

871 Transient hinge opening would permit DNA's escape from the E-S compartment (Fig. 9E top
872 pathway) while transient dissociation of one or another, or indeed both, kleisin-head
873 interfaces would permit escape from the E-K compartment (Fig. 9E bottom pathway). In both
874 cases, the subsequent closing of these exit gates would lead to entrapment of DNA within
875 the S-K ring. According to these scenarios, exit via the hinge or via a SMC-kleisin interface
876 without head disengagement would lead, at least initially, to the selective loss of E-S and E-
877 K entrapment respectively. If true, clamping of the DNA would provide the opportunity to
878 open gates without losing grip of the DNA while doing so. It is interesting in this regard that
879 whereas E-K entrapment does not increase between 2 and 40 min when both Scc2 and
880 Scc3 are present, E-S entrapment continues to increase in parallel with the rise in S-K
881 entrapment. Whether this asymmetry is a hint that Scc3 promotes entrapment within S-K
882 rings by opening an SMC-kleisin interface will require far more rigorous types of
883 experiments. Though EQEQ mutants reduce S-K entrapment, they do not eliminate it,
884 suggesting that DNA entry can in principle occur without head disengagement, as depicted
885 in Fig. 9E.

886
887 The notion that a key function of Scc3, dependent on its ability to bind DNA, is to facilitate
888 entrapment of DNA within S-K rings has an important corollary. S-K entrapment is thought
889 to be a crucial feature of sister chromatid cohesion. Hitherto, direct evidence for this
890 mechanism has been confined to the observation of small circular minichromosomes
891 entrapped within S-K rings in vivo. We show here that a function of Scc3, not shared by
892 Scc2, is to facilitate entrapment within S-K rings. If this is also an essential function of Scc3
893 in vivo, it follows that S-K entrapment must also be an essential cohesin function and one
894 that applies to proper chromosomes as well as small circular ones.

895
896 We have known for two decades that Scc2 and Scc3 have different roles in promoting
897 cohesin's association with chromosomes (Ciosk et al., 2000; Tóth et al., 1999). The various
898 topological assays described in this paper have finally revealed some of these. Scc2
899 promotes entrapment of DNA in E-S/E-K compartments by promoting its binding to engaged
900 SMC ATPase heads, while Scc3 promotes entrapment inside S-K rings. These findings are
901 supported by our cryo-EM structure that reveals the molecular identify of the clamped E-
902 S/E-K state and also by recent cryo-EM structures containing cohesin, DNA, and
903 Scc2^{NIPBL/Mis4} as well as Scc3^{SA2/Psc3} (Higashi et al., 2020; Shi et al., 2020). Crucially, our
904 assays reveal the topology of DNA's association with cohesin and the path of the kleisin for

905 the clamped E-S/E-K state, at least when formed by Scc2 alone and head engagement.
906
907 Entrapment of DNAs within E-S compartments has not hitherto been detected in vivo,
908 emphasizing the value of in vitro systems in revealing reactions that are otherwise difficult
909 to detect. Future work will be required to address whether E-S/E-K entrapment also occurs
910 inside cells, to elucidate the mechanism of S-K entrapment, and to reveal conditions that
911 promote J-K entrapment, a form that has been detected in vivo but not yet efficiently in vitro.
912

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922

923 **AUTHOR CONTRIBUTIONS**

924 J.E.C and K.A.N. conceived the entrapment study. J.E.C. conducted most the in vitro exper-
 925 iments and analysed the data, with contributions from M.B.R., S.Y., and M.V. In vivo exper-
 926 iments were carried out by M.B.R, N.J.P, and J.M. Cohesin purification protocol was devel-
 927 oped by B.-G.L. and J.L. Cryo-EM, processing and analysis was performed by B.-G.L and
 928 to a minor extent by A.G.L. and J.L. The manuscript was written by J.E.C., A.G.L., B.-G.L,
 929 J.L. and K.A.N.

