Investigating the function and regulation of cohesin ATPase

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Abstract

Cohesin is essential for the spatial organization of the genome through its ability to extrude loops on the DNA. Structural information indicates that the cohesin's Smcs fold at the elbow, bringing the hinge domain close to the ATPase heads and also can dissociate from each other to adopt an unzipped coils conformation. In all proposed loop extrusion models, cohesin needs to undergo changes to its conformational state, with unzipping or unfolding of the complex being necessary for its ATP hydrolysis driven loop extrusion. In this work, I investigate the role of cohesin's conformation in relation to its ATPase activity. Specifically I am looking at its the folding and the zipping up of the coiled coils. I examine the changes that occur in those conformational states during cohesin's ATP hydrolysis cycle while also considering their regulatory role. My research shows that both unzipping and unfolding occur during the hydrolysis cycle, while only unzipping is necessary for ATP hydrolysis. I find that the zipped-up coils inhibition is lost in cohesin trimers with mutations at the hinge that do not fold. The unfolded cohesin trimers hydrolyse ATP more readily than WT, indicating that the folded state is required for the stability of the zipping up of the coils. My results lead me to the conclusion that cohesin's rigid APO state inhibits ATP hydrolysis due to the zipped up coils that are stabilized by the folding at the elbow. ATP hydrolysis therefore, requires Scc2 and DNA to ensure the unzipping of the coils in the clamped state.

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Contributions

Some aspects of this work were carried out in collaboration. Calibrated chip-Seq was performed by Naomi Petela. James Collier assisted with the original setup of the crosslinking assay. Adam Costin and Errin Johnson trained me and assisted with the Electron microscopy. Nathan Cowieson performed the SAXs experiment at the Diamond SAXS facility. Christophe Chapard shared the cysteine pair for the zipped-up state. Jean Metson and Antonio Harding shared their cysteine pair for the folded state. The Gruber lab shared their plasmid with the rad50 zinc-hook domain sequence.

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Chapter I: Introduction

DNA to chromosomes

All living cells rely on their DNA to store the necessary information for their survival. During cell division it is imperative that DNA has been faithfully duplicated and the two copies equally segregated to the daughter cells. This intricate process of duplication of the genetic material along with its compaction and faithful segregation is highly regulated and precisely controlled during the eukaryotic cell cycle, leading up to mitosis.

The first problem the cell needs to overcome before addressing the process of DNA replication and chromosome segregation is the lengthwise compaction of the DNA in order for it to fit within the confines of the nucleus. This is a herculean task, since the DNA molecules need to be condensed close to 8.000 times in length to take up space small enough for the cellular nucleus (Paulson *et al.*, 2021). This requirement becomes even more apparent during mitosis, where chromosomes need to condense further to form rigid, autonomous structures that can withstand the pulling forces of the microtubules without

unwinding during the process of segregation (Ou *et al.*, 2017; Paulson *et al.*, 2021)

The second problem is that DNA in eukaryotes is not one long molecule but is rather divided in chromosomes. Given that the length of each chromosome vastly exceeds the diameter of the nucleus it is logical to conclude that DNA molecules within the nucleus would be entangled with each other. This poses a significant challenge for the cell which must ensure that during anaphase, each chromosome is duplicated, aligned at the spindle, and disentangled form any other part of the genome in order to be separated from its sister and move towards the pole of the cell (Nasmyth, 2001). Hence the cell needs to ensure the individualization of each chromosome, in a process that requires disentanglement from other chromosomal DNA while still maintaining the ability to condense and compact itself (Yatskevich, Rhodes and Nasmyth, 2019).

A third problem is related to gene expression and the organization of chromosomes to accommodate for transcription. It has long been known that gene promoters require the collaboration with an enhancer, usually located in distant loci on the chromosome. The process of bridging enhancers together with promoters while ensuring that the correct pair meets each other is also essential for the survival of the cell (Hnisz, Day and Young, 2016).

Despite a multitude of theories that have been proposed to explain each one of these problems, it is becoming increasingly accepted that all these processes are regulated by the function of one group of proteins. Specifically, it is proposed that DNA compaction, genome organization, chromosome condensation and chromosome individualization are all results of one process called loop extrusion (van Ruiten and Rowland, 2018; Mirny, Imakaev and Abdennur, 2019).

According to the loop extrusion model, a motor protein can bind to DNA, hydrolyse ATP and, through a process that is not yet fully understood, extrude increasingly bigger DNA loops (Figure 1a) (Datta, Lecomte and Haering, 2020). The first and most apparent effect of such an activity is the lengthwise compaction of DNA as multiple motor proteins can bind to the genome, build a plethora of loops and in such a way compact the DNA (Figure 1b) (Banigan and Mirny, 2020). Further smaller loops within loops, as well as the processive and robust efficiency of these motor proteins ensure the even higher levels of compaction sought after during the formation of mitotic chromosomes (Golfier *et al.*, 2020).

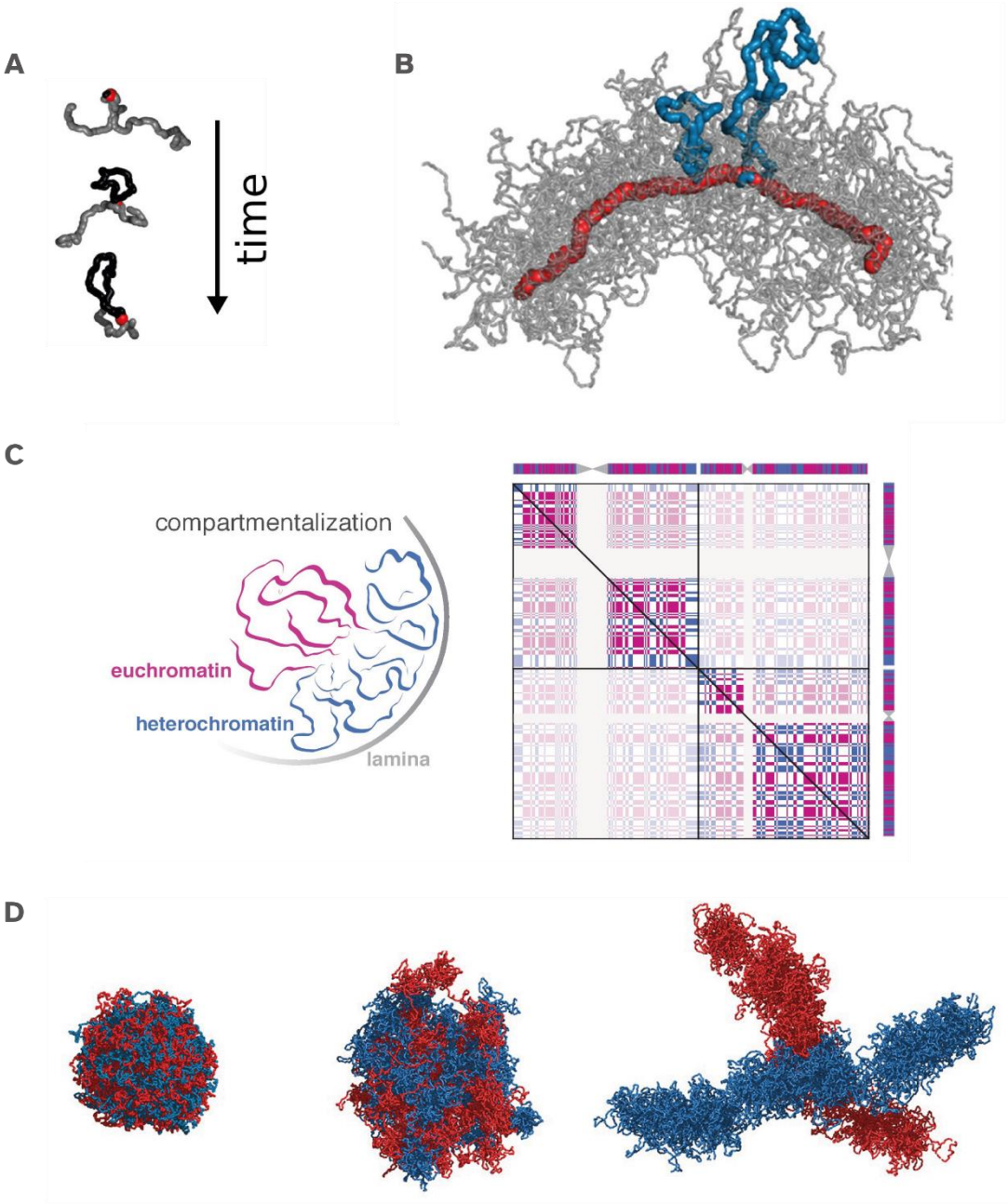
The continuous building and loss of loops can not only achieve the required levels of compaction for the DNA but also creates cis contacts within DNA

molecules. This allows distant parts of the genome such as enhancer promoter pairs to be brought together, which is imperative for many modes of genome regulation and gene expression (Hnisz, Day and Young, 2016). The formation of these contacts can partially interfere with genome compartmentalization, which is driven by differences in the physical properties between compartments separating transcriptionally active [euchromatin] and inactive [heterochromatin] chromatin (Figure 1c) (Mirny, Imakaev and Abdennur, 2019; Hildebrand and Dekker, 2020).

Chromosome individualization is also achieved through the process of loop extrusion (Nasmyth, 2001). This is due to the cis nature of the process, which extrudes a loop in a processive manner rather than capturing loops by bridging DNA molecules that are close in space. Hence, the extrusion process can pull DNA strands out of trans entanglement while building the cis loop. Molecular simulations have shown the process by which loop extrusion can build individual chromosomes out of entangled DNA molecules (Figure 1d) (Goloborodko *et al.*, 2016). In addition, the process of loop extrusion can guide the function of topoisomerase II, exposing trans DNA catenates to be resolved by the enzyme (Dyson *et al.*, 2021).

Finally, the genome needs to undergo segregation simultaneously when the microtubules begin the depolymerization process. This means that all the chromosomes must be duplicated, paired with their sister, individualized, and aligned at the mitotic spindle before the cell progresses to anaphase and chromosome segregation. However, perfect synchronization of all these stages between each chromosome is not possible, due to the intricacy of the processes involved. The solution to that problem is a molecule that can hold the two copies of each chromosome together allowing them to resist the microtubules and wait until the checkpoint, signalling that all other chromosomes have reached the final stage of alignment at the spindle, and only then initiate their coordinated release (Yatskevich, Rhodes and Nasmyth, 2019; Paulson *et al.*, 2021).

Cohesin is the member of the group responsible for solving the synchronization problem of chromosome segregation. Beyond its function as a loop extruder, cohesin has been shown to be essential for sister chromatid cohesion before anaphase, with its cleavage by Separase, being the event that initiates chromosome segregation (Nasmyth, 2005b).



Introduction Figure 1
(**A**) Loop extrusion by SMC complexes (Goloborodko et al., 2016)
(**B**) Loop extrusion compacting DNA (Goloborodko et al., 2016)
(**C**) HiC map showing the compartmentalization of the genome (Mirny et al., 2019)
(**D**) Molecular simulation experiment showing how chromosome individualization can be achieved by loop extrusion (Goloborodko et al., 2016)

SMC protein complexes

The group of motor proteins that perform the aforementioned essential cell cycle functions are called the SMC complexes (Yatskevich, Rhodes and Nasmyth, 2019). These complexes are long proteinaceous ATPases and it has been proposed that they adopt a ring structure capable of topologically entrapping DNA (Figure 2a) (Craig Peterson, 1994; Cobbe and Heck, 2004).

Each complex consists of a pair of Structural Maintenance of Chromosomes (SMC) proteins that form a dimer, connected by a third protein called the kleisin. The SMC proteins contain an ATPase head, forming one half of an ABC ATPase, a rod shaped long coiled coil spanning approximately 50nm in length and a hinge domain that serves as the dimerization domain between the complex's pair of SMCs. The ATPase head is formed by the N and C-terminus of the amino-acid chain, with the intramolecular coiled coil emanating from the head, folding on itself half the way through its length to form the hinge dimerization domain (Gligoris and Löwe, 2016a). The two SMCs stably interact through the hinge but can also transiently associate through ATPase head engagement driven by ATP binding. The kleisin protein asymmetrically bridges the SMC dimer by binding to the ATPase head on one of the two SMCs of the complex and the base of the

coiled coil on the other. The kleisin is mostly unstructured, while it plays the important role of being the docking site for other accessory proteins of the complex (Nasmyth and Haering, 2005; Marston, 2014)

Between the hinge dimerization and the kleisin bridging the SMC heads, the complexes are forming a tripartite ring like structure that can entrap DNA within its lumen (Gruber, Haering and Nasmyth, 2003). This entrapment has been shown to take place in a topological manner with the DNA held within the ring until the dissociation of one of the interfaces or the cleavage of one of the proteins (Gruber, Haering and Nasmyth, 2003; Nasmyth, 2005b, 2009)

The SMC complexes are highly conserved, from bacteria to humans and they exhibit very similar characteristics throughout evolution(Cobbe and Heck, 2004). In bacteria, the complexes consist of a homodimer of SMCs but despite the apparent symmetry of a homodimer, the kleisin still bridges the two SMCs in an asymmetric manner, similar to the heterodimers present in the higher eukaryotes (Bürmann *et al.*, 2013). The mechanism through which this asymmetry is created in bacteria has been elucidated in MukBEF in E. coli but is still unclear for the rest of the bacterial SMC complexes (Bürmann *et al.*, 2021). It is nevertheless a strong indicator of the structural conservation of those

complexes throughout evolution (Fennell-Fezzie *et al.*, 2005; Gligoris *et al.*, 2014; Rajasekar *et al.*, 2019).

Beyond the structural similarities, from MukBEF in E. coli to the B. subtilis SMC homodimers to the human cohesin, condensin and SMC5/6, all of the complexes in the SMC family exhibit similar functional characteristics. However, while the bacterial SMC complexes seem to be fully capable of organizing the DNA (Gruber, 2018), the eukaryotic complexes have a division of labour allowing them to focus on separate intricate tasks (Hassler, Shaltiel and Haering, 2018). Specifically, cohesin is responsible for stably entrapping sister chromatids during S-phase and holding them until their segregation in mitosis, while it is also shown to translocate on DNA and extrude loops throughout interphase contributing to the organization and compartmentalization of the genome (Nasmyth, 2005a). Condensin is mostly responsible for loop extrusion mediating the compaction of the genome and chromosome individualization during the early stages of mitosis leading up to the metaphasic chromosomes (Hirano, 2016). Finally, SMC5/6 is implicated in DNA damage repair and the resolution of replication induced DNA supercoiling (Solé-Soler and Torres-Rosell, 2020).



Introduction Figure 2
(**A**) Architecture of the SMC complexes adapted from (Wells et al., 2017)

Cohesin

In the cohesin complex, the two Smc proteins are Smc1 and Smc3, while the kleisin is Scc1 (Guacci and Koshland, 1997; Michaelis and Ciosk, 1997). Scc1's N terminus forms a helical bundle that binds to the coiled-coil above the ATPase head of Smc3 while the C-terminus forms a winged helix domain that binds to the bottom of the ATPase head of Smc1 (Haering *et al.*, 2004; Gligoris *et al.*, 2014).

The Saccharomyces cerevisiae cohesin trimer which is the model organism used for this study, is regulated by a series of accessory factors that mediate and promote different activities of the complex. An important group of those regulatory factors has been named HAWKS (HEAT Proteins Associated with Kleisins) and consists of Scc2, Pds5 and Scc3 (Figure 3a). All of those co-factors share structural features, as they all contain multiple HEAT (Huntingtin / EF3 / PP2A / Tor1) repeats and exhibit potential DNA binding activity (Wells *et al.*, 2017).

Scc2 together with Scc4 forms a complex named the loading complex (Ciosk *et al.*, 2000). They are recruited to cohesin through Scc2's interaction with Scc1 and are essential for life. This is due to their important role in activating the

ATPase of Cohesin, a process required not only for loading but also translocation and loop extrusion. Scc2, similar to the rest of the proteins included to the HAWK family, adopts a U shape formed by a series of consecutive HEAT repeats. Its N terminus however is a flexible unstructured region that is inserted within the structure of Scc4 forming their interaction (Chao *et al.*, 2015, 2017; Kikuchi *et al.*, 2016). Loading driven by Scc2 and Scc4, has been shown to take place predominately at the centromere, while other secondary cohesin loading sites have been identified throughout the genome (Hu *et al.*, 2011a; Ocampo-Hafalla and Uhlmann, 2011; Hinshaw *et al.*, 2015; Litwin and Wysocki, 2018). Even though cohesin loading happens in certain loci, the complex's translocation ability allows for a wider cohesin distribution along the chromosome (Kogut *et al.*, 2009; Hu *et al.*, 2011b). Mutations on either Scc2 or indeed a residue on the Smc1 hinge allow for the depletion of Scc4, while the yeast cells maintain viability and cohesin distribution in the chromosome arms remains the same (Petela *et al.*, 2018a, 2021). This result indicates that it is Scc2 that is necessary for the loading reaction and Scc4 has an accessory role related to localization on the centromere. Furthermore, the mutation in the hinge region points to a possible interaction of the hinge with the loader, a notion supported by the structural studies.

Another HAWK that is important to cohesin's function is Pds5 (Michaelis *et al.*, 2000). Pds5 has also been found to get recruited to the cohesin complex through its interaction with Scc1 (Lee *et al.*, 2016; Muir *et al.*, 2016). Pds5's function is twofold, as it is necessary for the release function of the complex while it is also required for cohesin maintenance on the chromosome (Chan *et al.*, 2013; Beckouët *et al.*, 2016; Elbatsh *et al.*, 2016). Furthermore, Pds5 and Scc2 have been shown to compete for binding to Scc1, proposing a mutually exclusive association (Petela *et al.*, 2018a). This way, pds5 not only promotes release via its collaboration with Scc3 and Wapl, but also ensures that while this takes place, there is no Scc2 bound to the complex, activating the loading reaction. Finally, Pds5 supports cohesion establishment by promoting acetylation of Smc3 by Eco1 and preventing the deacetylation by Hos1 (Chan *et al.*, 2013).