930

931 **DECLARATION OF INTERESTS**

932 The authors declare no competing interests.

933

MATERIALS AND METHODS

Recombinant yeast cohesin complex cloning

The *S. cerevisiae* genes *SMC1*, *SMC3*, *SCC3*, *SCC2*, *SCC1*, and *SCC4* were codon optimised for expression in *Spodoptera frugiperda* cells and synthesised using the Genescript Thermo Fisher service. These were then cloned into MultiBac vectors. Tag introduction and mutagenesis was achieved through Gibson assembly (New England Biolabs) to generate *SMC1*-His, 2xStrepII-*SCC3*, *SCC1*-2xStrepII, *SCC2*-2xStrepII, *SCC2C*¹³³*SCC2*¹³³⁻¹⁴⁹³-2xStrepII, and His-*SCC4*. *SMC3* *SMC1*-His, 2xStrepII-*SCC3*, *SCC2*-2xStrepII, and *SCC2*¹³³⁻¹⁴⁹³-2xStrepII were cloned into pACEbac1 vectors, and *SCC1*-2xStrepII and His-*SCC4* cloned into pIDC vectors. *SMC1*-His and *SMC3* were then combined into the same vector via cloning to create a pACEbac1 *SMC1*-His *SMC3*. Vectors containing cohesin trimers were generated by combining pACEbac1 *SMC1*-His *SMC3* with pIDC *SCC1*-2xStrepII by a Cre recombinase reaction (New England Biolabs). The vector for the *Scs2/4* expression was also created by combining the pACEbac1 *SCC2*-2xStrepII with pIDC His-*SCC4* using Cre recombinase.

Virus generation and protein expression

DNAs were first transformed into DH10Bac (Thermo Fisher) cells and bacmids containing the expression vector screened for by blue-white selection. DNA was then extracted and 2 µg of bacmid DNA was transfected into 2 ml *S. frugiperda* Sf9 cells (Thermo Fisher) at a cell density of 1x10⁶ cells ml⁻¹ using FuGENE HD reagent (Promega), grown in Sf900 II SFM media (Thermo Fisher). These were then incubated at 27°C for 5 days to create P1 virus. P2 virus was then amplified by infecting 50 ml Sf9 cells at a density of 2x10⁶ cells ml⁻¹ with 500µl P1 virus and incubating in the dark at 27°C for 3 days with shaking at 100 rpm. P2 virus was then harvested by pelleting cells by centrifugation at 4000g and decanting into 5% FBS (Sigma), and then stored in the dark at 4°C. Typically, proteins were then expressed by adding 5 ml P2 virus to 500 ml Sf900 cells at a density of 2x10⁶ cells ml⁻¹ and incubating in the dark at 27°C for 2 days with shaking at 100 rpm. Cells were then harvested by centrifugation at 1000g, washed with PBS, and then frozen in liquid nitrogen and stored at -80°C.

Protein purification

Cells were thawed in Buffer A (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP (Thermo Fisher), 5% glycerol) supplemented with 1 Complete Protease Inhibitor (EDTA-free) tablet

(Roche), 70 µg RNase A (Roche), and 100 U ml⁻¹ Supernuclease (Sino Biological) and then lysed by sonication. Following sonication, cell lysate was supplemented with 1 mM PMSF (Sigma). Proteins were then purified via a three strep purification protocol. First, proteins were purified via affinity pulldown of their StrepII tags using a StrepTrap HP column (Fisher Scientific) and eluted into Buffer A supplemented with 2.5mM desthiobiotin (Fisher Scientific). Scc2 constructs were eluted into 50 mM Tris pH 8.0 rather than 50 mM HEPES pH 7.5. Proteins were then further purified by anion exchange chromatography using a 5 ml HiTrap Q HP column (GE Healthcare) across a gradient of 100 mM to 1M NaCl. Scc2 constructs were eluted across a gradient of 0 mM to 1 M NaCl. Finally, proteins were purified via size exclusion chromatography using a Superose 6 increase 10/300 GL column (VWR) for cohesin trimers and a HiLoad 16/600 Superdex 200 column (GE Healthcare) for Scc3 and the Scc2 constructs.

980

981 **Protein gel electrophoresis and western blotting**

982 Samples were mixed with 4xLDS sample buffer (Thermo Fisher), loaded onto a 3-8% Tris-
983 acetate gel (Thermo Fisher) and separated at 150 V for 50 min. Gels were then either
984 stained with Quick Coomassie stain (Generon) or transferred onto a 0.2 µm nitrocellulose
985 membrane using a Trans-blot Turbo transfer pack (Bio-Rad). The antibodies used for
986 western blotting were anti-His (Sigma), anti-Strep HRP conjugated (iba) and anti-Smc3
987 (Bethyl Laboratories). Primary antibodies were probed with anti-mouse HRP conjugated
988 antibodies (Thermo Fisher).

989

990 **Purification of pUC19 plasmid DNA**

991 pUC19 plasmid was transformed into TOP10 (Thermo Fisher) cells and grown overnight at
992 37°C. The next day a single colony was inoculated into 250 ml SOB++ media and grown at
993 37°C overnight for 16 hours. DNA was then purified via MaxiPrep (Qiagen) using precooled
994 reagents and equipment and eluted into 50 mM HEPES pH 7.5. DNA was then further
995 purified by CsCl₂ density gradient centrifugation in the presence of EtBr (Thermo Fisher).
996 The DNA was then extracted, and the EtBr removed by washing several times with butanol
997 saturated with 50 mM HEPES pH 7.5 and then the butanol phase discarded. The CsCl₂ was
998 then removed by dialysis against 2 L 50 mM HEPES pH 7.5 buffer over 24 hours at 4°C,
999 with two buffer changes. The DNA was then collected and stored at -20°C.

1000

1001 **Protein crosslinking assay**

For protein crosslinking assays, 10 µl reactions were prepared containing 570 nM protein (buffered with 50 mM HEPES pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM TCEP, 5% glycerol). If added, ATP (Sigma) and pUC19 were added to a concentration of 5 mM and 60 nM respectively. Reactions mixes were first incubated on ice for 5 min before adding 1 µl BMOE (Thermo Fisher) to a final concentration of 0.64 mM. Reactions were then incubated on ice for 6 min. Samples were denatured by adding 4xLDS buffer and heating at 70°C for 10 min before being separated in 3-8% Tris-acetate gels (Thermo Fisher) run at 150 V for 3 hours. Gels were stained with Quick Coomassie stain (Generon).

DNA entrapment assay

For DNA entrapment assays, 13 µl reactions were prepared containing 165 nM protein and 9.3 nM supercoiled pUC19 (buffered with 50 mM HEPES pH 7.5, 20 mM NaCl, 1 mM MgCl₂, 5% glycerol). Scc2C was added to a concentration of 40 nM. These were incubated on ice for 5 min before reactions were initiated by addition ATP (Sigma) to a final concentration of 5 mM (1 µl ATP added to 12 µl protein DNA mix). Typically, reactions were then incubated at 24°C for either 40 min or 2 min depending on the compartment being assessed. To these, 1.5 µl BMOE (Thermo Fisher) was added to a final concentration of 0.64 mM and samples were incubated on ice for 6 min. Samples were then denatured by addition of 1.5 µl 10% SDS and heated at 70°C for 20 min. DNA loading dye was added and mixtures separated in a 0.8% agarose gel, run at 50 V for 17 hours at 4°C. The gel was stained with EtBr (Thermo Fisher) and visualised by UV light. Images shown are representative of 2 independent experiments.

ATPase assay

ATPase experiments were carried out as described in (Petela et al., 2018).

Calibrated ChIP-sequencing

ChIP sequencing experiments were carried out as described in (Petela et al., 2018).

Electromobility shift assay (EMSA)

A FAM-labelled 39 base pair HPLC purified DNA oligo (Invitrogen, GAATTCGGTGCGCATAATGTATATATTATGTTAAATAAGCTT) was annealed to a complementary DNA oligo to form dsDNA by heating to 95°C for 5 min and then decreasing the temperature to 4°C in 0.1°C increments (buffered in 100 mM potassium acetate, 50 mM

1036 HEPES pH 7.5) to a final concentration of 45 μ M. The reactions were then prepared by
1037 adding 0.3 μ M FAM-dsDNA to increasing concentrations of protein (buffered in 50 mM
1038 HEPES pH 7.5, 75 mM NaCl, 1 mM TCEP, 10% glycerol) in a final volume of 10 μ L. The
1039 reaction mix was then incubated on ice for 30 min in the dark. These were then separated
1040 in 5% acrylamide gels prepared with 0.5% TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA)
1041 run at 100 V for 1 hour at 4°C in the dark. FAM-labelled DNA was visualised directly on a
1042 Fujifilm FLA7000 scanner with the LD473/Y[520] filter.

1043

1044 **Cell viability of Scc3 mutants**

1045 Mutant *scc3* alleles (under their native promoter) were incorporated at the *leu2* locus in
1046 heterozygous *SCC3*/ Δ *scc3* cells. Diploids were sporulated and tetrads dissected onto YPD
1047 plates. The genotype of the resulting haploids was determined by replica plating. All
1048 mutations were confirmed by DNA sequencing.