Scc3 is another significant cofactor of the cohesin trimer. It has been shown to remain bound to Scc1 throughout the cohesin cycle (Tóth et al., 1999a)with a role both in release and loading of the complex. Structural work with the Scc3-Scc1-DNA complex has shown that Scc3 binds close to the C-terminus of Scc1 and can also bind DNA through a positively charged patch (Collier *et al.*, 2020b). Charge reversal mutations of this patch of Scc3 show a loss of Cohesin's distribution on the chromosome but for the core centromere, indicating a vital

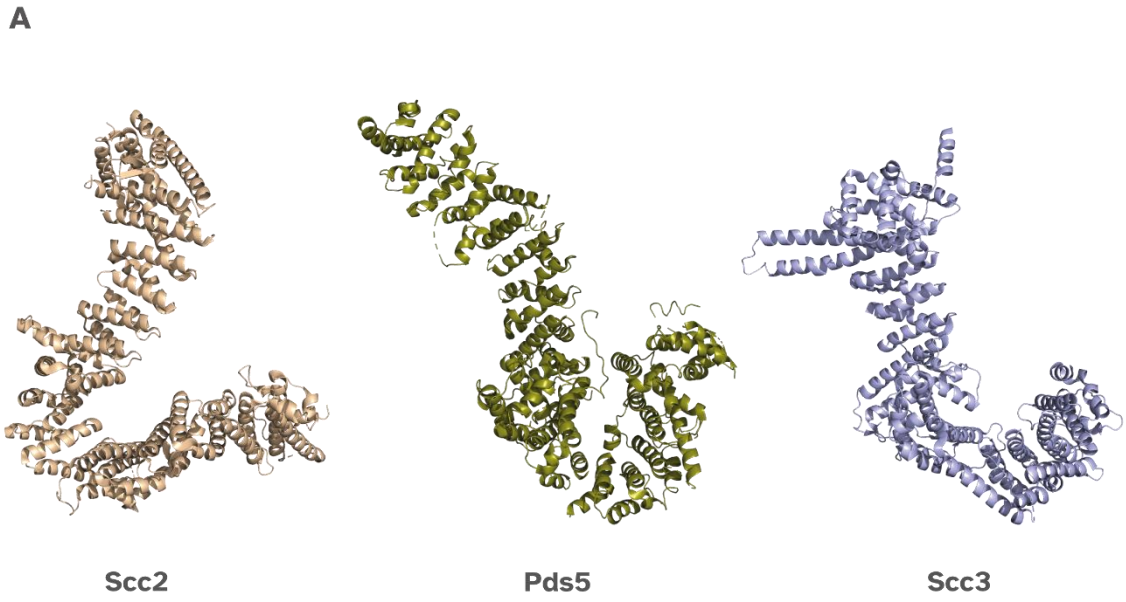
role of this association of Scc3 with DNA on the loading of cohesin (Roig *et al.*, 2014a; Li *et al.*, 2018). Moreover, the recent cryo-EM structure revealed a DNA binding site on Scc3 that differs from the one previously described by the crystal structure, possibly indicating two different modes of Scc3/DNA association (Shi *et al.*, 2020). Scc3 has been shown to bind to Wapl and through this interaction help recruit it to the complex and subsequently facilitate release. Mutations on the residue 404 of Scc3 can cause release defects and can therefore rescue Eco1Δ cells (Beckouët *et al.*, 2016). Finally, Scc3 associates with the cohesin hinge, as shown by the cryo-EM structure obtained of the clamp state of cohesin (Higashi *et al.*, 2020; Shi *et al.*, 2020).

Mediating the release of the cohesin complex from the DNA is Wapl. Wapl has been shown to form a complex with pds5 and Scc3 and through that to associate with cohesin to cause the release of DNA from the cohesin trimer (Gandhi, Gillespie and Hirano, 2006; Kueng *et al.*, 2006; Lopez-Serra *et al.*, 2013; Elbatsh *et al.*, 2016). Its function is crucial to ensure the dynamic nature of cohesin's population on the DNA. Depletion of Wapl has been shown to increase the amount of cohesin on to the chromosomes and its residence time (Tedeschi *et al.*, 2013). Finally, Wapl has an important role after the cleavage of Scc1 by Separase. Pertinent to the recycling of the cleaved cohesin trimers is the release

of the N-term Scc1 fragment from Smc3. To achieve this, Wapl binds to the complex and drives the release of the fragment, allowing for the SMC dimer to bind to a new full length Scc1 (Beckouët *et al.*, 2016; Srinivasan *et al.*, 2019).

Eco1 is promoting cohesion establishment by acetylating the complex (Tó *et al.*, 1999b). The acetylation of Smc3 K112K113 in yeast cohesin is necessary to protect the complex from release and hence ensure that it will remain bound to the sister chromatids until the cleavage by Separase in mitosis (Ben-Shahar *et al.*, 2008). Depletion of Eco1 results in segregation defects due to the susceptibility of non-acetylated cohesin to release from Wapl. Henceforth the simultaneous depletion of Eco1 and Wapl alleviates the effect of Eco1's loss and restores viability (Beckouët *et al.*, 2016). After mitosis Hos1, is the deacetylase removing the signal from the lysine pair allowing for the recycling of the SMC dimer (Borges *et al.*, 2010).

Finally, Separase has been shown to be responsible for the proteolytic cleavage of Scc1. Activated in a cell cycle dependent manner, Separase's activity is the event that, by the cleavage of cohesin, initiates the sister chromosome segregation (Peters and Nasmyth, 2013; Boland *et al.*, 2017).



Introduction Figure 3
(**A**) Structures of the cohesin HAWKS. Scc2 (5ME3), Pds5 (5F0O), Scc3 (4UVK)

Sister chromatid cohesion

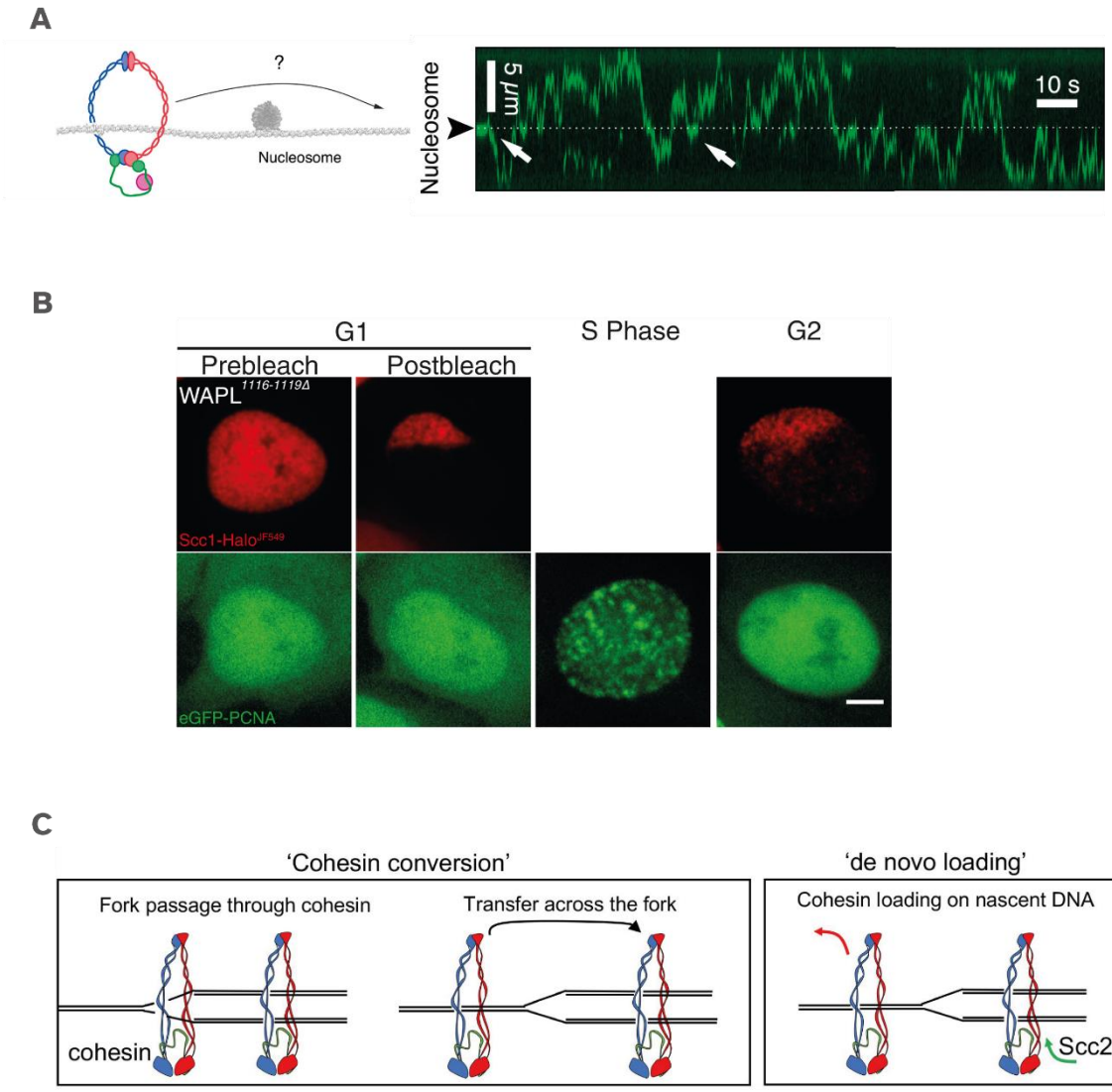
Sister Chromatin Cohesion is built during the S phase and is retained during G2 to mitosis (Uhlmann and Nasmyth, 1998). Sister chromatid cohesion is accomplished by entrapping the two copies of each chromosome and holding them together until the DNA has been compacted to rigid metaphasic chromosomes, microtubules have latched onto all chromosome pairs, and the checkpoint is met for segregation of the two copies of the genome. It is during this process, that Separase cleaves Scc1, allowing for the two copies of the genome to be pulled to each pole of the cell through the depolymerization of the microtubules (Uhlmann, 2003). Failure of cohesin to perform its function can result in uneven segregation of the DNA, causing aneuploidy to the daughter cells, which can result in a number of fatal outcomes.

Successful SCC requires established cohesin holding the sister chromatids. This enforces the requirement of cohesin complexes catenating two sister chromatids to be acetylated by Eco1 and thus protected from release. Eco1's colocalization with the replication machinery, ensures that the cohesin entrapping sister chromatids is therefore acetylated (Lengronne *et al.*, 2006).

The mechanism by which cohesin remains topologically bound to DNA when met with the replication fork is still unknown.

In vitro single molecule experiments suggest that cohesin can bypass roadblocks such as nucleosomes while translocating (Figure 4a) (Stigler *et al.*, 2016). *In vivo* FRAP microscopy data have shown that even the replication fork can pass through cohesin, while building established cohesion that holds both sister chromatids (Figure 4b) (Rhodes *et al.*, 2017).

However, a recent study proposed two distinct mechanisms for cohesion establishment. A *de novo* pathway that takes place after replication, where cohesin loads onto both replicated sisters and holds them together and a conversion pathway, where cohesin molecules holding one chromatid before the fork are transferred with the help of replisome associated proteins after the fork ending up holding both sisters (Figure 4c) (Srinivasan *et al.*, 2020).



Introduction Figure 4
(**A**) *In vitro* DNA curtain experiment showing cohesin translocating through a nucleosome (Stigler et al., 2016)
(**B**) *In vivo* cell imaging showing cohesin remaining associated with the DNA past replication (Rhodes et al., 2017)
(**C**) Schematic representation of the two mechanisms of cohesion establishment (Srinivasan et al., 2020)

The ATPase activity of the cohesin complex

The cohesin complex contains a pair of ABC type ATPase heads (Haering *et al.*, 2002). Each one of them formed by each SMC, they are capable of binding and hydrolysing ATP in a cooperative fashion. Similar to other ABC type ATPases, cohesin's heads consist of a Walker A motif, responsible for the binding of ATP, a Walker B motif that contains the glutamate which attacks the third phosphate group that leads to hydrolysis and a signature motif, present in both ATPase heads that ensures the cooperative nature of the ATPase activity by recognising the nucleotide bound to the opposite head (Figure 5a,b) (Löwe, Cordell and van den Ent, 2001; Lammens and Hopfner, 2010; Petela *et al.*, 2018a).

In vitro studies of the ATPase activity of cohesin have measured a rate of hydrolysis consistent with that measured in condensin and the bacterial SMC complex (Petela *et al.*, 2018b). As it was described originally in the bacterial SMC complex, the SMC ATPase heads assume an engaged state while bound to the nucleotide, whereas in the APO state the heads are facing towards opposite directions and assume a juxtaposed state, where the coiled coils emanating from them are zipped up (Soh *et al.*, 2015; Muir *et al.*, 2020). From the structures solved for either the Smc3 or the Smc1 head we know that they

can bind the nucleotide in the absence of any other co-factor (Haering *et al.*, 2004; Gligoris *et al.*, 2014). Moreover, cysteine crosslinking experiments have shown that the cohesin trimer can assume the heads-engaged state just by the addition of ATP to the complex (Collier *et al.*, 2020b). Despite the complex binding ATP and orienting its ATPase heads to the engaged position, *in vitro* experiments have shown that there is no hydrolysis taking place in the presence of only the cohesin trimer and ATP (Petela *et al.*, 2018a; Davidson *et al.*, 2019; Gutierrez-Escribano *et al.*, 2019; Kim *et al.*, 2019).

The factor essential for cohesin's ATP hydrolysis is the loading complex (Petela *et al.*, 2018b). No significant hydrolysis rate has ever been measured with Cohesin in the absence of Scc2-Scc4. Specifically, it is Scc2 that activates the ATPase, as addition of only the truncated version of Scc2 that lacks the N-terminal region responsible for Scc4 binding is sufficient to activate the ATPase activity at full rates (Petela *et al.*, 2018b; Shi *et al.*, 2020).

However, the presence of only Scc2 is still not enough. Along with Scc2, DNA is also required to ensure the full activation of the cohesin ATPase. It has been hypothesised that this is because DNA binds on the top of the SMC heads, stabilizing the engaged state, while Scc2-DNA-Cohesin-ATP form a stable

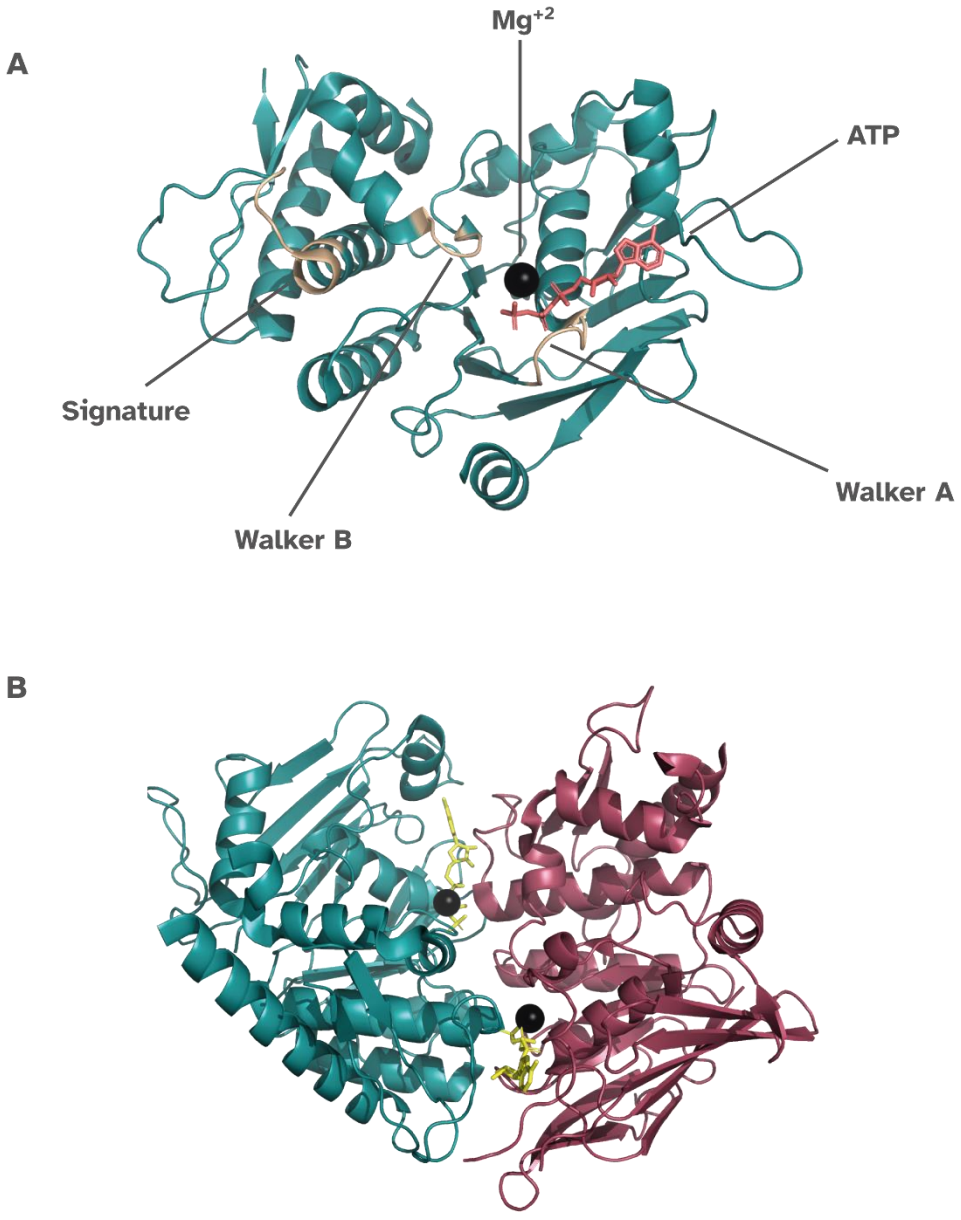
complex that structurally adopts the pre-hydrolysis state (Petela *et al.*, 2018a; Davidson *et al.*, 2019; Gutierrez-Escribano *et al.*, 2019; Kim *et al.*, 2019).

In the absence of DNA, cohesin can still hydrolyse ATP if Scc3 is present, albeit at approximately half the rate (Petela *et al.*, 2018a; Davidson *et al.*, 2019). There is no explanation for this function yet and it remains unknown whether Scc3 somehow replaces the need for DNA in ensuring the formation of the stable clamp state, or it works through another mechanism.

None of the rest of the cofactors that associate with the cohesin trimer have been described to have any direct effects on cohesin's ATP hydrolysis, as measured by the *in vitro* assays. However, pds5 has been shown to reduce the rate when added alongside Scc2, an effect that is undoubtedly a result of the competition between Pds5 and Scc2 for Scc1 binding (Petela *et al.*, 2018a).

This necessity of an accessory factor to activate the ATPase activity appears to be conserved and has been shown on multiple SMC complexes. Bacterial SMCs also require DNA and the presence of the KITE family proteins (**k**leisin **i**nteracting **t**andem winged-helix (WH) **e**lements) to hydrolyse ATP at full rate, while condensin's hydrolysis rate is likewise dependent on Ycs4 and

DNA (Palecek and Gruber, 2015; Terakawa *et al.*, 2017; Vazquez Nunez, Ruiz Avila and Gruber, 2019).



Introduction Figure 5
(**A**) SMC ATPase domain, with the Walker A, Wlker B and signature motifs highlighted
(**B**) Structure of the cohesin engaged heads bound to ATP. (6zz6)

Cohesin's structure and architecture

Several structural and biochemical studies have been done to determine the structure, protein interactions and the overall architecture of the complex. Significant similarities have been shown between all the SMC complexes, further establishing the commonalities among them.

Due to the unique structure of the SMC proteins and the apparent flexibility of the coiled coils, along with the unstructured nature of the kleisin, X-ray crystallography has failed to provide a high-resolution structure of the holocomplex. However, several truncated constructs have been solved, providing unique insights on essential parts of the complex. These include all three interfaces that close the cohesin ring (Gligoris and Löwe, 2016b).