1049

1050 **Cryo-EM sample preparation**

1051 1 μ M purified *S. cerevisiae* cohesin EQ/EQ trimer (Smc1E1158Q, Smc3E1155Q, Scc1-
1052 2xStreptII) was incubated for 30 min at 4°C with 1 μ M Scc2C2(151-1493), forming cohesin
1053 tetramer, 5 mM ATP, and 1.3 μ M of a 40 bp dsDNA (5'-GAATTCGGTGCG-
1054 CATAATGTATATTATGTTAAATAAGCTT-3', 5'-AAGCTTATTTAACATAA-
1055 TATACATTATGCGCACCGAATTC-3') or relaxed plasmid DNA. The plasmid was 1789 bp,
1056 derived from pUC19, containing a single site for Nt.BspQI nicking endonuclease. Nicking
1057 was performed according to the manufacturer's instructions for 60 min at 50 °C (NEB) and
1058 the product was purified using a PCR purification kit (Qiagen) and eluted in water. For vitri-
1059 fication, 3 μ l of sample were applied to glow-discharged Quantifoil Au 2/2 holey carbon 200
1060 mesh grids or Ultrafoil Au 2/2 holey gold 200 mesh grids (Quantifoil), and flash frozen in
1061 liquid ethane using an FEI Vitrobot Mark IV (Thermo Fisher Scientific) and a liquid-ethane
1062 cryostat set to -180 °C (Russo et al., 2016).

1063

1064 **Cryo-EM data collection**

1065 All cryo-EM images were collected on a Titan Krios electron microscope operated at 300 kV
1066 (Thermo Fisher Scientific). Images for the cohesin tetramer:40 bp dsDNA complex were
1067 collected with a Quantum energy filter (GIF) in front of a K3 Summit direct electron camera
1068 in super-resolution mode (both Gatan). The nominal defocus range was set to 1.5 ~ 3.3 μ m.
1069 Each image was dose-fractionated over 55 frames with a dose rate of 1 electrons per Å per

image. A total 11,944 micrographs were collected in three separate sessions using Krios III at LMB (calibrated pixel size: 1.069 Å / pixel at nominal magnification of 81,000) and the Krios microscope at the Biochemistry Department at the University of Cambridge (pixel size: 1.07 Å / pixel at nominal magnification of 81,000). Because the images showed strong orientation bias, 6,580 micrographs of the datasets were collected at tilts of 25° or 30°. For the tetramer:plasmid DNA complex, 535 images were collected using the Volta phase plate (VPP) (Danev and Baumeister, 2016) and a Gatan K2 Summit direct electron camera in counting mode on Krios II at LMB (calibrated pixel size: 1.00 Å / pixel at magnification of 105,000) with total doses of 45 electrons per Å², dose fractionated into 40 movie frames using a nominal defocus range of 0.6 ~ 1.0 µm.

Cryo-EM data processing and reconstruction

RELION 3.1 was used for all data processing unless otherwise specified (Scheres, 2012). The resolution was determined based on gold standard Fourier shell correlation (FSC) using the 0.143 criterion (Rosenthal and Henderson, 2003). Using RELION's own motion correction implementation, movie frames were aligned and combined with dose weighting using 7 × 5 patches or 5 × 5 patches for K3 and K2 datasets, respectively. CTF parameters were estimated with CtfFind4 (Rohou and Grigorieff, 2015) for un-tilted images. Focus-gradient patch CTF estimation was performed for tilted images using the programme Warp (Tegunov and Cramer, 2019). For particle picking, RELION and crYOLO were used (Wagner et al., 2019).

For the cohesin tetramer:40 bp DNA complex, initially particles were picked from a subset of ~1,000 images with a Laplacian-of-Gaussian blob as a template using RELION, followed by particle extraction and reference-free 2D classification. An initial 3D model was obtained using particles from selected 2D class images showing different orientations. Then, particle coordinates from images that formed good 2D classes were used to train a model in crYOLO, and particle picking was performed with the trained model. Picked particles were extracted using a box size of 320² pixels followed by 3D classification. After several rounds of 3D classification, 588,164 particles with clear density for the Smc1/3 heads and Scc2 were selected and 3D refined to 3.8 Å resolution. Further CTF refinement and Bayesian polishing were performed in RELION followed by another round of 3D auto-refinement, which resulted in a final 3.4 Å map of the cohesin Smc1EQ, Smc3EQ, Scc1, Scc2, ATP, 40 bp DNA complex showing the head part only.

1104

1105 For processing of the tetramer:plasmid DNA complex, picked particles from crYOLO were
1106 binned and extracted in a box of 160² pixels (2 Å per pixel), followed by several rounds of
1107 2D/3D classifications with the map of the tetramer:40 bp DNA complex as initial 3D model.
1108 23,704 particles showing the head part clearly were selected and re-extracted using a box
1109 size of 320² pixels (1.07 Å/pix) and were 3D refined to 7.3 Å resolution.