The cohesin hinge structure was solved as a construct that contained the mouse hinge dimerization domain and a short part of the protruding coiled coils. The structure exhibited a doughnut shape with a positively charged lumen in the middle and the coiled coils emanating in opposite directions (Kurze *et al.*, 2011). Structural work on the condensin hinge however, showed the coiled coils adopting a zipped-up conformation. This difference is possibly due to the longer coiled coil included in the condensin construct allowing for enough inter-coil

interactions. In the same study, even though a high-resolution structure of the cohesin hinge with the longer coiled coil was not attained, SAXs experiments allowed for a predicted structural envelop of the construct, which clearly confirmed the juxtaposed coils conformation. Finally in both complexes, the hinge appeared to be in an angle in respect with the plain of the coils, suggesting a bent (Soh *et al.*, 2015).

The positively charged lumen of the hinge was hypothesised as a possible DNA binding site. In support of this theory, the structure was also solved in a state where the dimerization domain was half open, providing for a possible mechanism for the DNA to enter the lumen. In more recent studies the human hinge has been solved with a ssDNA oligo bound to the outside of the hinge as well as half open hinge structures for both the north and south pole. However, to this day no structure has been shown to exhibit DNA binding inside the hinge lumen (Shi *et al.*, 2020).

The Smc1-Scc1C interface showed that indeed cohesin forms an ABC type ATPase with the nucleotide binding domain being formed by the N and C terminal lobes of Smc1. Also, the structure was solved as a nucleotide dependent homodimer of SMCs, providing insight in the engagement of the cohesin ATPase heads (Haering *et al.*, 2004).

The Smc3-Scc1N interface showed the Scc1 helical bundle that stably associates with the Smc3 neck region (the coiled coil above the ATPase head), confirming the evolutionary conserved asymmetry of the complex. Furthermore, the coiled coil region above the Scc1N binding site exhibited a break in its continuity, forming a feature named the joint. Structural alignment allowed for a heterodimer model base on the Smc1 engaged head homodimer, showing that as an ABC ATPase the cohesin complex's heads dimerize in an ATP dependent manner (Gligoris et al., 2014; Shi et al., 2020). Moreover, the structure allowed for the visualization of K112 and K113 that are the targets of Eco1 acetylation during cohesion establishment. Both lysins are a significant distance away from the Scc1N binding site, suggesting an indirect establishment mechanism where release is prohibited not by strengthening of the interface. Finally, more recent studies of the Smc1-Smc3 heads heterodimer have confirmed the existing model and due to significant changes between the APO versus the nucleotide bound state, suggested a transient dissociation of the Scc1 N-terminus following nucleotide binding (Muir *et al.*, 2020). A similar mechanism was also described for the condensin Smc2/Smc4 heterodimer (Hassler *et al.*, 2019).

Recent EM studies have shed light on, not only the structure of the holocomplex but also the architecture and conformational states it adopts. Starting with

negative stain EM, it was shown that cohesin adopts a folded conformation with the coiled coil exhibiting a break in its continuity, named the elbow, close to the middle point which allows it to fold down with the hinge region now contacting the joint region (Bürmann *et al.*, 2019). This feature was first shown in E.Coli's MukBEF where the structure of the coiled coil break was solved (Bürmann *et al.*, 2019). Sequence analysis and subsequent holocomplex cryo-EM structural studies have provided evidence for coiled-coil breaks at varying locations between each complex making up the elbow in MukBEF, cohesin and condensin, whereas X-ray crystallography and EM studies have identified no such region in the B. subtillis SMC complex or Smc5/6 (Diebold-Durand *et al.*, 2017; Lee *et al.*, 2020; Yu *et al.*, 2021).

Cryo-EM studies of cohesin revealed the folded conformation of the cohesin complex in the apo state while the coiled coils assumed a zipped-up conformation from the heads to the hinge (Figure 6a). The complex therefore seemed to adopt a folded rod state. The holocomplex structure also confirmed the existence of the joint region on Smc1, which had not been solved in the previous structural studies, showing that it is indeed a symmetrical feature as predicted by its existence in the bacterial homodimers.

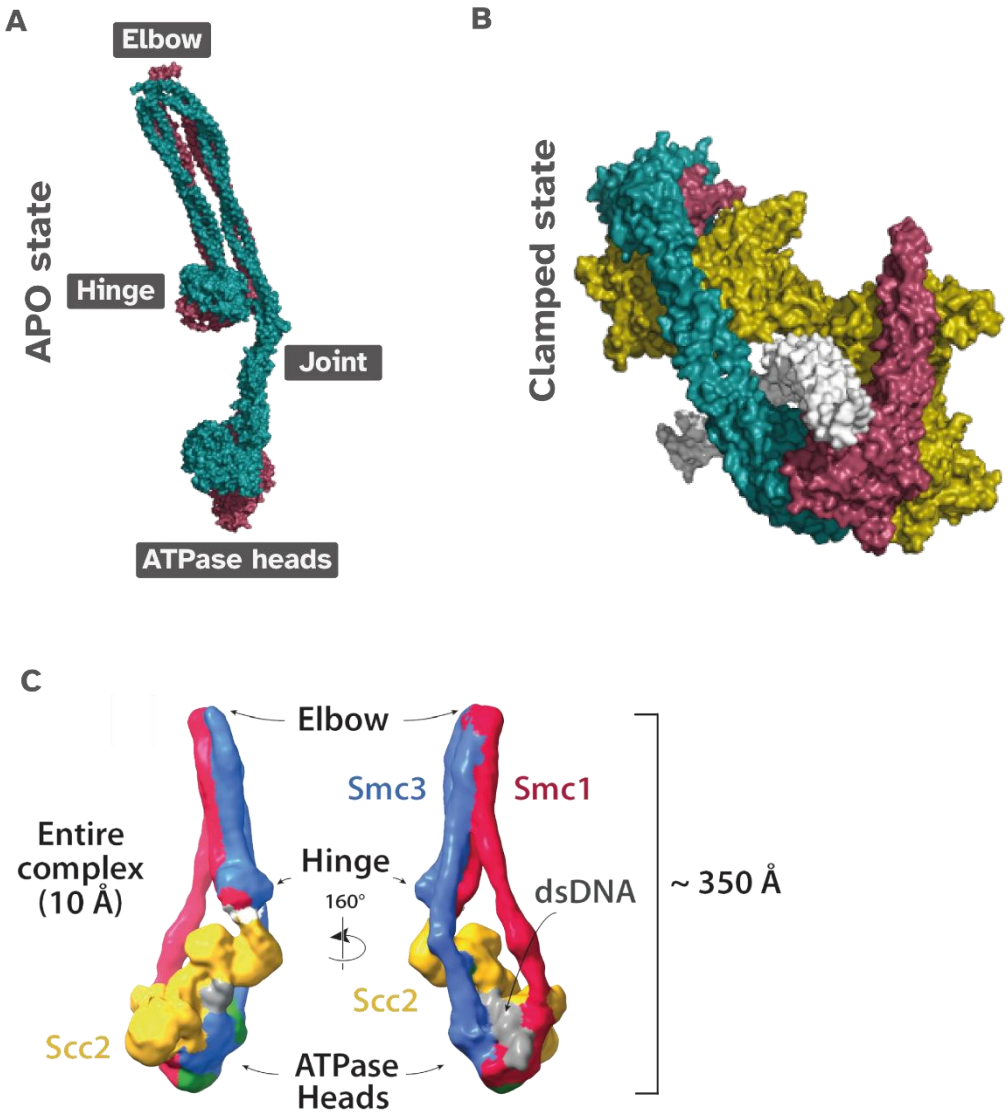
The structure of the ATP and DNA bound state of the complex with Scc2 (clamped state) showed significant conformational changes compared to the APO state (Figure 6b). The coiled coils emanating from the ATPase heads were no longer juxtaposed and were both facing away from each other. This allowed for the formation of a lumen between the engaged heads and the joint region, large enough for the DNA molecule to sit on top of the ATPase heads. Regarding the rest of the coiled coil, low resolution structural information from the same studies clearly show that the coils come together in the region south of the elbow and still assume a bent rod shape from the elbow onwards (Figure 6c). This structure suggests that the hinge remains associated with the ascending coil during the formation of the clamped state, showing the complex still folded. However, due to the nature of the Cryo-EM imaging which selects for homogeneous populations it is still uncertain whether this represents the majority of cohesin particles as any unfolded cohesin trimers would be excluded due to their heterogeneity. Hence the final particle selected for classification and analysis would preferentially represent the subpopulation that is more rigid and adopts a homogeneous structure obscuring the average state of the molecule in solution. Further structural and biochemical information is required to determine whether the clamped state is still stably folded, or the appearance

of a fold is just a preferential population selected for the solution of the structure (Collier *et al.*, 2020b; Higashi *et al.*, 2020; Shi *et al.*, 2020).

Both structural studies that first showed the clamped state included Scc2 and Scc3 in the structure. Scc2 has clearly been shown to bind to both the Smc1 and Smc3 engaged heads, helping them come together, while also sandwiching the DNA between itself and the top of the heads. Scc3 binds to the complex through an interaction with Scc2, while it seems to be in contact with the folded hinge region. The N-terminal region of Scc2, even though absent from the structure, would have extended very close to the density occupied by the hinge (Higashi *et al.*, 2020; Shi *et al.*, 2020). Considering that Scc4 binds to the N terminal region of Scc2 it is proposed that either Scc4 only, or both Scc2 and Scc4 contact the hinge while the complex is in the folded conformation. This idea is supported by the cryo-EM structure of the yeast cohesin apo state, where the N terminus of the Scc2 bound to the cohesin trimer comes close to the hinge (Petela *et al.*, 2021).

Another important gain from the clamped state structure was the resolution of part of Scc1. Previous studies have solved small peptides of Scc1 as part of a complex with the respective regulatory subunit (Collier *et al.*, 2020b; Higashi *et al.*, 2020; Shi *et al.*, 2020). Specifically, a part of Scc1 was solved with Scc3, a

part with pds5 and one with Separase (Roig *et al.*, 2014b; Lee *et al.*, 2016; Lin, Luo and Yu, 2016; Muir *et al.*, 2016; Boland *et al.*, 2017; Li *et al.*, 2018). In the cryo-EM structure further information were gathered regarding Scc1's interaction region with Scc2, confirming previous genetic studies that had showed deletions in Scc1 that affect Scc2 binding to the complex (Petela *et al.*, 2018a). Still, a significant part of Scc1 remains unresolved due to its flexible unstructured nature.



Introduction Figure 6
(**A**) Structure of the cohesin Apo state (Petela et al., 2021)
(**B**) Structure of the cohesin Clamped state (6zz6)
(**C**) 10A resolution structure of theclamped state cohesin with the hinge still folded down (Collier et al., 2020)

DNA association and entrapment

Cohesin's functions rely upon the complex's ability to stably bind to DNA and topologically entrap one or two DNA molecules. As described before, DNA association with the cohesin complex happens through multiple co-factors as well as the cohesin trimer itself. The ATPase heads have been shown to bind to DNA when in the engaged state (Collier *et al.*, 2020b; Higashi *et al.*, 2020; Shi *et al.*, 2020), while further DNA binding has been described for the hinge, where both dsDNA and ssDNA have been shown to associate with it *in vitro* (Chiu, Revenkova and Jessberger, 2004). On top of those sites, Scc3 has two separate DNA binding sites described in the literature (Roig *et al.*, 2014b; Li *et al.*, 2018; Shi *et al.*, 2020), while Scc2 binds to DNA as shown by EMSA and has recently been described to bind DNA in complex with the engaged ATPase heads (Collier *et al.*, 2020b; Higashi *et al.*, 2020; Shi *et al.*, 2020). Despite the structural similarities with Scc2 and Scc3, no DNA bound state has been described for pds5. All the aforementioned DNA binding sites are not sequence specific and rely on positively charged residues to associate with the DNA. This is a strong indicator for cohesin's ability to slide on DNA, that could be allowing it to translocate along the genome with no specific sequence limiting its movement.

Further from the simple interactions with DNA, cohesin has been long believed to entrap a single or even two DNA molecules within its ring (Nasmyth, 2005b). This theory is supported by *in vivo* experiments, where minichromosomes within a yeast cell were pulled down through the immunoprecipitation of covalently circularised cohesin trimers (6C cohesin, containing a crosslinkable cysteine pair on each interface) and visualised using western blotting. The result was 2 separate band shifts on top of the free supercoiled minichromosome plasmid. One band represents the catenated monomers where cohesin is bound to one molecule of DNA and the other the catenated dimers where cohesin holds 2 plasmid DNAs, causing significant retardation to its migration on the gel (Figure 7a) (Haering *et al.*, 2008; Gligoris *et al.*, 2014).

Following the *in vivo* experiments, purification of S.*p.* Cohesin allowed for the *in vitro* proof of topological DNA binding in the cohesin ring. Purified cohesin was shown to bind DNA in an ATP, Scc3 and Scc2/4 dependent manner, resisting salt concentrations of up to 500mM (Figure 7c) (Murayama and Uhlmann, 2014, 2015). However, these experiments could not definitively claim topology, as the result can also be explained by strong DNA-protein interactions or pseudo topological entrapment of a large DNA loop. To address this, a recent *in vitro* experiment used a purified 6C S.c. cohesin in a DNA entrapment assay and

showed that indeed DNA is entrapped topologically in a crosslinker dependent manner (Figure 7d) (Collier *et al.*, 2020b). Although these *in vitro* results seem to corroborate the *in vivo* data, both groups have described partial but significant activity by the EQ complex, something that is directly contradicted by the *in vivo* results, where mere ATP binding without hydrolysis is not enough for any type of stable association of cohesin with DNA.

Both of the *in vitro* results supported the idea of a single plasmid entrapped withing the cohesin ring but failed to provide any evidence on the two DNA molecules entrapment, required for cohesion according to the one ring model. *In vitro* evidence for the two DNAs entrapped within one ring have since been presented in two different assays. First, a follow-up experiment to the salt resistant DNA entrapment assay showed that for cohesin to sequentially entrap 2 DNA molecules, first a double stranded DNA gets captured and then the second molecule needs to be single stranded, a condition present at the replication fork (Murayama *et al.*, 2018). Second, an experiment using optical tweezers showed that cohesin not only is able to bridge 2 DNA molecules aligned in proximity to each other, but that the bridging is resistant to significant force, prompting the authors to suggest that it is topological entrapment and not an ionic protein-DNA interaction (Figure 7e) (Gutierrez-Escribano *et al.*,

2019). This entrapment was shown to be a result of a single-capture step, as opposed to the sequential entrapment described before that requires the second DNA molecule to be single stranded.

Topological entrapment of DNA within the cohesin ring requires an entry gate. An opening of one of the 3 cohesin interfaces to allow for DNA to enter the ring (Nasmyth, 2011; Gruber, 2017). One theory is that the entry gate is the Scc1-Smc3 interface. This was proposed as the Scc1-Smc3 interface has already shown to open during release by Wapl (Chan *et al.*, 2012). The hinge has also been proposed to be the entry gate, as structural studies have identified half opened isolated hinges, pointing to a possible opening of the interface under certain conditions. In support to that hypothesis the lumen within the hinge structure has been shown to be highly positively charged, which would promote association with DNA in a possible open conformation (Kurze *et al.*, 2011). Finally, dual dissociation of Scc1 from both SMCs has also been proposed, supporting a bicycle lock mechanism for DNA entering the cohesin ring.

A first attempt to conclusively answer the question of Cohesin's entry gate was the introduction of fusion linkers to each Scc1-Smc interface in yeast, blocking

any opening of those interfaces. Both strains proved viable, indicating that no one specific Scc1-Smc interface is the entry gate, but leaving the bicycle lock scenario as a plausible explanation. The linker closing the Smc3-Scc1 interface also rescued eco1Δ cells, alluding to a successful prohibition of release and therefore confirming that the interface is indeed closed. To address the question of the hinge being the entry gate, a Frb-FKBP-12 dimerization module was inserted in either side of the hinge dimerization domain. The covalent bond formed in the presence of rapamycin, caused lethality to the yeast strain, indicating to the opening of the hinge as an essential aspect of cohesin's function. However, the use of the bulky Frb-FKBP-12 dimerization module has been criticized as it could interfere with the viability of the complex in ways beyond the closing of the hinge (Gruber *et al.*, 2006). More recent experiments have confirmed the formation of both CMs and CDs for the strains containing the Scc1-Smc linkers but were unable to answer the question of the hinge being the entry gate in yeast, as no linker can be inserted on the interface and *in vivo* cysteine crosslinking would hinder cell viability and function affecting the entrapment assay. For that reason, purified xenopus cohesin was used in xenopus cell extracts and after crosslinking the hinge, it was shown that the

complex still associated with chromatin in a stable manner (Srinivasan *et al.*, 2018).