1110 In order to obtain a map of the entire complex, particles from the first 3D classification during
1111 the processing of the tetramer:40 bp DNA complex (above) containing clear DNA density
1112 were re-centred on the joint region of the complex and re-extracted in a box size of 320²
1113 pixels (2 Å per pixel). After initial 2D classification, an initial model was generated with a few
1114 class averages in different orientations, followed by 3D classification. After several rounds
1115 of 3D classification, 21,343 particles containing well-ordered coiled coil and hinge density
1116 were 3D refined to 10 Å resolution.

1117 **Model building**

1118 A homology model of yeast Scc2 was obtained from SWISS-MODEL (Waterhouse et al.,
1119 2018) using a crystal structure of Scc2 from *E. gossypii* (PDB 5ME3) as the template (Chao
1120 et al., 2017). Crystal structures of yeast Smc1 head (PDB 1W1W; (Haering et al., 2004))
1121 and Smc3 head (PDB:4UX3; (Gligoris et al., 2014)) and the Scc2 homology model were
1122 docked into the tetramer:40 bp DNA cryo-EM density map using UCSF Chimera X (Goddard
1123 et al., 2018). MAIN (Turk, 2013) and COOT (Emsley et al., 2010) were used for manual
1124 rebuilding, followed by refinement using Phenix.real_space_refinement (Afonine et al.,
1125 2018). Manual-rebuilding and refinement were repeated for several cycles. The cryo-EM
1126 map and the atomic model of the cohesin head segment at 3.4 Å resolution (Table 1) were
1127 deposited in the EM Data Bank (EMDB) and Protein Data Bank (PDB) with accession
1128 numbers EMD-11585 and PDB 6ZZ6.

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1344

Fig S1

Figure S1. Related to Figures 1 and 2

(A) Left hand panel - Coomassie stain of cohesin trimers. Individual subunits were then probed for by western blotting. Right hand panel – Coomassie stain of Scc2C and Scc3. (B) Left hand panel - ATPase activity for purified cohesin trimers incubated with DNA, Scc2, and ATP in the presence of Scc3 (tetramer) or absence (trimer). Right hand panel – ATPase activity for either WT cohesin tetramer, Smc1E1158Q Smc3E1155Q (EQEQ), Smc3K38I (KI) or Smc3K112Q K113Q (QQ). (C) Entrapment of DNA in S-K rings in the presence of Scc2 and Scc3 comparing WT cohesin to Smc3K112Q K113Q (QQ) (I = input DNA), or (D) entrapment of linear DNA (L) or circular supercoiled DNA (C) in the presence or absence of protein (Trimer, Scc2 and Scc3) (* = damaged open circular DNA).

Fig S2

Figure S2. Related to Figure 3

(A) EMSA comparing the ability of Scc3 to bind dsDNA against Scc3-Scc1²⁶⁹⁻⁴⁵¹ complexes, or (B) of Scc3-3E(1)-Scc1²⁶⁹⁻⁴⁵¹ (Scc3K224E K225E R226E) against Scc3-3E(2)-Scc1²⁶⁹⁻⁴⁵¹ (Scc3K423E K513E K520E) complexes. (C) Average calibrated ChIP-seq profiles 60 kb either side of *CENs* of Scc1-PK6 in the presence of WT Scc3 (KN27542) or Scc3-3E(1) (KN27547), taken in cycling cells at 25°C. (D) Average calibrated ChIP-seq profiles of Scc1-PK6 in the presence of WT Scc3 (KN27542) or Scc3-3E(2) (KN27697), taken in cycling cells at 25°C. (E) Vertical tetrad dissection of spores from diploid cells heterozygous for deletion of the endogenous *SCC3* gene, expressing a single copy of either WT Scc3 (KN21273), Scc3-3E(1) (KN27539), Scc3-3E(2) (KN27696), or Scc3-6E (KN27763) from an ectopic locus. WT Scc3, Scc3-3E(1), Scc3-3E(2) are able to support viability but Scc3-6E is not. Further marker selection, protein expression and genotype sequencing confirmed that Scc3-6E causes lethality. (F) Average calibrated ChIP-seq profiles 10 kb either side of *CENs* of Scc1-HA3 either expressing TIR necessary for auxin inducible degradation (KN20783) or not (KN20785). Samples were arrested in G1 with α -factor at 25°C prior to release into auxin containing media to deplete endogenous Scc3. ChIP-seq samples were taken 60 min after release.