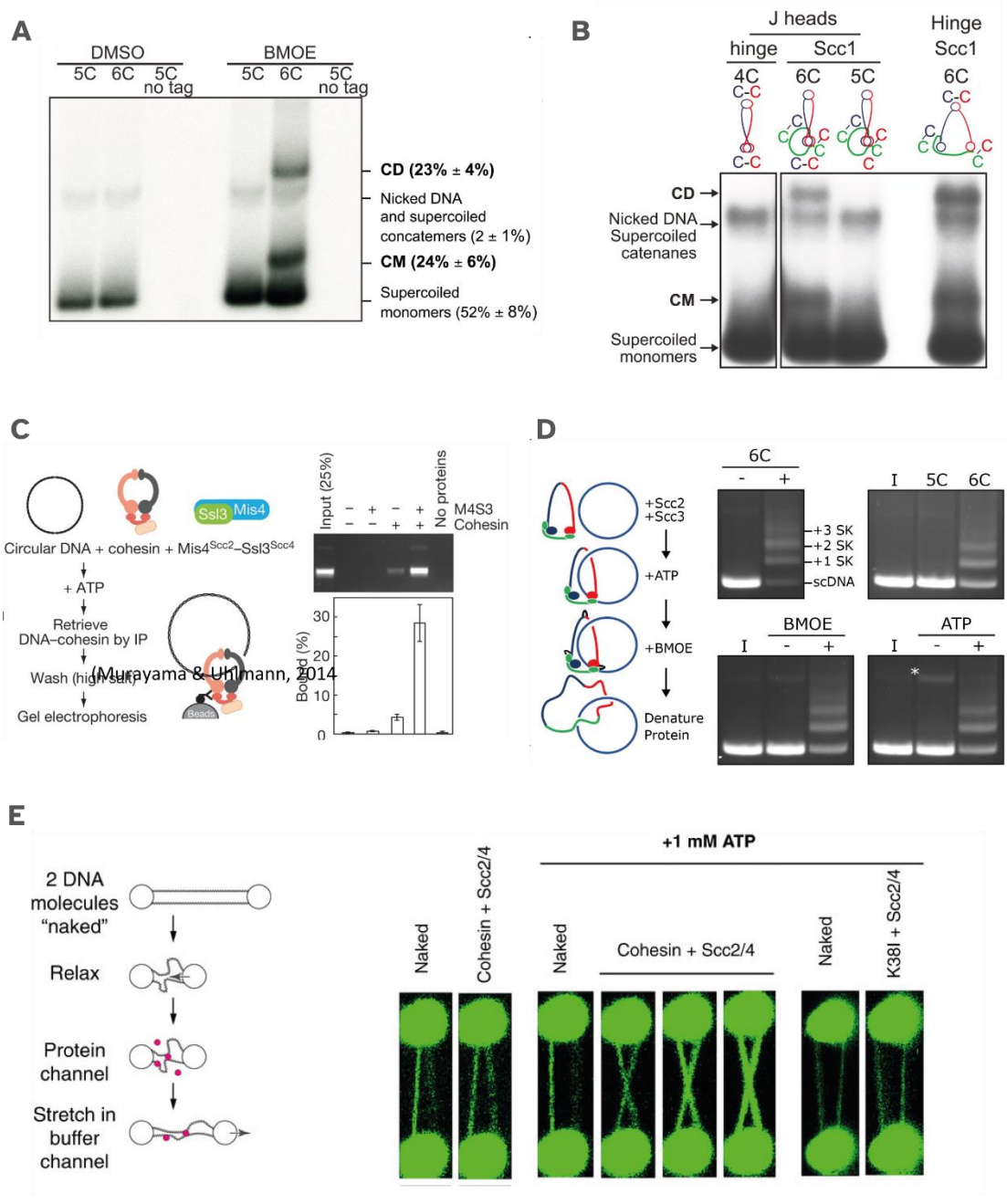
Contradicting these *in vivo* experiments, the salt resistant entrapment vitro assay, also showed that pds5 and Wapl can cause topological DNA binding to cohesin, presumably by opening the “exit gate”. This function is ATP dependent and requires hydrolysis, as ATPγS inhibits it (Murayama and Uhlmann, 2015). This explanation seems to fall short in that neither pds5 nor Wapl nor cohesin on its own are able to induce any ATP hydrolysis, a process which as was discussed is activated by Scc2. However, relying on structural information recent studies have proposed a mechanism for the opening of the Smc3-scc1 gate based on ATP binding and the subsequent structural changes occurring on the Smc3 head (Muir *et al.*, 2020). Building on this hypothesis, along with DNA binding studies of the Scc1 N terminus and mass spec data mapping the DNA contacts with Cohesin, allowed the proposal for new model for loading. According to this new model, ATP binding switches the heads to the engaged state while opens the Smc3-Scc1 interface. DNA enters through the coils, reaching the binding site on top of the heads, where together with Scc2 they form the clamped state. Scc2’s association with DNA and the engaged heads drives a conformational state which pushes the Scc1 N term back into the

interface, closing the gate and topologically entrapping the DNA molecule. Subsequent ATP hydrolysis disengages the heads allowing the DNA to fall into the kleisin compartment (Higashi *et al.*, 2020).

This model of opening of the "exit gate" by ATP binding could also be the mechanism that drives release. As for the role of pds5 and Wapl, according to a recent finding, they are not directly responsible for release which can take place in their absence if only one depletes the cells of Scc2, whose role is to block it (Srinivasan *et al.*, 2019). Hence Pds5 and Wapl might be responsible for the preservation of the interface opening during release and competing with Scc2 for the binding to cohesin.

One further question that needs to be answered is where the DNA molecules are held within the cohesin ring. For many years the assumption was the complete opening of the cohesin trimer with the SMCs coming apart and together with the kleisin forming an open circular structure reminiscent of a ring (Nasmyth, 2009). However, recent structural information concerning not only cohesin, but all other SMC complexes support the adoption of a zipped-up rod conformation by the two SMC proteins (Diebold-Durand *et al.*, 2017; Lee *et al.*,

2020). Even when the DNA resides on top of the ATPase heads forcing the coiled coils to rotate outwards, they tend to adopt a closed associated rod state higher up on their length. Hence to address these questions the cohesin complex was divided in sub-compartments during different states and tested for CM and CD formation. The compartments were the Kleisin [K] compartment, circularized by covalently closing the Smc3-Scc1 and Smc1-Scc1 interfaces along with the two ATPase heads and the SMC [S] compartment which includes the lumen between the hinge and the heads. Regarding the covalent fusion of the heads, cysteine pairs for either the Engaged state (E state - ATP bound) or the juxtaposed state (J state - Apo) were used giving rise to the ES, EK, JS and JK compartments. Testing all of those compartments for DNA entrapment in budding yeast, showed only the kleisin compartment entrapped DNA. Specifically, the EK compartment seemed to form CMs while CDs were only found in the JK compartment (Figure 7b) (Chapard *et al.*, 2019a). Addressing the formation of CMs *in vitro* using the cysteines for the ES, EK, JS, JK, showed that ES as well as EK can entrap DNA (Collier *et al.*, 2020b).



Introduction Figure 1

(**A**) *In vivo* DNA minichromosome entrapment assay with 6C circularized cohesin (Srinivasan et al., 2018)
(**B**) *In vivo* entrapment assay showing that DNA is entrapped in the JK compartment (Chapard et al., 2019)
(**C**) *In vitro* salt resistant DNA entrapment assay (Murayama & Uhlmann, 2014)
(**D**) *In vitro* 6C cirrularized cohesin loading assay (Collier et al., 2020)
(**E**) *In vitro* DNA tethering assay (Gutierrez-Escribano et al., 2019)

The mechanism of loop extrusion

Cohesin has long been believed to contribute to genome organization and compaction. It shares many characteristics with condensin, a protein complex that has been shown to be responsible for the active condensation of chromatids. The mechanism that was proposed for SMC proteins, to explain their ability to compact DNA was that of active, ATP dependent processive loop extrusion. According to this model, cohesin, as with the Condensin complex, is capable of extruding loops in order to establish higher order structures in the genome (Uhlmann, 2016; Nasmyth, 2017).

Cohesin's function as a motor protein was first proposed as the mechanism for translocating on the DNA (Craig Peterson, 1994). *In vivo*, cohesin is distributed along the chromosome arms, in a manner indicative of a translocation mechanism from the loading sites of the complex (Hu *et al.*, 2011a). The first piece of direct evidence supporting the active translocation theory came from *in vitro* single molecule experiments that showed that cohesin can translocate along double tethered DNA, in a process that appears to be active and powered by ATP hydrolysis (Stigler *et al.*, 2016). It was later proposed that translocation paired with an anchoring mechanism of cohesin to the DNA could constitute a

viable model for loop extrusion (Hassler, Shaltiel and Haering, 2018; Banigan and Mirny, 2020).

In vivo experimental evidence supporting cohesin's loop extrusion function came with the development of HiC, a technique that allowed for the visualisation of contacts within the genome. Study of chromosome contact maps revealed conserved distant cis contacts that were named Topologically Associated Domains (TADs) that were believed to represent loops formed within the chromosome. The most distant points within the domain appeared to have stronger interactions supporting the theory that the contacts where the result of loop formation (Sanborn *et al.*, 2015; Fudenberg *et al.*, 2016). Depletion of cohesin caused a dramatic loss of TADs and loop anchor points within the genome (Figure 8a) (Rao *et al.*, 2017).

Moreover, it was shown that cohesin mediated loops were limited by boundary sequences inhabited by CTCF, a transcription regulatory factor (Wendt *et al.*, 2008; Busslinger *et al.*, 2017; Wutz *et al.*, 2017). This result provided with an explanation for the non-stochastic loop sizes identified in cells and the overall pattern that was maintained between experiments. CTCF was not only responsible for the insulation of loop extrusion, but its directionality plays a crucial role in stabilizing the cohesin formed loop. Specifically, convergent CTCF

sites tend to constitute the base of the loop, blocking cohesin from further extending it. On the contrary divergent or unidirectional ones are no barrier to cohesin, which continues the extrusion process, until it meets the next convergent CTCF site (de Wit *et al.*, 2015). Recent structural and biochemical data could shed light onto the mechanism driving the process, with both Scc3 and pds5 shown to bind to CTCF, which could potentially explain the convergence rule (Li *et al.*, 2020; Nora *et al.*, 2020).

Final proof came with the *in vitro* single molecule experiments that showed that cohesin can form DNA loops in the presence of ATP and Scc2 (Figure 8b). While ATP hydrolysis is a requirement for the extrusion process as it is for loading onto DNA, topological entrapment is not. Moreover, cohesin's loop extrusion was found to be symmetrical and 2 sided in its nature while it was proposed that it requires the formation of a small loop to initiate the extrusion process, enlarging the loop symmetrically (Davidson *et al.*, 2019; Kim *et al.*, 2019).

A series of different loop extrusion mechanisms have been proposed, with none of them yet proven (Figure 8c). According to the pumping model, cohesin binds ATP, which causes head engagement which in turn unzips the coiled coils opening the S compartment. The open S compartment can engulf a DNA loop and by hydrolysing ATP, drive the zipping up of the coils from the hinge to the

heads which pushes the DNA to the K compartment. Multiple steps of this process would lead to a progressively bigger loop held in the K compartment.

On the other hand, the scrunching model proposes the use of two DNA binding sites, one on top of the ATPase heads and one at the hinge. Using the folding of the coils at the elbow, cohesin captures DNA at the hinge and by folding down moves it towards the heads. Due to the DNA already being bound to the heads a few bases downstream, this movement pushes towards the bending of the DNA to form a loop. By releasing the DNA and repeating the process of capture and fold, cohesin processively enlarges the looped DNA. For cohesin to extrude loops in such a fashion, the ATP hydrolysis cycle should be able to cause the unfolding of the coils, and thus drive the conformational changes required for this mechanism.

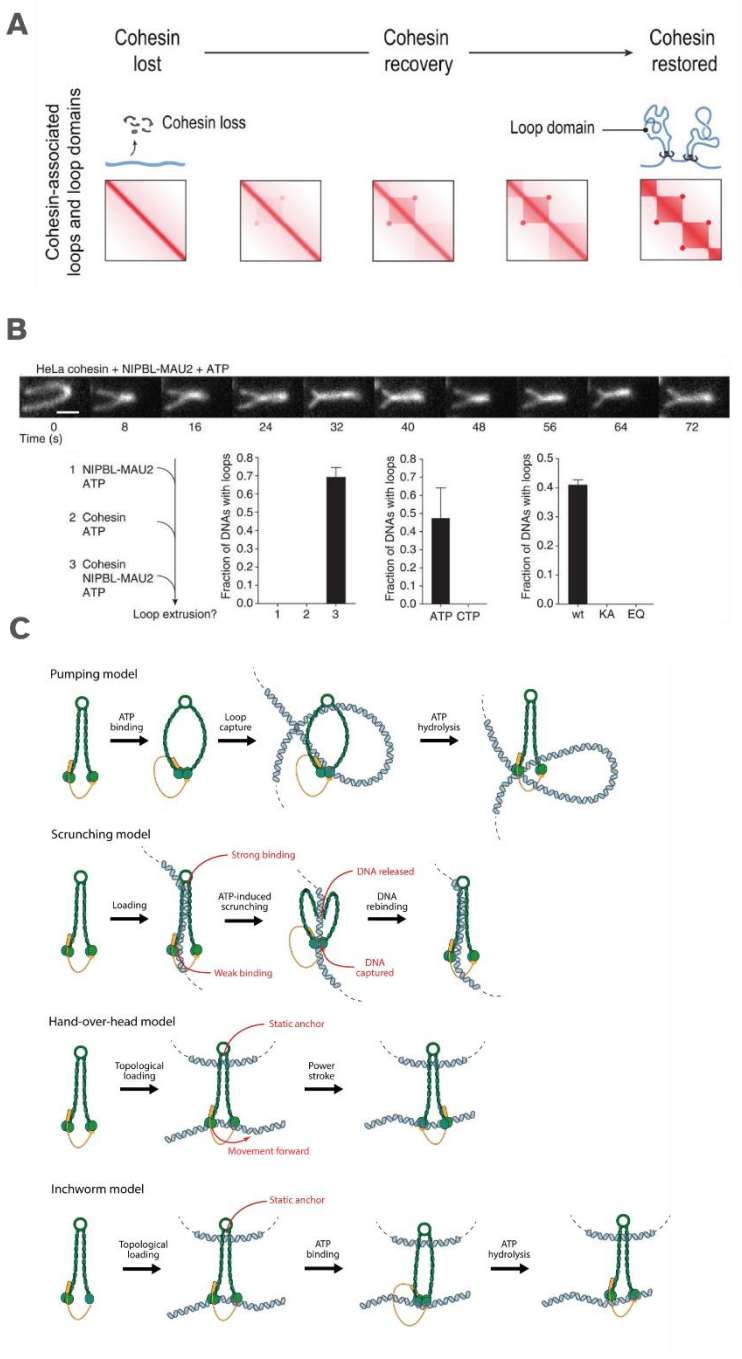
Then, the hand over head model proposes a mechanism by which cohesin captures a loop at its base in a topological manner, leading to two strands of DNA entrapped within cohesin. One of them is stably bound to the hinge forming an anchoring site and the other is transiently binding on the heads, free to move. Assuming a unidirectional movement of cohesin towards the base of the loop, the DNA would continuously enter the looped region.

Finally, a variation of the hand over head model, named the inchworm model, proposes the same entrapped loop with the anchoring on the hinge, only in this case the head binding site, does not translocate on the DNA, but actively pushes the DNA in the loop using a power stroke driven by ATP binding and hydrolysis (Hassler, Shaltiel and Haering, 2018; Sedeño Cacciatore and Rowland, 2019; Yatskevich, Rhodes and Nasmyth, 2019; Datta, Lecomte and Haering, 2020).

The last two models rely on a unidirectional mechanism, to ensure the enlargement of the loop rather than a back and forth. However, the *in vitro* experiments clearly described cohesin mediated loop extrusion as symmetrical. An explanation for this contradiction could be a pair of cohesin molecules working unidirectionally but towards opposite directions. In this case the loop will be enlarged in a symmetrical fashion while each cohesin moves or strikes towards one direction. It is worth mentioning that both single molecule loop extrusion experiments attempted to quantify the number of cohesin molecules at the extrusion site, but came up with contradicting results, with one of them identifying a single molecule, where the other found two.

Finally, an *in vitro* study most recently showed that loop extrusion by cohesin can take place even in the presence of barriers 5 times the size of the cohesin complex, excluding any mechanism that assumed either topological or pseudo

topological interactions with DNA. Hence, the only model that remains possible from the ones proposed so far, is the scrunching model where the cohesin can extrude loops by merely associating with the DNA (Pradhan *et al.*, 2021).



Introduction Figure 8
(**A**) Cohesin depletion experiment. TADs are restored apon cohesin recovery. (Rao et al., 2017)
(**B**) *In vitro* loop extrusion by the cohesin complex (Davidson et al., 2019)
(**C**) Proposed models for the mechanism of loop extrusion by cohesin (Yatskevich et al., 2019)

Thesis objective

Several mechanisms have been proposed to explain cohesin's ability to extrude loops. For all of them, the coils need to either unfold, unzip or both in an ATP hydrolysis cycle driven manner, in order to extrude and enlarge loops. In this work, I am aiming to determine whether unzipping up and unfolding are caused by the ATP hydrolysis cycle of cohesin in order to allow it to perform functions such as loop extrusion. I am planning to use structural techniques and Cysteine crosslinking to measure the folded and the zipped up state, in conjunction with ATPase activity measurements. I am asking whether the clamped state of the complex is mutually exclusive with the folded and zipped-up conformations that are present in the APO state. Furthermore, I am aiming to determine the role of such rigid conformation that the complex assumes in the APO state especially with regards with regulating the ATPase activity of cohesin. Answering these questions would allow for a better understanding of the ATPase cycle of cohesin and can elucidate the functional importance of ATPase driven conformational changes for the Cohesin complex.

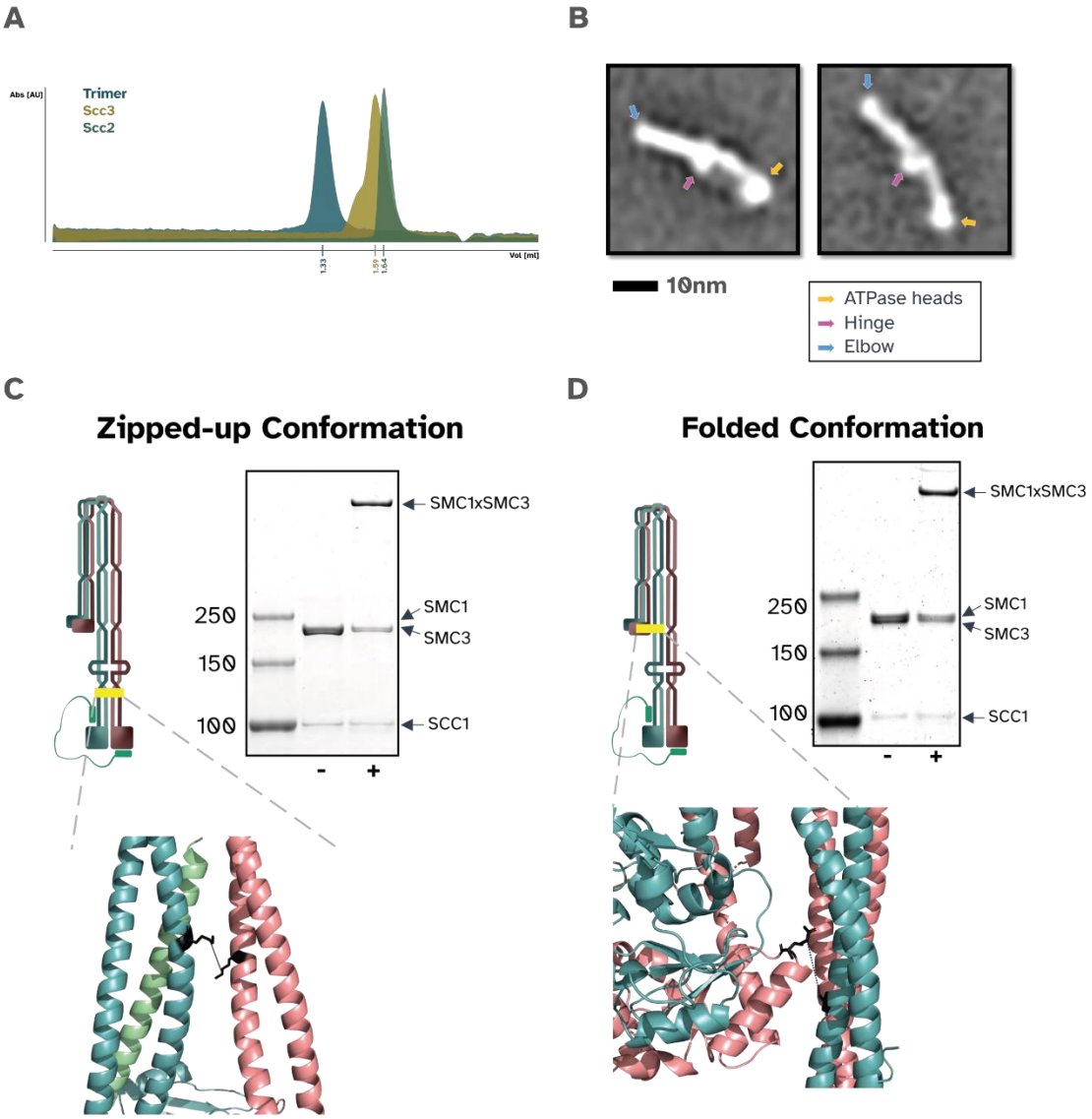
Chapter II: Cohesin's conformational states change during hydrolysis.