Fig S3

Figure S3. Related to Figure 4

(A) EMSA comparing the ability of full length Scc2/4 and Scc2/4-Scc1¹⁵⁰⁻²⁹⁸ complexes to bind dsDNA or (B) Comparing Scc2-2E(1) (Scc2S717E K721E) and Scc2-2E(2) (Scc2K788E H789E). (C) Average calibrated ChIP-seq profiles 10 kb either side of *CENs* of cells expressing WT (KN24185), S717L K721E double mutant (KN27010) or no ectopic copy of *SCC2* (KN22390), over endogenous *scc2-45*. Cells were arrested in G1 with α -factor at 25°C before release into medium containing nocodazole at 37°C. Samples were taken 75 min after release. *scc2-45* is a temperature sensitive allele.

Fig S4

Figure S4. DNA is never entrapped in J-S and only rarely in J-K compartments

(A) BMOE crosslinking of J-K compartments, or (B) of J-S compartments, with cysteine pairs in the designated interfaces. CC = circular cohesin. (C) Crosslinking of J-S or J-K compartments in the presence of DNA, Scc2, Scc3 and ATP (D/S/A) or absence of all four. (D) Entrapment of DNA in either S-K rings, or J-S/J-K compartments in the presence of Scc2, Scc3, or both Scc2 and Scc3. Incubated for 40 min (* = damaged open circular DNA; I = input DNA).

Fig S5

Figure S5. Related to Figure 5

(A) BMOE crosslinking of cohesin containing a cysteine pair for E head association, in the presence of ATP, DNA, or Scc2 in various combinations and in the presence of 1mM MgCl₂. (B) Crosslinking of E-K compartments, or (C) of E-S compartments, with cysteine pairs in the designated interfaces. CC = circular cohesin. (D) Crosslinking of cohesin containing a cysteine pair for E head association, comparing WT cohesin against Smc3K38I (KI) in the presence of ATP, DNA, or Scc2 in various combinations. (E) Crosslinking of cohesin containing a cysteine pair specific for engaged heads, in the presence of ATP, DNA, or Scc2 in various combinations and in the absence of 1mM MgCl₂. (F) BMOE crosslinking of cohesin with a cysteine pair specific for J head association, in the presence of ATP, DNA, or Scc2 in various combinations.

Figure S6. Comparison of cryo-EM structures

(**A**) Cohesin tetramer (yeast, this study), (**B**) pentamer (human, 6WG3) (Shi et al., 2020) and (**C**) condensin (yeast, 6YVU) (Lee et al., 2020) are shown in two orientations (same or similar to Fig. 7A and 8A).

Movie 1. A model for the formation of the clamped E-S/E-K state of cohesin. According to this model cohesin transitions from a putative ‘bridged state’ (modelled on the same state of yeast apo condensin as observed by cryo-EM) (Lee et al., 2020) in which Scc2, analogous to Ycs4 in condensin, bridges the Smc1/3 heads. In the bridged state Scc2’s DNA binding surface becomes accessible for DNA to attach without impediment and positions it for the next step, namely clamping. ATP-binding driven head engagement, achieved through a rotation of the Smc3 head relative to the rest of the complex, and with Scc2’s and Smc1’s relative orientations staying the same, results in entrapment in the E-S compartment. Because the disordered kleisin chain has to be pushed upwards during the clamping, the DNA is also in the E-K compartment. The initial binding of DNA to the Scc2 DNA binding site guides the DNA through the large opening of the heads generated by the bridged state and leads to the final clamped state that has been described in this study. The movie is a simple morph between a putative bridged state of cohesin modelled on the same state in condensin and the high-resolution cryo-EM structure of the clamped state determined in this study, with a few clashes removed manually because cohesin and condensin subunits, in particular Scc2 and Ycs4 are not completely homologous structurally.

Table 1.

<i>S. cerevisiae</i> cohesin	+ 40 bp DNA		+ circular DNA
	Tetramer Smc1E1158Q, Smc3E1155Q, Scc1, Scc2C2, ATP	Tetramer head segment Smc1E1158Q, Smc3E1155Q, Scc1, Scc2C2, ATP (EMD-11585, PDB 6ZZ6)	T. head segment Smc1E1158Q, Smc3E1155Q, Scc1, Scc2C2, ATP
Data collection and processing			
Magnification	81,000	81,000	105,000
Voltage (kV)	300	300	300
Electron exposure (e ⁻ /Å ²)	55	55	40
Defocus range (μm)	1.5 ~ 3.3	1.5 ~ 3.3	0.6 ~ 1.0
Pixel size (Å)	1.07	1.07	1.0
Symmetry imposed	C1	C1	C1
Initial particle images (no.)	1,516,413	2,314,881	65,442
Final particle images (no.)	21,343	588,164	23,728
Map resolution (Å)	10	3.35	7.3
FSC threshold	0.143	0.143	0.143
Map resolution range (Å)	10 – 50	3.2 – 50	7.3 – 50
Refinement			
Initial model used (PDB)		1W1W, 4UX3, 5ME3	
Model resolution (Å)		3.35	
FSC threshold		0.143	
Model resolution range (Å)		3.2 – 4.5	
Map sharpening B factor (Å ²)		-88.25	
Model composition			
Non-hydrogen atoms		16556	
Protein residues		1909	
		Smc1(2-71, 87-195, 1044-1224); Smc3(1-224, 997-1071, 1104-1222); Scc1(67-103, 502-510, 519-555); Scc2(221-236, 250-262, 278-291, 307-322, 336-373, 386-589, 597-634, 647-662, 678-617, 927-1049, 1059-1078, 1090-1184, 1203-1342, 1355-1398, 1413-1434, 1448-1456, 1466-1475)	
Nucleotide residues		68 (poly-A)	
Ligands		2 ATP, 2 Mg	
B factors (Å ²)			
Protein		11.21	
Nucleotide		68.00	
Ligand		19.62	
R.m.s. deviations			
Bond lengths (Å)		0.002	
Bond angles (°)		0.556	
Validation			
MolProbity score		1.80 (85 th percentile)	
Clashscore		8.36	
Poor rotamers (%)		0.06	
Ramachandran plot			
Favored (%)		94.98	
Allowed (%)		5.02	
Disallowed (%)		0.00	

REAGENTS	SOURCE	IDENTIFIER
Antibodies		
Anti-His (mouse)	Sigma	Cat# SAB1305538-400UL
Anti-mouse HRP	ThermoFisher	Cat# 62-6520
Anti-Smc3 (mouse)	Bethyl Laboratories	Cat# A300-060A
Anti-Strep HRP	iba	Cat# 2-1502-001
Bacterial Strains		
DH10Bac	ThermoFisher	Cat# 10361012
PIR1	ThermoFisher	Cat# C101010
TOP10	ThermoFisher	Cat# C404010
Cell Lines		
Sf9	ThermoFisher	Cat# 11496015
Chemicals and Recombinant Proteins		
ATP Lithium Salt	Sigma	Cat# 11140965001
Bismaleimidoethane (BMOE)	ThermoFisher	Cat# 22323
Complete EDTA free protease inhibitor cocktail	Roche	Cat# 4693132001
Cre Recombinase	New England Biolabs	Cat# M0298S
Desthiobiotin	Fisher Scientific	Cat# 12753064
EtBr	ThermoFisher	Cat# 15585011
Fetal Bovine Serum	Sigma	Cat# 12303C
FuGENE HD Transfection reagent	Promega	Cat# E2311
Gibson Assembly Mix	New England Biolabs	Cat# E2611L
HiLoad 16/60 Superdex 200	GE Healthcare	Cat# GE28-9893-35
HiSpeed Plasmid Maxi Kit	Qiagen	Cat# 12663
HiTrap Q HP	GE Healthcare	Cat# GE29-0513-25
Immobilon Western ECL	Millipore	Cat# WBLKS0500
NuPAGE 3-8% Tris-Acetate Protein Gels	ThermoFisher	Cat# EA0378BOX
PMSF	Sigma	Cat# 329-98-6
Quick Coomassie Stain	Generon	Cat# GEN-QC-STAIN-1L
RNase A	Roche	Cat# 10109169001
Sf900 II SFM	ThermoFisher	Cat# 10902104
StrepTrap HP	Fisher Scientific	Cat# 11540654
Supernuclease	SinoBiological	Cat# SSNP01
Superose 6 Increase 10/300 GL	VWR	Cat# 29-0915-96
TCEP	ThermoFisher	Cat# 20490
4xLDS	ThermoFisher	Cat# NP0007
Critical Commercial Assays		
EnzChek phosphate assay kit	Invitrogen	Cat# E6646

Recombinant DNA
pACEbac1 <i>SMC1-His</i>
pACEbac1 <i>SMC3</i>
pACEbac1 <i>SMC1-His SMC3</i>
pACEbac1 <i>SCC2¹³³⁻¹⁴⁹³-2xStrepII</i>
pACEbac1 <i>SCC2-2xStrepII</i>
pACEbac1 <i>2xStrepII-Scs2¹⁵¹⁻¹⁴⁹³</i>
pACEbac1 <i>2xStrepII-SCC3</i>
pIDC <i>SCC1-2xStrepII</i>
pIDC <i>Scs1²⁶⁹⁻⁴⁵¹-2xStrepII</i>
pIDC <i>Scs1¹⁵⁰⁻²⁹⁸-2xStrepII</i>
pIDC <i>His-SCC4</i>