The native conformational states of the cohesin trimer

Structural and biochemical studies have shown that cohesin adopts a zipped up and folded conformation. The experiments directly showing this were negative stain electron microscopy experiments where cohesin trimers were treated with BS3, a chemical crosslinker that fixed the molecules at one conformational state (Bürmann *et al.*, 2019; Gutierrez-Escribano *et al.*, 2019). Due to the nature of the treatment, even though it still indicated a common conformational state of cohesin, one could not conclude that this was the native state of the complex and not an artificial outcome of the crosslinking. To address this, I expressed and purified cohesin trimers from insect cells and imaged them in their native state using negative staining Electron microscopy. The resulting images were sorted manually with approximately 1000 particles picked for analysis and 2D classification using the EMAN2 software. As shown in the selected 2D class images, the cohesin trimer indeed adopts a folded and zipped up conformation in its native state, without the need of a crosslinker to stabilize it (Figure 1a, b).

To further demonstrate the native conformational state of cohesin, while being able to discriminate between the zipped up and the folded state, I used cysteine crosslinking *in vitro*. By mutating a pair of residues located on either side of the interaction to cysteines I could induce covalent crosslinking in solution using a bifunctional molecule named BMOE. The crosslinked SMCs would remain covalently linked during SDS-Page electrophoresis, appearing as a high molecular weight band that can be quantified to assess the efficiency of the reaction. To assay the zipped-up conformation I mutated a pair of residues located near the joint region on each coiled coil, Smc3 K198C and Smc1 K201C (Chapard *et al.*, 2019a). For the folded state, I used a cysteine on the Smc1 hinge paired with a cysteine on the Smc3 ascending coil, Smc3 V933C and Smc1 R578C (Petela *et al.*, 2021).

Upon introducing those mutations to separate cohesin trimers, I was able to assess the crosslinking efficiency in the native state of the complex. The results confirmed the Apo state EM observations with both pairs producing an Smc1-Smc3 crosslinked band representing >50% of the population (Figure 1c).



Results Figure 1

(**A**) Size exclusion chromatography profile of purified cohesin trimer (blue), Scc3, (yellow) and Scc2 Δ150 (green) (**B**) 2D classes of APO state WT cohesin trimers from negative stain Electron Microscopy showing the folded and zipped up conformation of the compolex. The ATPase head domain (yellow), the Hinge (magenda) and the elbow (blue) are indicated by arrows. (**C**) *In vitro* cystein crosslinking of Smc1K201C and Smc3K198C measuring the coil-coil association required for the zipped up confromation -/+ crosslinker(**D**) *In vitro* cystein crosslinking of Smc1R578C and Smc3V933C measuring the Smc3 hinge - Smc1 coil association required for the folded confromation -/+ crosslinker

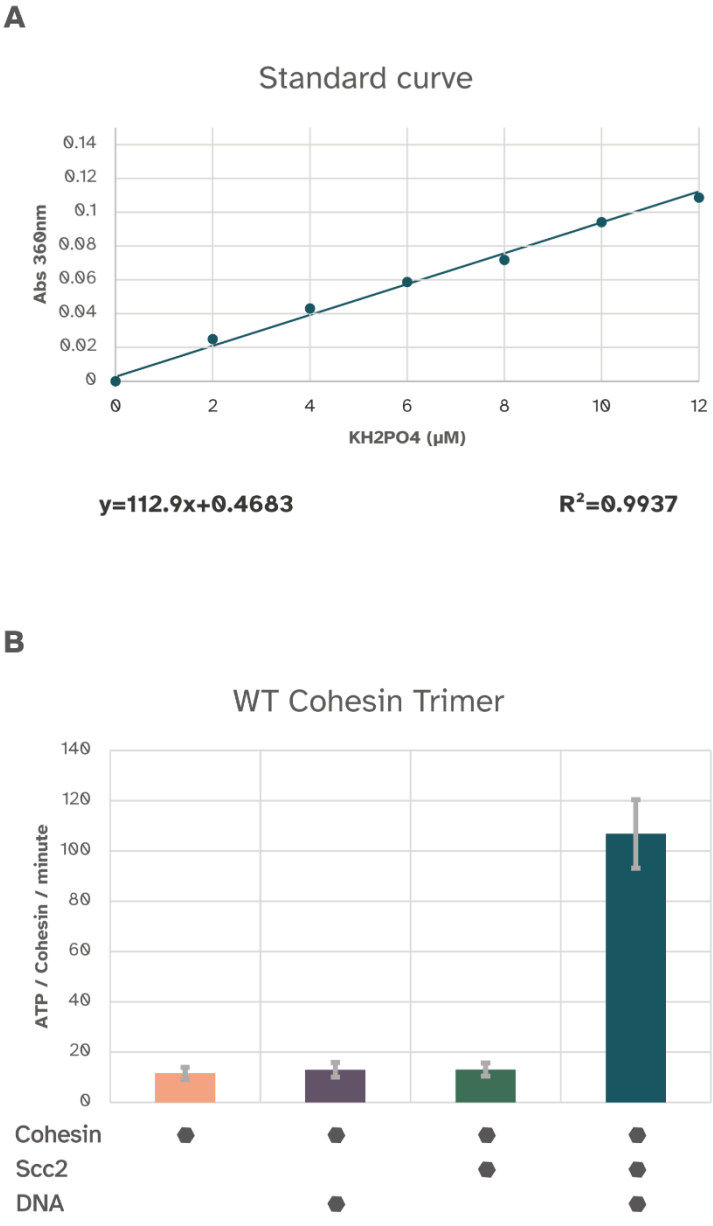
The cohesin trimer hydrolyses ATP in the presence of Scc2 and DNA

Cohesin's ATP hydrolysis has been measured in several different cases, all of which found Scc2 to be essential for the process. To be able to evaluate ATP hydrolysis myself, I expressed and purified a truncated version of Scc2 missing the N-terminal 150aa which has been shown to be sufficient for activation of ATP hydrolysis in cohesin (hereafter called Scc2). Using an assay to measure ATP hydrolysis, I confirmed that the cohesin trimer along with Scc2 and a 40bp dsDNA can hydrolyse ATP at a rate consistent with the published data in the literature (Voulgaris and Gligoris, 2019).

To acquire the hydrolysis rate, first a standard curve was attained using a serial dilution of phosphate standard along with the enzyme and substrate provided with the kit (EnzCheck, E6646) measuring absorbance at 360nM (Figure 2a).

Following that, I measured the ATP hydrolysis of the cohesin trimer in four different conditions. In accordance with previously published data, only the condition that included the trimer together with Scc2 and DNA hydrolysed ATP. The rate of hydrolysis as calculated using the standard curve was at 110 (±15) ATP molecules/cohesin/min. On the other hand, the cohesin trimer on its own,

together with only DNA or only Scc2 exhibited no measurable hydrolysis (Figure 2b).



Results Figure 2
(**A**) Standard curve generated using KH_2PO_4 as a source of inorganic phosphate. Absorbance was measured for a series of concentrations to generate the standard curve (**B**) WT cohesin trimer ATPase activity measurements following the protocol by the EnzCheck Phosphate assay kit (E-6646). ATP hydrolysis by the cohesin complex was measured in 4 different conditions.

The clamped state induces conformational changes to the cohesin trimer

The structural differences seen between the Apo state cohesin trimer and the clamped state with ATP, Scc2 and DNA indicate conformational changes disrupting the zipping up of the complex. However, due to the nature of procuring cryo-EM structure one cannot conclude whether folding is disrupted while it is clear that unzipping occurs for the formation of the clamp state. A low resolution cryo-EM structure shows cohesin still capable of adopting the folded conformation while in the clamped state. However, cryo-EM selects for the particles that present the most stable and homogeneous architecture. Hence, unfolded cohesin complexes would be excluded due to their heterogeneity. Therefore, to address the effect of the folding, I set out to evaluate the conformational state of the clamped complex in solution.

Cohesin's ABC type ATPase contains the well described Walker B motif that drives hydrolysis after ATP binding and recognition (Löwe, Cordell and van den Ent, 2001; Hopfner, 2016). Mutations on the Walker B motif can affect ATP hydrolysis by blocking cohesin in a pre-hydrolysis state, where ATP is bound in the nucleotide binding domain, but the complex is unable to hydrolyse it. One

such mutation is the E to Q mutation, which, if introduced on both SMCs, can create a hydrolysis incapable complex. As described in (Petela *et al.*, 2018a) indeed the budding yeast Smc3E1158Q / Smc1E1155Q cohesin trimer is not capable of hydrolysing ATP despite the presence of Scc2 and DNA while also causing lethality *in vivo*. However, the EQEQ cohesin can still bind ATP and subsequently assume the pre-hydrolysis state, where the ATPase heads are engaged with Scc2 and DNA bound to them(Shi *et al.*, 2020). To demonstrate this biochemically in solution, I used a pair of cysteines that can crosslink when the heads are in the engaged state (E-state) as described in (Chapard *et al.*, 2019a; Collier *et al.*, 2020a)

Using the EQEQ cohesin trimer with the E-state cysteines I compared the apo EQEQ cohesin with that in the presence of ATP, Scc2 and DNA and found that the second can successfully crosslink with ~45% efficiency. The formation of engaged heads in the presence of ATP, Scc2 and DNA confirms that the complex adopts the pre-hydrolysis state as shown in the cryo-EM structure, with the ATPase heads bound to ATP and oriented properly (Figure 3a).

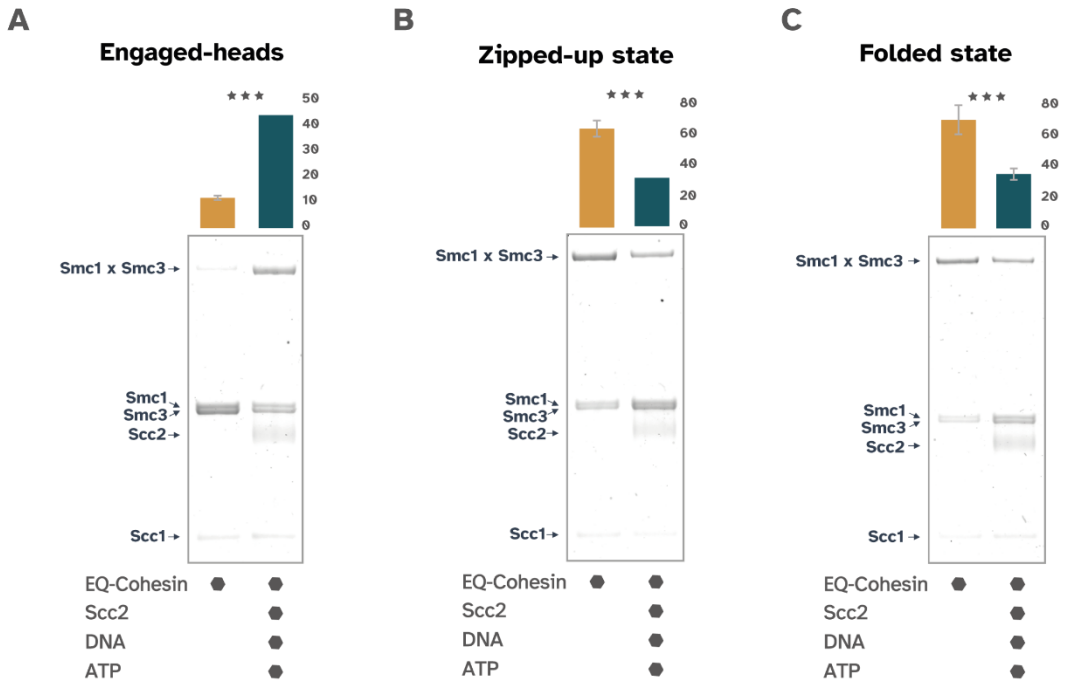
Having established the conditions where cohesin stably adopts the clamped state in solution, I moved forward to measuring the folded and zipped up conformations comparing the Apo to the pre-hydrolysis state cohesin.

The EQEQ cohesin trimer on its own crosslinked at the zipped-up state with the same efficiency measured for the wild type cohesin trimer, confirming that the EQEQ mutations are not affecting the complex's ability to zip-up in the Apo state. However, upon addition of ATP, Scc2 and DNA and the adoption of the pre-hydrolysis state, the complex exhibited a ~50% reduction in crosslinking efficiency (proportional to the E-state formation) demonstrating the effect upon cohesin's zipped-up conformation seen in the structure (Figure 3b). The same effect was observed for the folding where the adoption of the pre-hydrolysis state again caused the same level of reduction in the efficiency of crosslinking suggesting the dissociation of the hinge from the ascending coil (Figure 3c).

It is clear from the clamped state structure that the coils adopt an unzipped conformation upon the formation of the clamped state. Since the folding is equally reduced to the zipping up, I can safely conclude that the formation of the pre-hydrolysis state also results in the unfolding of the complex.

These results supplement the observations from the structural studies, further proving that cohesin is unzipped and unfolded upon the formation of the clamped state. This begs the question whether these conformational changes are required for or caused by hydrolysis. In other words, is the conformational

change necessary for cohesin to adopt the clamped state and hydrolyse ATP or is the hydrolysis cycle that leads to the conformational change.



Results Figure 3
(**A**) *In vitro* cystein crosslinking to measure the formation of the Engaged heads in the APO versus the clamp state that includes Scc2, DNA and ATP. The experiment was repeated 3 times and the quantification is shown in the bar graphs above the gels. Student t-test gave a $p<0.001$. (**B**) *In vitro* cystein crosslinking to measure the formation of the the zipped-up conformation in the APO versus the clamp state that includes Scc2, DNA and ATP. The experiment was repeated 3 times and the quantification is shown in the bar graphs above the gels. Student t-test gave a $p<0.001$. (**C**) *In vitro* cystein crosslinking to measure the formation of the the folded conformation in the APO versus the clamp state that includes Scc2, DNA and ATP. The experiment was repeated 3 times and the quantification is shown in the bar graphs above the gels. Student t-test gave a $p<0.001$.

Zipping-up of the coils inhibits ATP hydrolysis

In order to determine whether unzipping and unfolding are required for ATP hydrolysis, I developed a protocol combining the cysteine crosslinking with the ATPase assay. In this experiment, I incubated the cohesin trimer with the crosslinker, and after the formation of the covalent bond, I quenched the remaining crosslinker molecules using excess DTT. The pre-crosslinked cohesin is then added to the mix containing all the other factors necessary for the ATPase assay and activity is subsequently measured. This allowed me to determine whether the disruption of any of the conformational states is required for hydrolysis in which case the pre-crosslinked complex would be unable to hydrolyse ATP. Depending on the efficiency of crosslinking I expected a corresponding proportional reduction in ATPase activity for the complex.

As a positive control, I used a cohesin trimer with the J-state cysteines which are representative of the APO state and trap the heads in an orientation mutually exclusive to the E-state (Chapard *et al.*, 2019b). The J-state control confirmed that by crosslinking the complex, I inhibit ATP hydrolysis at a level proportional to the crosslinking efficiency (Figure 4a). On top of that, the negative control with DMSO instead of crosslinker proved that any effect on the ATPase activity

is dependent on successful crosslinking. I also observed no effect with the WT trimer lacking any crosslinking pair in the presence of BMOE, showing that the crosslinker has no adverse effects on the activity of the complex unrelated to the targeted crosslinking.

Following the validation of the assay, I ran the experiments with the cohesin trimers containing the folded and the zipped-up state cysteines respectively. Crosslinking the complex in the folded state did not affect its ATPase activity, with the crosslinked trimer hydrolysing ATP at the same level as the DMSO control (Figure 4a). On the other hand, the zipped-up state crosslinking led to a 70% drop in ATPase activity, proportional to the crosslinking efficiency (Figure 4a). All samples were visualised on an SDS-page gel to quantify crosslinking efficiency and confirm successful formation of the covalent bond (Figure 4b).

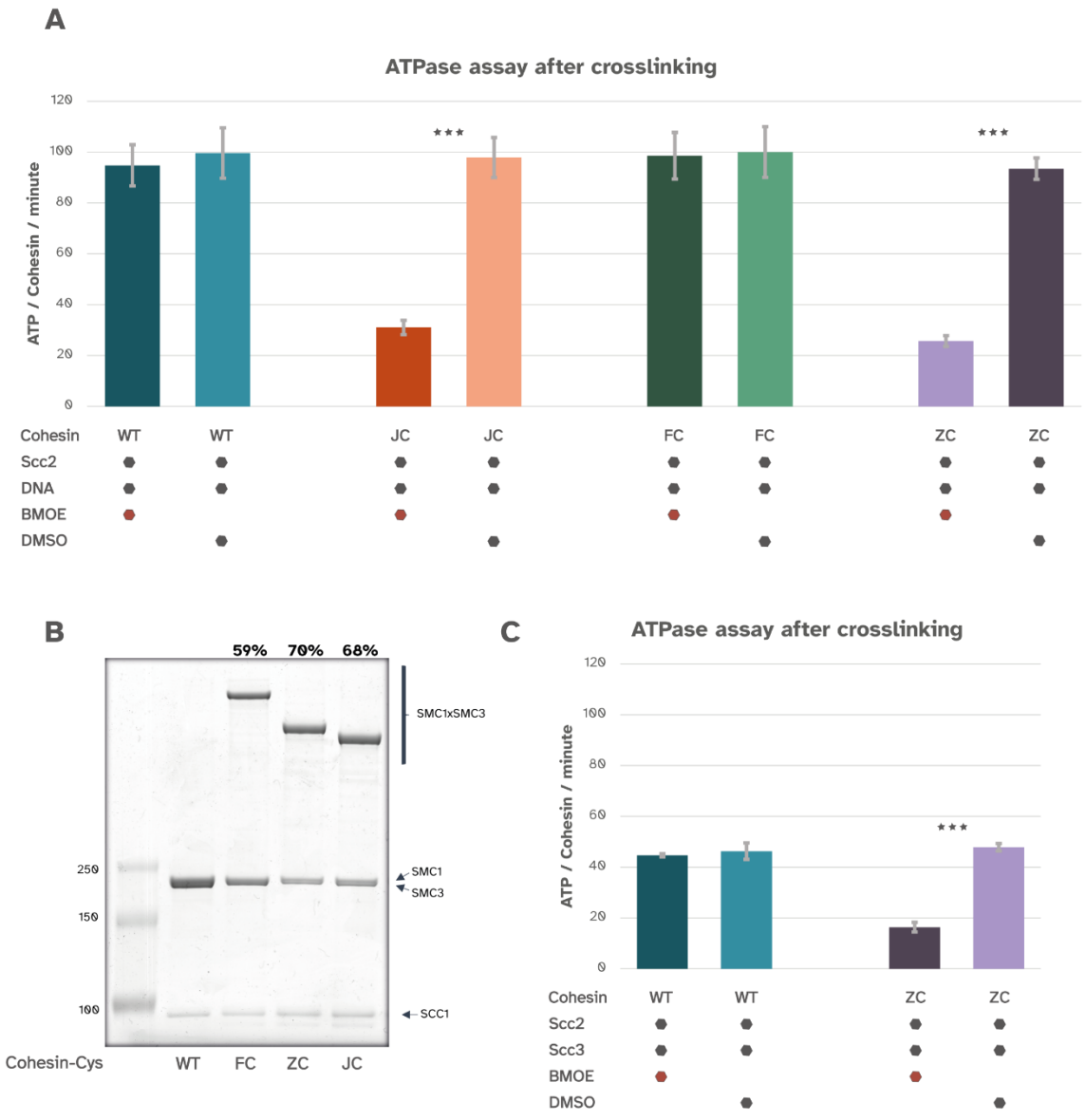
These results demonstrate that the unzipping of the coiled coils is a prerequisite for cohesin to hydrolyse ATP, while the unfolding of the complex is not. Considering that unfolding of the complex is caused by the formation of the clamped state, it only follows that unfolding is an outcome of the hydrolysis cycle of Cohesin.

DNA binding on the ATPase heads is not the reason why unzipping is necessary for hydrolysis

In the clamped state, the coiled coils emanate from the heads facing away from each other, creating a compartment large enough for DNA to enter and sit on top of the engaged heads. It is tempting to assume then, that the requirement for the unzipping of the coils is a condition to allow for enough space to accommodate DNA binding.

To address this, I measured the ATPase activity of pre-crosslinked zipped up cohesin tetramers containing Scc3. The addition of Scc3 enables cohesin to hydrolyse ATP even in the absence of DNA albeit at a lower rate (Petela *et al.*, 2018a). Hence, this allowed me to test whether the DNA independent ATPase activity is still affected by crosslinking the complex in the zipped-up state. Indeed, even in the absence of DNA, there is a reduction in activity, proportional to the efficiency of crosslinking between the coil, just as I observed for the Scc2+DNA sample. This means that the unzipping of the coils is important to allow for hydrolysis by SMC heads, but it is not directly linked to DNA binding on top of them.

In conclusion, cohesin requires the unzipping of the coils to hydrolyse ATP, a precondition unrelated to DNA binding. Unfolding of the complex is, on the other hand, only an outcome of formation of the pre-hydrolysis state.



Results Figure 4
(**A**) ATPase activity measurements after crosslinking with BMOE in comparison with DMSO controls. Cohesin trimers with cysteine pairs capturing the J-head state (JC) the folded conformation (FC) and the zipped-up confromation (ZC) were tested along with (WT). Each experiment was repeated 3 times and the student t-test gave a $p<0.001$.
(**B**) SDS-page gel showing the crosslinking efficiency of each cohesin trimer prior to ATPase measurements
(**C**) ATPase activity measurements after crosslinking with BMOE in comparison with DMSO controls. Cohesin tetramers were tested for the zipped-up crosslinking (ZC) along with (WT)

Chapter III: Disruption of the folded state, impacts cohesin's ATPase activity.

A truncated Cohesin trimer, exhibits a gain of function in its ATPase activity

My observations so far point to the unzipping of the coils being a requirement for ATP hydrolysis. However, I was unable to establish whether the zipped-up state present in the APO cohesin is indeed an inhibitory mechanism of the ATPase activity of the complex. My experiment with the pre-crosslinked cohesin, created an artificial condition where the coils are covalently held together, forming a rigid association that would not be present in WT conditions. I thereby asked whether the WT zipping-up also forms a rigid enough state to inhibit ATP hydrolysis.

To answer this question, I attempted to elucidate what the requirements for the formation of the zipped-up state are. My first hypothesis was that the association between the Smc1 and Smc3 coiled coils is sufficient to establish a zipped-up state. Since the coiled coil continuity breaks at the elbow where cohesin folds, I asked whether an engineered cohesin with coiled coils

extending up to the elbow region would be able to adopt a zipped-up state capable of inhibiting ATP hydrolysis. I therefore expressed and purified a truncated cohesin trimer, where both SMCs extend up to the elbow region with their N terminal ascending and the C terminal descending coils connected with a flexible GC linker. To evaluate the extent of the zipping up I tested the crosslinking efficiency for the zipped-up state, comparing the truncated to the full-length trimer. The results reveal that the truncated cohesin remains in an unzipped conformation (Figure 5a). The lack of zipping up in the truncated complex proves that the possible contacts between the coiled coils below the elbow are not sufficient to support the stable association between the coils, present in WT cohesin trimers.

Nevertheless, the truncated cohesin is natively unzipped, which allowed me to test the ATPase activity of the complex in the absence of this conformational state. I tested the same four conditions as with the WT. The result was a striking gain of function in the ATPase activity of the complex consistent with the premise that the zipped-up state is a mode of inhibition for hydrolysis. The truncated cohesin showed Scc2 independent activity, with the trimer capable to hydrolyse ATP on its own while DNA did not affect the rate. More importantly,

the addition of Scc2 only, allowed the complex to reach its maximal hydrolysis rate, bypassing the need for DNA (figure 5b).

This result demonstrates that cohesin's native state includes an internal constraint that is lost in the truncated construct. A constraint that originates in the zipped-up conformation of the coiled coils.

Formation of a stable zipped-up state requires the full-length coiled coils along with the proper transition between the coils and the hinge

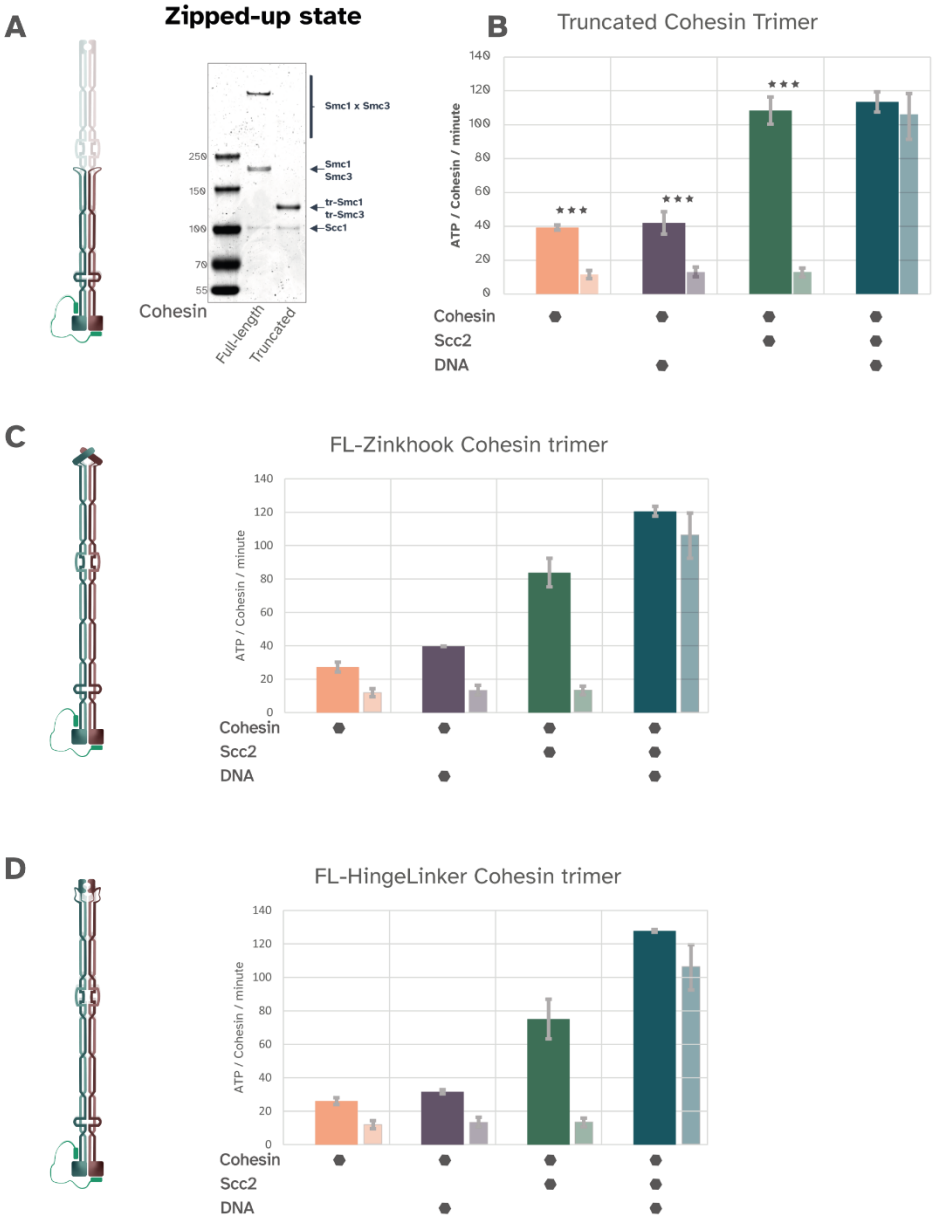
Since the truncated version of cohesin was unable to form the zipped-up conformation, I tested whether the full-length coiled coils are required instead. I wanted to discriminate between the contribution of the coiled coils and the hinge domain, so I engineered a complex that contains the full-length coils but lacks the hinge. Unfortunately, this was not possible to purify due to extensive aggregation (data not shown). I reached the same outcome with a full complex containing the hinge, but with a pair of mutations on the interphase that disrupt its dimerization function (data not shown).

Therefore, in order to evaluate the role of the full-length coils independently of the hinge, I inserted the rad50 zinc-hook domain to each SMC coiled coil instead, connected via a small Gly-Ser linker. This way, I could achieve the dimerization necessary for the purification of the full-length coils but still omit the hinge domain to test merely the contribution of the coiled coils. I measured the ATPase activity of the full length-zinc-hook complex and determined that it can still hydrolyse in the absence of DNA and partially in the absence of Scc2. The hydrolysis levels reached by the full length-zinc-hook complex were slightly lower than those of the truncated cohesin, indicating only a small part of the WT inhibition being present (Figure 5c). Hence, even the full length of the coiled coils is not enough to establish sufficient zipping up to regulate the ATPase activity.

Since the sum of the contacts between the coils was not sufficient to establish the zipped-up conformation I turned to examining the role of the hinge domain. I intended to assess the hinge as separately from the coiled coils as possible, so I introduced a long GC linker between the hinge and the coils, allowing the hinge to be flexible and capable to form interactions while still disrupting the continuity with the coils. The cohesin trimer with the hinge linker also hydrolysed ATP in the absence of DNA and partially in the absence of Scc2 at levels similar

to the full-length zinc-hook complex (Figure 5d). Hence the hinge domain itself is also not sufficient to restore the zipped-up state inhibition.

I consequently conclude that the formation of the rigid zipped up state that regulates the cohesin ATPase requires the full coiled coils, the hinge dimerization domain and it is imperative that they are connected in the proper way. This raised the question, why does the hinge need to interact with the emanating coils in a particular manner in order to support the zipping up of the complex?



Results Figure 5

(**A**) SDS-page gel showing the zipped-up crosslinking efficiency of the truncated cohesin versus WT. (**B**) ATPase activity measurements for the truncated cohesin complex. The opaque bars represent the WT rates for comparison. Each experiment was repeated 3 times and the student t-test gave a $p<0.001$. (**C**) ATPase activity measurements for the full-length cohesin with the zinc-hook domain replacing the hinge. The opaque bars represent the WT rates for comparison. Each experiment was repeated 3 times and the student t-test gave a $p<0.001$. (**D**) ATPase activity measurements for the full-length cohesin with a GS linker between the hinge and the coiled-coils. The opaque bars represent the WT rates for comparison. Each experiment was repeated 3 times and the student t-test gave a $p<0.001$.

Charge mutations at the coiled coils proximal to the hinge abolish DNA binding

In all the engineered complexes I tested, the transition region between the coiled coils and the hinge was either absent or disrupted by a linker. Hence, I focused our attention to determining the importance of that region.

First, I examined the cryo-EM structure of cohesin and determined that the transition region between the hinge and the coils is precisely the part of the hinge domain that forms the interaction with the ascending coils thereby leading to the folded state (Figure 6a). Potential disruptions at that region could prohibit cohesin from forming a stable folded state. A possible explanation of the effect I see in the ATPase regulation then would be that lack of the folded state has a downstream effect on the zipping up of the coils. In its APO folded state, the hinge associates with both Smc1 and Smc3 coils which could promote the zipped-up state by holding the two coils in proximity.

Second, it is reported in the literature that this region of the hinge is required for double stranded DNA binding. *In vitro* DNA binding experiments for both bovine cohesin and mouse condensin have found that removing the transition region from purified hinge constructs, leads to complete loss of dsDNA binding.

My findings with the truncated cohesin show that while DNA is necessary to alleviate the ATPase inhibition in WT cohesin, in the unzipped truncated cohesin DNA becomes obsolete for hydrolysis, suggesting that DNA binding plays an important part in the unzipping to overcome the inhibition. One way DNA binding promotes ATP hydrolysis could be by stabilizing the engaged state of the ATPase heads and prohibiting the coils from zipping back up. However, a second role of DNA could conceivably involve binding to a separate site to alleviate the APO state inhibition. One such possibility is the binding to the hinge transition region, whereby binding of DNA would lead to unfolding.

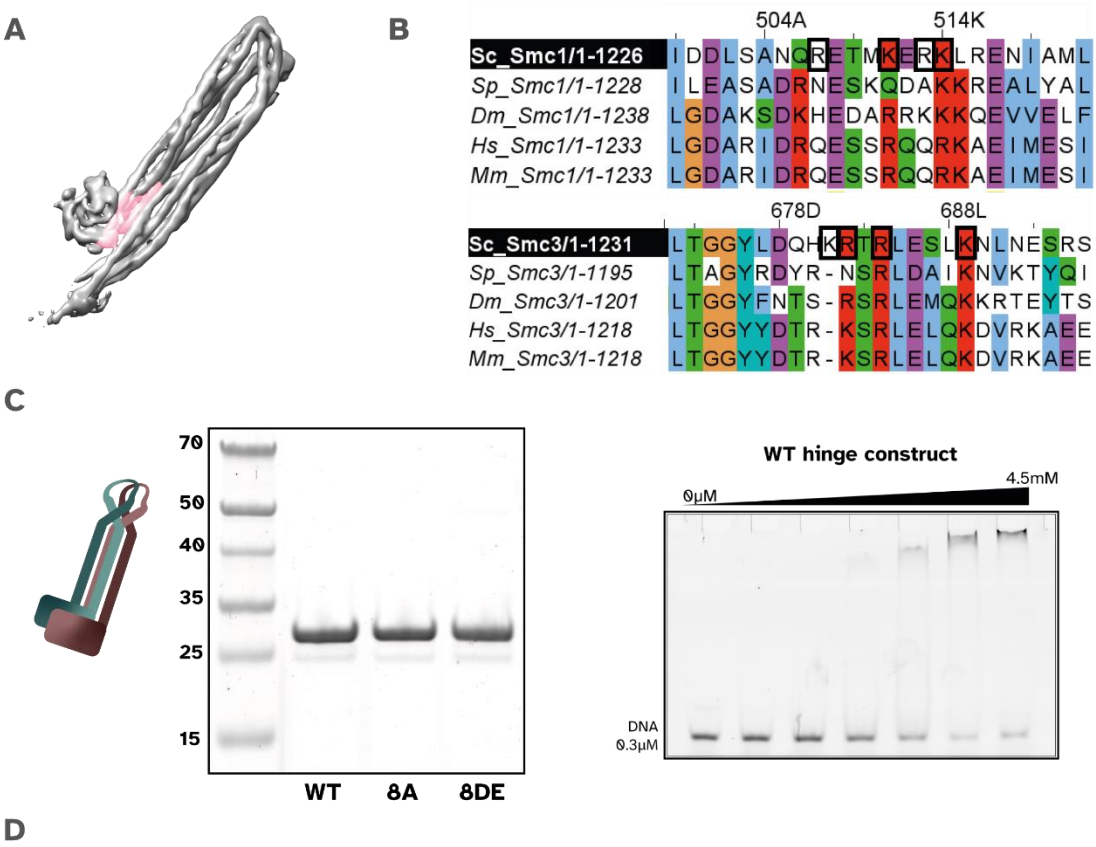
I therefore proceeded to determine the role of DNA binding to the transition region of the hinge. Does binding of DNA to this region lead to unfolding which in turn destabilizes the zipped-up state allowing for hydrolysis? Hence, I introduced mutations to affect the charge of this positively charged patch located at the transition region of the hinge.

I aligned the sequences and considered structural information for cohesin, condensin and the bacterial SMCs to identify 8 conserved residues, 4 on each SMC that could be part of the positive patch involved in folding and DNA binding (Figure 6b).

According to a study by (Vazquez Nunez, Ruiz Avila and Gruber, 2019), when the same DNA binding site in the B.s. SMC is occupied by DNA, it leads to the unzipping of the coils. Furthermore, mutating the charge on this basic patch, not only abolished DNA binding, but also led to a natively unzipped complex. The proposed mechanism was that coiled coil association is supported by electrostatic interactions between the coiled coils emanating from the hinge and DNA binding to this positive patch breaks the interaction and leads to the unzipping of the coiled coils. It is worth pointing out that the B.s. SMC complex does not have an elbow region and hence does not exhibit a folded state (Diebold-Durand *et al.*, 2017; Bürmann *et al.*, 2019). It follows therefore, that these mutations on cohesin might not affect the coiled coil association through the same mechanism. However, their position at the interaction site that mediates the folded state could indicate a similar mechanism where unfolding and potential DNA binding could lead to a conformational change affecting the zipping-up of the complex. First, I set out to test whether the positively charged region is indeed a DNA binding site and whether it is involved in the regulation of the ATPase.

To address the DNA binding question, I engineered and purified protein constructs with the isolated cohesin hinge, including the proximal coiled coils

that include the positive patch (Figure 6c). Using a labelled 50bp dsDNA, I measured DNA binding to the hinge construct with an Electrophoretic Mobility Shift Assay (EMSA). The hinge showed DNA binding as expected from the literature (Figure 6c) (Chiu, Revenkova and Jessberger, 2004; Hirano and Hirano, 2006; Griese, Witte and Hopfner, 2010; Vazquez Nunez, Ruiz Avila and Gruber, 2019). Then, I introduced 2 sets of mutations targeting the 8 positive residues, one including charge reversal mutations exchanging the Lys with Asp and the Arg with Glu (8DE) and one neutralizing the charge by exchanging the Lys and Arg with Ala (8A). Following purification of both mutated hinge constructs I measured their affinity for DNA using EMSA. Both exhibited loss of DNA binding, in contrast with the WT (Figure 6d).