S. cerevisiae strains	
MATa ura::ADH1 promoter-OsTIR1-9myc::URA3 Scc3-PK3-aid::KanMX4 SCC1-HA3::HIS3	KN20783
MATa Scc3-PK3-aid::KanMX4 SCC1-HA3::HIS3	KN20785
MATa/alpha scc3::NatMX4/WT	KN21079
MATa/alpha scc3::NatMX4/WT, leu::Scc3-HA3::LEU	KN21273
MATa Scc1-PK9::KanMX scc2-45::natMX (L545P D575G)	KN22390
C. glabrata Mata, SCC1-PK9::NATMX4	KN23308
MATa Scc1-PK9::KanMX scc2-45::natMX (L545P D575G) lys2::Scc2-HyGMX	KN24185
C. glabrata Mata, SCC1-HA3::NATMX4	KN25532
MATa Scc1-PK9::KanMX scc2-45::natMX (L545P D575G) LYS2::Scc2(S717L,K721E)-HyGMX	KN27010
MATa/alpha scc3::NatMX4/WT, leu::Scc3 (K224E, K225E, R226E)-HA3::LEU	KN27539
MATa scc3::NatMX4, Scc1-PK6::TRP1, leu::Scc3-HA3::LEU	KN27542
MAT alpha scc3::NatMX4, Scc1-PK6::TRP1, leu::Scc3 (K224E, K225E, R226E)-HA3::LEU	KN27547
MATa/alpha scc3::NatMX4/WT, leu::Scc3 (K423E, K513E, K520E)-HA3::LEU	KN27696
MATa scc3::NatMX4, Scc1-PK6::TRP1, leu::Scc3 (K423E, K513E, K520E)-HA3::LEU	KN27697
MATa/alpha scc3::NatMX4/WT, leu::Scc3 (K224E, K225E, R226E, K423E, K513E, K520E)-HA3::LEU	KN27763
MATa ura::ADH1promoter-OsTIR1-9myc::URA3, Scc3-PK3-aid::KanMX4, leu::Scc3-HA3::LEU	KN27796
MATa ura::ADH1 promoter-OsTIR1-9myc::URA3, Scc3-PK3-aid::KanMX4 leu::Scc3 (K224E, K225E, R226E, K423E, K513E, K520E)-HA3::LEU	KN27802
MATa ura::ADH1promoter-OsTIR1-9myc::URA3, Scc3-HA3-aid::KanMX4, Scc1-PK6::TRP1, leu::Scc3 (K224E, K225E, R226E, K423E, K513E, K520E)-HA3::LEU	KN27804
MATa ura::ADH1promoter-OsTIR1-9myc::URA3, Scc3-HA3-aid::KanMX4, Scc1-PK6::TRP1, leu::Scc3-HA3::LEU	KN27821
MATa ura::ADH1promoter-OsTIR1-9myc::URA3, Scc3-HA3-aid::KanMX4, leu::Scc3-HA3::LEU, Scc2-PK9::NatMX	KN28075
MATa ura::ADH1 promoter-OsTIR1-9myc::URA3, Scc3-HA3-aid::KanMX4, Scc2-PK9::NatMX, leu::Scc3 (K224E, K225E, R226E, K423E, K513E, K520E)-HA3::LEU	KN28287

software, algorithm	
RELION 3.1	doi:10.1016/j.jsb.2012.09.006
CtfFind4	doi:10.1016/j.jsb.2015.08.008
Warp	doi:10.1038/s41592-019-0580-y
CrYOLO 1.5	doi:10.1038/s42003-019-0437-z
Chimera	https://www.cgl.ucsf.edu/chimera/
ChimeraX 1.0	https://www.cgl.ucsf.edu/chimerax/
COOT	doi:10.1107/S0907444910007493
MAIN	doi:10.1107/S0907444913008408
Phenix.real_space_refinement	doi:10.1107/S2059798318006551
PYMOL 2	https://pymol.org/2/
SWISS-MODEL	https://swissmodel.expasy.org

Other	
Quantifoil Au 2/2 holey carbon 200 mesh cryoEM grids	Quantifoil GmbH
Ultrafoil 2/2 holey gold 200 mesh cryoEM grids	Quantifoil GmbH