Results Figure 6

(**A**) Cryo-EM structure of the APO cohesin trimer focusing on the coiled-coils above the joint including the hinge. The transition region of the hinge is indicated in red (**B**) Sequence alignments of SMC1 and SMC3 showing the conservation of the mutated residues. SMC1 R507,K511,R513,K514 and SMC3 K681,R682,R684,K689 (**C**) Purification of isolated hinge dimers and EMSA showing DNA binding. (**D**) EMSA of 8A mutant hinge and 8DE mutant hinge. Both abolish DNA binding

ATPase activity of the 8DE and 8A mutants

After confirming that the mutations abolish DNA binding, I expressed and purified full length cohesin trimers containing both sets of mutations, 8DE and 8A. According to the hypothesis, DNA binding to the transition region of the hinge is essential for the complex to overcome the inhibition and hydrolyse ATP. Since the mutants completely abolish DNA binding in that region, a cohesin trimer with those mutations would be unable to overcome the inhibition and therefore lack any ATPase activity

First, I measured the ATPase activity of the 8A mutant, which proved almost similar to WT. There is a small increase on the Trimer + Scc2 condition, albeit only marginally above WT (Figure 7a). Since its ATPase activity is like WT, despite the abolishment of DNA binding to the hinge by the 8A mutations, it is safe to conclude that DNA binding in that region of the hinge is not correlated with the ATPase activity of the complex.

The 8DE on the other hand had a more striking result. The Trimer + Scc2 condition measured ATPase activity at more than half the maximal rate, exhibiting a substantial gain of function, while in the absence of Scc2 a small

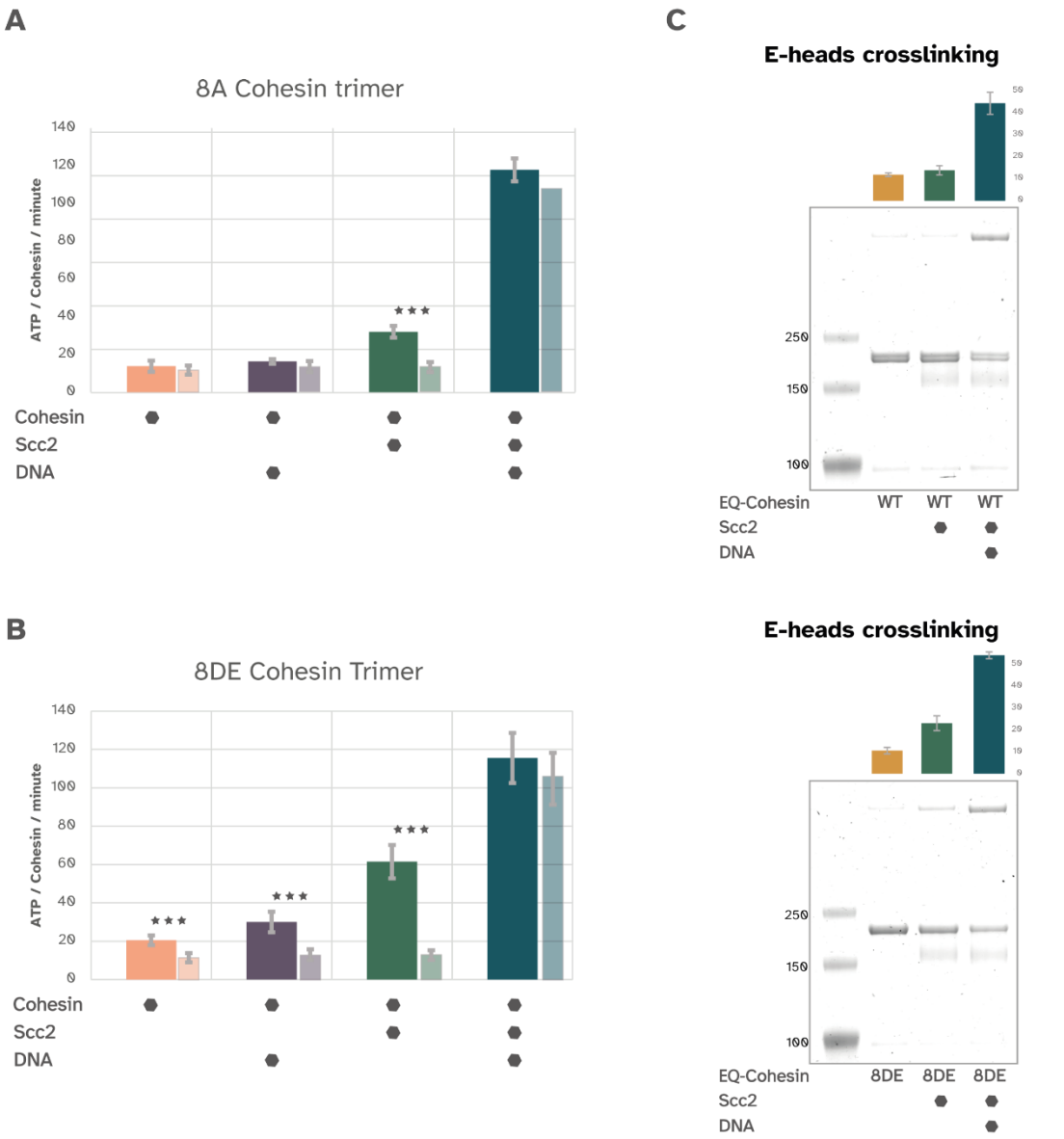
but significant amount of activity was observed (Figure 7b). This result indicates that the transition region of the hinge when mutated is affecting cohesin in a way that relieves its ATPase constraint.

One orthogonal way to confirm the striking ATPase activity result of the 8DE mutant is to evaluate the conditions and the efficiency by which it can form the pre-hydrolysis state. Same as with the WT trimer, in order to measure the pre-hydrolysis state, I introduced the EQEQ mutations to the 8DE trimer along with the E-state cysteines that can measure the head engagement indicative of the pre-hydrolysis state.

In the case of WT Cohesin, the formation of the pre-hydrolysis state requires ATP along with both Scc2 and DNA. Consistent with its ATPase activity, the Scc2 condition for the 8DE mutant showed increased efficiency at approximately 43% of the Scc2 + DNA condition (Figure 7c).

So, I showed that DNA binding in the hinge transition region is not involved in regulating the ATPase. However, the charge reversal mutations in the 8DE cohesin have a significant effect in the ATP hydrolysis regulation of the complex. How is this group of residues located at the hinge affecting the enzymatic activity of the ATPase heads?

As discussed before, this region of the hinge is involved in the formation of the folded state. Is it therefore possible that the association of the hinge with the ascending coiled coils is formed by ionic interactions that are disrupted by the charge reversal mutations? If folding is, as I proposed, involved in the stabilization of the zipped-up state, then an unfolded 8DE mutant would be defective in zipping up and by extension in regulating its ATPase activity.



Results Figure 7

(**A**) ATPase activity measurements for the 8A cohesin trimer. The opaque bars represent the WT rates for comparison. Each experiment was repeated 3 times and the student t-test gave a p<0.001. (**B**) ATPase activity measurements for the 8DE cohesin trimer. The opaque bars represent the WT rates for comparison. Each experiment was repeated 3 times and the student t-test gave a p<0.001. (**C**) *In vitro* cystein crosslinking to measure the formation of the Engaged heads in the APO, the Scc2 only and the clamp state that includes Scc2, DNA and ATP. The experiment was repeated 3 times and the quantification is shown in the bar graphs above the gels. Student t-test gave a p<0.001. WT cohesin trimers (top) and 8DE cohesin trimers (bottom) were tested.

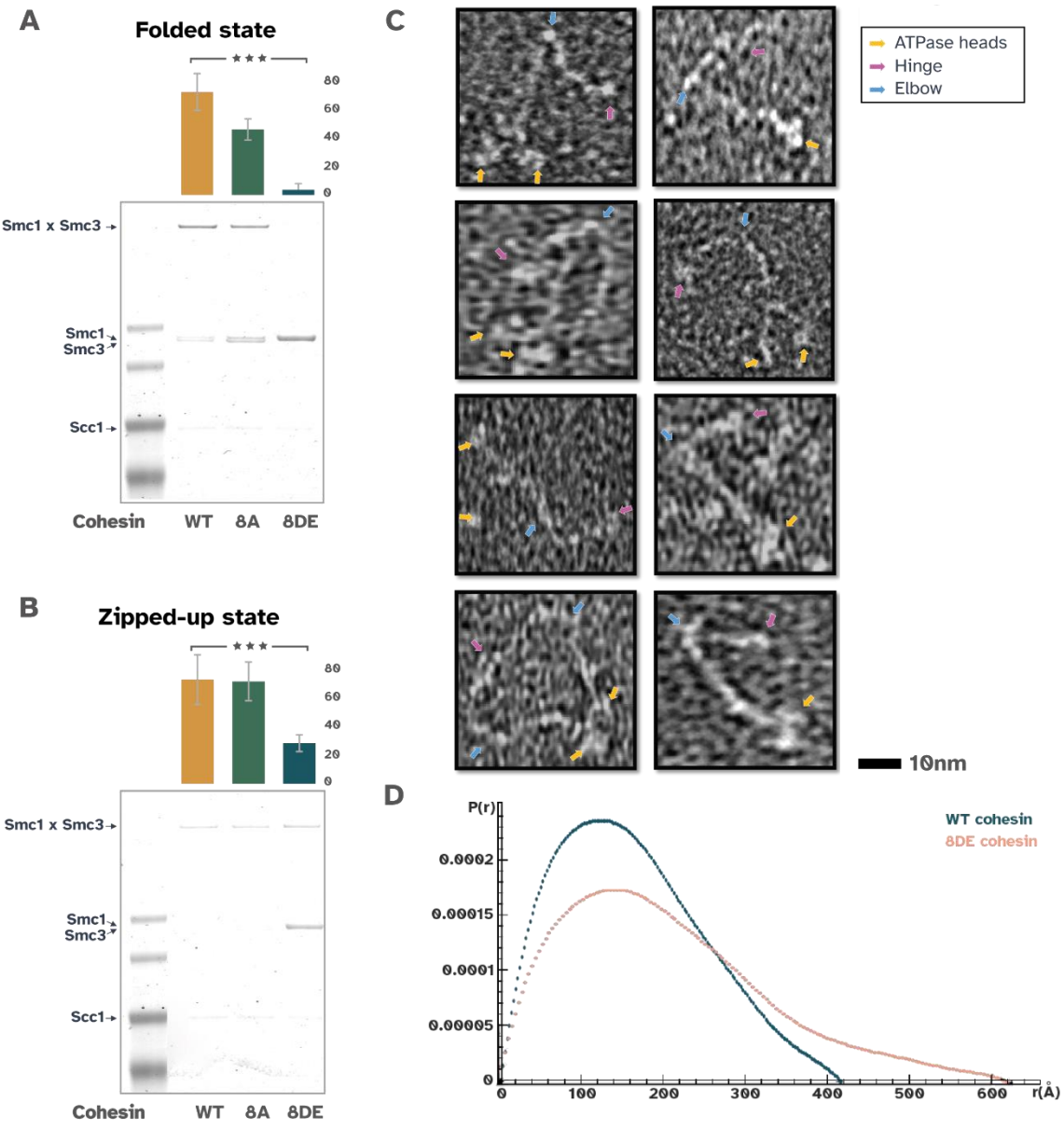
The effect of the charge mutations to the conformational states of cohesin

To address the effect of the mutations on the conformational state of the complex, I introduced the cysteine pairs previously used to measure the fold and zipped-up associations for WT and compared WT cohesin trimer with the 8A and the 8DE trimers. Both 8A and 8DE exhibit a reduction in fold crosslinking efficiency, with 8A reduced only by 35% while 8DE was reduced by 94.9% effectively abolishing folding of the complex (Figure 8a). Then, I tested whether there was any downstream effect to the zipping up of the complexes. Using the cysteine pair to measure the zipped-up state, I detected no change for the 8A mutant, but a 61.1% reduction for the 8DE (Figure 8b).

In order to confirm these results, I imaged the 8DE mutant in negative stain electron microscopy and found that it indeed almost exclusively appears unfolded, and a significant percentage of the molecules appear to have reduced or non-existent coiled coil association (Figure 7c). Analysis of the imaged molecules proved difficult due to the heterogeneity of conformations assumed by the unfolded complex, prohibiting the 2D classification.

Further confirmation regarding the folded state of the 8DE mutant was attained by SAXs, where native protein in solution was exposed to an X-ray beam, and based on the scatter profile, I could calculate the maximum dimension of the molecule. The result for the WT trimer were 42nm whereas the 8DE mutant was at 62nm, a difference consistent with unfolding of the cohesin complex (Figure 7d).

These results are in agreement with the ATPase profiles of the mutant complexes. First, the 8A is unaffected in zipping up which leads to ATP hydrolysis at rates similar to WT. Second the 8DE exhibits gain of function in its ATPase activity which agrees with the partial loss of zipping up caused by the unfolding of the complex. Furthermore, I conclude that the successful abolishment of folding in the 8DE cohesin leads to a significant effect on the zipping up of the complex eluding to a correlation between the two states of the complex. This phenotype is in agreement with the notion that the folded state of cohesin is important for the support and maintenance of the zipped-up state.



Results Figure 8

(**A**) *In vitro* cystein crosslinking to measure the formation of the folded conformation in the APO state comparing WT to 8A to 8DE cohesin trimers. The experiment was repeated 3 times and the quantification is shown in the bar graphs above the gels. Student t-test gave a p<0.001. (**B**) *In vitro* cystein crosslinking to measure the formation of the the zipped-up conformation in the APO state comparing WT to 8A to 8DE cohesin trimers. The experiment was repeated 3 times and the quantification is shown in the bar graphs above the gels. Student t-test gave a p<0.001. (**C**) Manually picked particles of APO state 8DE cohesin trimers from negative stain Electron Microscopy showing the unfolded and unzipped conformation of the compolex. The ATPase head domain (yellow), the Hinge (magenda) and the elbow (blue) are indicated by arrows. (**D**) SAXs profile of WT (blue) and 8DE (salmon) cohesin trimers

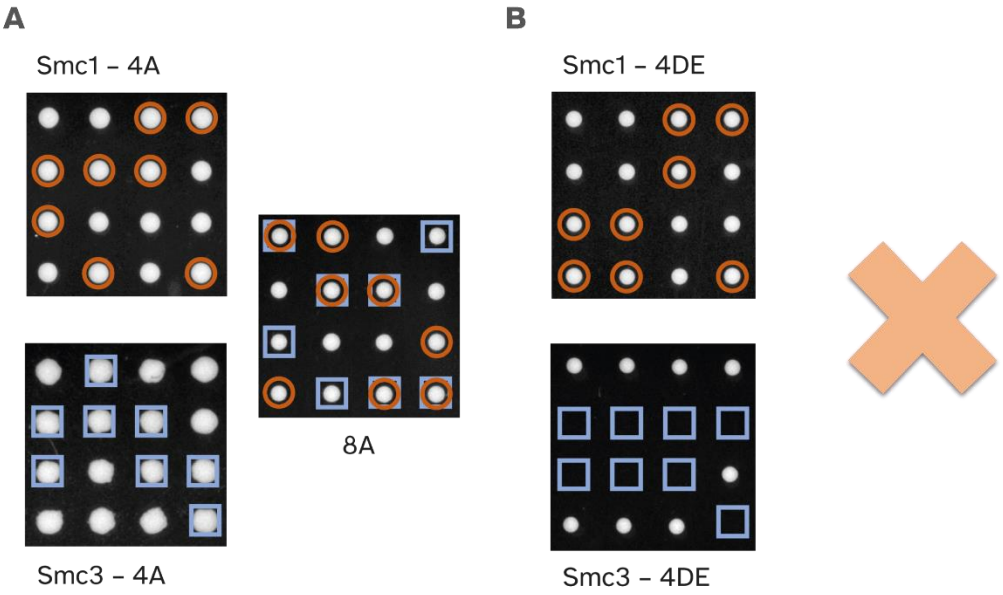
Chapter IV: *In vivo* effects of the 8DE and 8A mutants

Viability of the 8DE and 8A mutant

Both mutants, especially the 8DE, exhibited significant changes over the WT, regarding their conformational states and their enzymatic activity as ATPases. However, the effect on the ATPase activity is a gain of function raising the question of possible viability *in vivo* and effects of such a mutant in the cell.

To address this question, I first introduced the 4 mutations to each SMC on separate yeast strains, in the endogenous locus, using untagged genes. The diploid strains were dissected, and viability was evaluated on the spores.

Both SMCs were viable for the Ala mutations and their subsequent cross revealed viable spores with both mutant proteins (Figure 9a). Regarding the charge reversal mutations, they proved lethal on Smc3 while still viable for Smc1 (Figure 9b). This result indicates an essential function disrupted by these mutations.



Results Figure 9
(**A**) tetrad dissection of mutant yeast strains containing the A mutations in each SMC.
(**B**) tetrad dissection of mutant yeast strains containing the DE mutations in each SMC.

Distribution of the 8DE and 8A cohesin mutants on the chromosomes

Viability is an important marker for the effects of a mutation. However, cohesin has multiple functions and the fact that a mutation leads to lethality does not mean that the complex is not capable of some of its other functions.

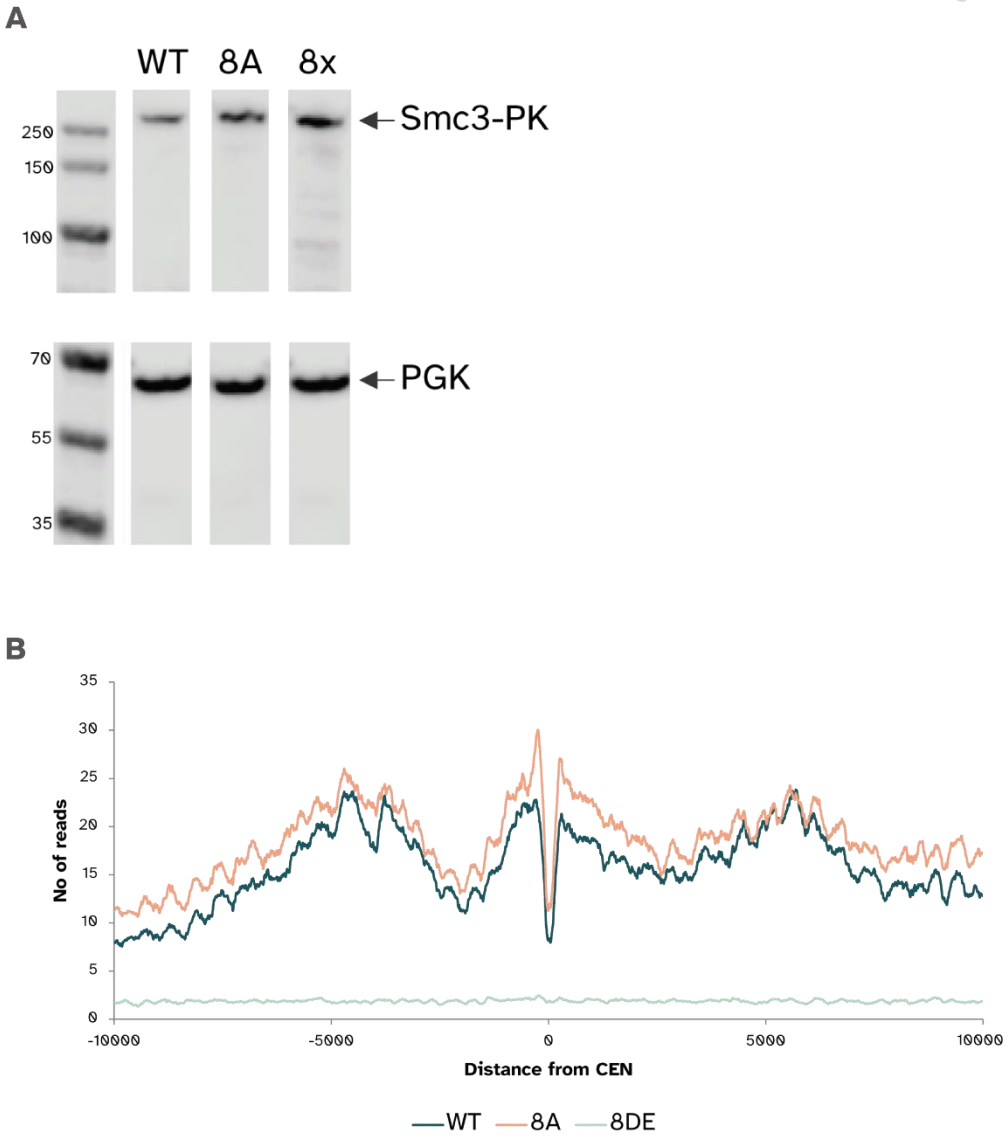
To address this, I introduced an ectopic copy of the mutant Smc3 gene in a distant locus under the same promoter as WT. Both Smc3 copies were expressed by the cells and while the WT protein ensures viability, the ectopic copy can perform any of its functions unhindered. The mutant Smc1 gene was expressed in the endogenous locus since it does not cause lethality. In my case, my intention was to do calibrated Chip-sequencing on the mutant cohesin. To that end, I inserted a PK tag on the ectopic copy and pulled down on cohesin through this tag, ensuring only mutant molecules will be immunoprecipitated and examined for their distribution on the genome.

Upon fixation, lysis of the cells and fragmentation of the DNA, I pulled down on the tag fused to the ectopic mutant version of Smc3, and with it the rest of the mutant cohesin to be processed. In order to ensure that no confounding variables could skew the results, I followed the exact same strategy for the WT

control and the 8A mutant despite their viability. This allowed for direct comparison of the profiles between the three samples.

Finally, before running the experiment, each strain was examined (WT, 8A, 8DE) to measure protein expression levels by western blotting using an antibody against the tag fused to the ectopically expressed protein. All strains illustrated measurable levels of protein expression equal with each other (Figure 10a).

Calibrated Chip-Sequencing results were obtained, showing similar levels of cohesin's genomic occupancy, between WT and 8A, while the 8DE mutant failed to associate with the DNA in any way (Figure 10b). This result is alluding to an essential disruption on the function of the 8DE cohesin despite retaining its ability to hydrolyse ATP, even more readily than WT. Furthermore, the distribution of the 8A cohesin constitutes proof that this DNA binding site is not essential for cohesin's function.



Results Figure 10
(**A**) Western-blot anti-pK tag showing the expression levels of ectopicly expressed Smc3. PGK expression for refference. (**B**) Calibrated chip sequencing of WT versus 8A versus 8DE cohesin. The graph shows the average reads of all the chromosomes.

Chapter V: Summary

The results of this work provide novel insights in cohesin's conformational states and address important regulatory mechanisms for its ATPase activity.

1. Using negative stain electron microscopy and *in vitro* cysteine crosslinking, I am demonstrating that in its APO state, the cohesin complex assumes a folded and zipped up conformation.

2. I am showing that the formation of the Scc2-ATP-DNA dependent pre-hydrolysis state confers conformational changes to the complex, including the unzipping of the coiled coils and their unfolding.

3. The ATPase assay measuring the activity of pre-crosslinked complexes conclusively shows that the unzipping of the coils is required for ATP hydrolysis, while the covalently folded complex can still hydrolyse at WT levels.

4. My results demonstrate that the DNA independent hydrolysis is equally diminished by the pre-crosslinking of the complex at the zipped-up state as it is for the DNA dependent one.

5. The truncated cohesin lacks the ability to adopt the zipped-up conformational state and presents with a gain of function in its ATPase activity, bypassing the

need for DNA and even hydrolysing in the absence of Scc2. This result alludes to an internal constraint, present in the WT cohesin trimer that has been lost in the truncated version.

6. The lack of WT inhibition for both the zinc-hook and the hinge-linker engineered cohesin trimers indicates that the zipping up requires the proper transition between the hinge domain and the coils emanating from it.

7. I discovered a group of 8 positively charged residues in the coiled coils emanating from the hinge, that are responsible for DNA binding. Both the 8DE mutant (charge reversal) and the 8A mutant (charge neutralization) abolish DNA binding to the hinge

8. The 8DE mutations abolish folding while severely disrupting the zipping up. The 8A mutant exhibits only a small reduction in folding while it remains fully zipped up.

9. The ATPase activity of the 8DE mutant exhibits a gain of function of the same nature as the engineered cohesin trimers. This is due to its unfolded state that affects the zipping up. The less efficient zipping up of the coils allows the complex to overcome the constraint.

10. In agreement with the ATPase assay, formation of the clamped state for the 8DE mutant is partially DNA independent.

11. The 8A mutant hydrolyses ATP at rates similar to WT, with only a marginal increase in the addition of only Scc2.

12. The 8DE mutant is lethal *in vivo* while the 8A is viable. Subsequent Chip-Seq analysis confirms that the 8A is distributed on the genome like WT cohesin while the 8DE does not associate with DNA.

Chapter VI: Discussion

In this work I am answering two questions regarding the role of cohesin's conformational states.

1st: Does ATP hydrolysis by the cohesin complex lead to unfolding?

2nd: Is the rigid zipped-up and folded conformation cohesin adopts in its APO state inhibitory of ATP hydrolysis?

My results demonstrate that cohesin has an internal constraint that inhibits the complex to hydrolyse ATP on its own and requires both Scc2 and DNA to be bypassed.

This ATPase activity inhibition stems from the zipping up of the coils which need to come apart for hydrolysis to occur. Given the cryo-EM structures capturing the complex with DNA residing on top of the ATPase heads, it is tempting to assume that the unzipping of the coils is necessary to allow enough space for DNA binding. However, my results showing inhibition of the ATPase activity even in the absence of DNA when the complex is crosslinked in the zipped-up state, contradict this claim. One explanation is that the

unzipping of the coils is necessary to allow for the rotation of the SMC heads and the assumption of the engaged state. DNA binding could be a subsequent step that stabilizes the head engagement by prohibiting the coils to return to the zipped-up state.

Regarding the folded state, it is clearly not directly inhibitory of ATP hydrolysis, however it appears to be disrupted by the formation of the clamped state. Since the formation of the clamped state requires the unzipping of the coiled coils and leads to the unfolding of the complex, it follows logically that unzipping might be causing the unfolding of the complex.

A mechanism through which such an event might occur can be proposed if one looks at the 8DE mutant. In the case of the 8DE mutant, the complex has lost its ability to assume the folded conformation. Even though folding is not directly inhibitory of the ATPase activity of cohesin, lack of it appears to unburden cohesin from its internal constraint mediated by the zipping up of the coils and allows it to hydrolyse in the absence of DNA. Crucially, the reduction to the zipped up conformational state of the 8DE complex can only be explained as a downstream effect of the unfolding, as the mutations introduced at the hinge are located very far from the coiled coils cysteines above the joint that are used to measure zipping-up. This downstream effect

could be a direct or indirect outcome of the dissociation of the hinge from the ascending coiled coil. The hinge in the folded conformation interacts with the ascending coils, contacting both Smc1 and Smc3. Assuming the rigid structure of the hinge interface, its association with both SMCs might lead to a direct mechanism of holding them together. Upon unfolding, the coils lose the support of the hinge keeping them together and can readily unzip. Alternatively, the folding of the complex might confer some rigidity to cohesin that is supported by the association of the hinge with the coils along with multiple contacts made by the bent part with the rest of the coil. Loss of such rigidity could make cohesin more susceptible to the unzipping of the coils.

In either case, the 8DE mutant demonstrates that folding of the cohesin complex is supporting the zipped-up state and when abolished can cause the unzipping of the coiled coils. If so, the reverse could be true as well, where the zipped-up state provides a more stable association of both the ascending coils with the hinge, ensuring a stronger association. Hence, unzipping of the coiled coils could lead to unfolding.

During the ATP hydrolysis cycle, the rotational force originating from the engaging heads is transferred to the coiled coils causing them to unzip, which in turn leads to the dissociation of the hinge from the ascending coils. This

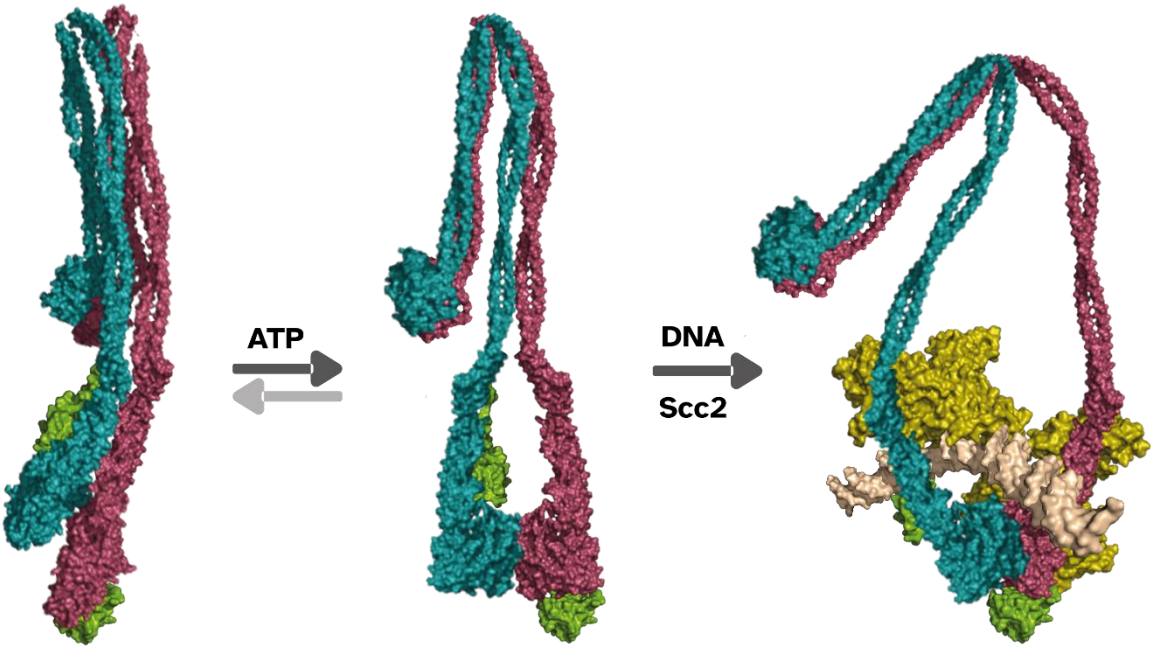
mechanism could explain the WT level hydrolysis in the covalently folded cohesin as the coiled coils can still unzip enough to allow for DNA to bind and hydrolysis to occur, despite the failure to confer a conformational change to the unbudging fold.

In conclusion, cohesin has an internal constraint prohibiting it to assume a stably engaged state and subsequently hydrolyse ATP. This constraint stems from the zipping up of the SMC coiled coils. The folded state supports the stable zipping up of the coils with unfolding leading to loss in zipped-up state. In the case of the truncated cohesin, the complete lack of zipping-up and the ATPase inhibition that comes with it, allows the complex to hydrolyse ATP on its own without the need of Scc2 or DNA. This shows that the role of Scc2 and DNA in promoting ATP hydrolysis is directly related to alleviating the inhibition stemming by the rigid conformation of the APO state, hence allowing cohesin to proceed with hydrolysis.

The model I propose to explain the role of the conformational states of cohesin is the following. Cohesin natively assumes both the folded and the zipped-up states. Binding of ATP to the NBDs of the ATPase heads can lead to a transient head engagement which is insufficient for ATP hydrolysis. The temporary head engagement and the movement of the heads is not enough to

bypass the rigidity stemming from the zipped-up state supported by the folding, forcing the heads to return to the apo state. However, upon addition of Scc2 and DNA, the head engagement is stabilized and reinforced, while the formation of the clamp state prohibits the coils from returning to the zipped-up state. This allows for persistent unzipping of the coils which in turn disrupts the folded state.

This model can provide support to the loop extrusion mechanisms that have been proposed for cohesin, that include unfolding of the complex. It also provides an explanation to the ATP hydrolysis requirement for such loop extrusion as it connects ATP hydrolysis to the unfolding. Finally, and most importantly, this claim is testable with the tools provided by this work. Namely, using the pre-crosslinked cohesin that can hydrolyse ATP, one can test loop extrusion *in vitro* and demonstrate weather cohesin can extrude loops while covalently folded. This would conclusively support or disprove the loop extrusion models that include the folding and unfolding of the complex as a part of Cohesin's mechanism of reeling in DNA.



Discussion Figure 1
Model proposed for ATP hydrolysis driven conformational changes of cohesin. Apo cohesin is zipped-up and folded. ATP leads to transient head engagement unable to unzip and unfold the complex. Addition of Scc2 and DNA, stabilizes the engegeed state and leads to stable unzipping of the coils, which in turn leads to dissociation of the hinge from the ascending coil breaking the fold.

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Chapter VIII: Materials and Methods

Recombinant yeast cohesin complex cloning

The S. cerevisiae cohesin genes were codon optimized for SF9 cell expression and synthesized using Thermo Fisher's Genescript service. Vectors from the multibac expression protocol were used for cloning. Smc1, 8His-Smc3, 6His-Scc2(139-1493)-2xStrepII, 2xStrepII-Scc3 and variations thereof were cloned in the pAceBac vector, while Scc1-2xStrepII was cloned in the piDC vector. pAceBac plasmids were amplified in Top10 cells while piDC plasmids required Pir+ cells. 8His-Smc3 and Smc1 were both inserted in one pAceBac vector and expressed together. Any truncations, mutations or domain insertions for constructs were done using the Gibson assembly protocol. A GS linker was used for the truncated complexes. Plasmids containing all 3 cohesin trimer proteins were made by cre recombination of the pAceBac plasmid containing both SMCs and the piDC containing Scc1.

Baculovirus generation

Vectors containing the genes of interest were transformed into DH10 cells where transposition into the bacmid was confirmed by blue white screening

and antibiotic selection. Following lysis of the cells, isopropanol and ethanol precipitation was used for the isolation of the bacmid at >2µg/ml concentration. 2µg of bacmid were transfected to 2ml of SF9 cells at 0.5 x 10^6/ ml concentration using the FugeneHD reagent. The cells were grown in SF900II SFM at 27°C in 6 well plates for 3 days. Upon visual examination and confirmation of viral infection and ceasing of cell growth, the cells were span at 2000g. The P1 virus was collected as the supernatant. Amplification for P2 virus was achieved by infecting 50ml of SF9 cells at 2x10^6/ml concentration with 500µl of P1. P2 was collected after 3 days upon confirmation of infection.

Protein Expression

For the expression of the protein of interest, 500ml of SF9 cells at 2x10^6/ml concentration were infected with 5ml of P2 virus. The cells were incubated at 27oC in suspension shaking at 120rpm for 2 days. After 2 days the culture was sampled and evaluated for viability and expression levels. Upon confirmation of protein expression the pellet was collected by centrifugation at 2000g for 15min washed ones with PBS buffer and then flash frozen with liquid nitrogen. The pellet was stored at -80°C.

Protein purification

Cell pellets were thawed at 4oC and suspended in 100ml buffer A (50mM Hepes pH 7.5, 150mM NaCl, 1mM TCEP, 5% Glycerol) supplemented with 1 complete EDTA free Protease inhibitor tablet, and 100units of Supernuclease. Lysis was achieved via sonication using the Vibra Cell Sonicator (VCX130PB-1) for 2 minutes (10 sec intervals) at 80% amplitude. 1mM PMSF was added after sonication and the lysed cells were incubated for 15min on ice. Finally the solution was centrifuged at 50.000g in 4^{o}C to pellet any cell debris while the supernatant was collected and passed through a sterile 0.8μM pore filter.

The protein of interest was then purified following a 3-step protocol, including Affinity chromatography, Anion exchange chromatography and Size exclusion chromatography. Affinity pulldown was performed using the 5ml Strep-Trap HP column, connected to an Akta Explorer apparatus. The column was washed with Buffer A and eluted with 15ml buffer A supplemented with 5mM d-desthiobiotin. The elution was then diluted to 30ml using Buffer B (50mM Hepes pH 7.5, 100mM NaCl, 1mM TCEP, 5% Glycerol) and was then loaded to the HiTrap Q HP anion exchange column. Elution was done across a gradient of 100-1000mM NaCl. Finally size exclusion chromatography was used to

select against any aggregates and the protein was collected and flash frozen in liquid nitrogen. In the case of the isolated hinge constructs, Hi-Trap Talon column was used to pull down on the 8His tag on Smc3 and the ion exchange step was omitted due to the high isoelectric point.

DNA oligo duplex

dsDNA was created by annealing a pair of complementary 40bp oligos (GAATTCGGTGCGCATAATGTATATTATGTTAAATAAGCTT , AAGCTTATTT AACATAATATACATTATGCGCACCGAATTC). The 100mM oligos were mixed at 45µl each, with 10µl NEB buffer 2. The annealing reaction was done in a thermocycler, first heating the sample at 95°C, and then gradually decreasing the temperature to 10°C in 0.1°C increments. The final concentration of the dsDNA was 45mM.

ATPase assay

ATPase activity was measured using the Enz-Chek phosphate assay kit as described in (Voulgaris and Gligoris, 2019).

Negative staining Electron microscopy

Cohesin trimers were applied to Carbon support film 300 mesh 3 mm grids, at 0.1mg/ml concentration and fixed using Uranyl Formate. The grids were imaged with a FEI Tecnai T12 Transmission Electron Microscope at the bioimaging facility in the Dunn school of pathology.

Protein Crosslinking Assay

Cohesin trimers with Cysteine pairs were incubated at 150nM concentration with oligo duplex DNA at 450nM, ATP at 10nM and Scc2 at 150nM. The reactions were incubated for 5 minutes in room temperature. BMOE was then added to a final concentration of 1mM and the reaction was incubated for 5 minutes on ice. The sample was then denaturated with 4xLDS buffer and boiled at 95°C for 5 minutes. The results were visualized at 3-8% Tris-Acetate gels stained with Quick Coomassie stain.

Sequence alignment

Protein sequences were aligned using the Clustal Omega tool in the EMBL-EBI website and visualized using the Jalview software.

ElectroMobility Shift Assay (EMSA)

An HPLC purified oligo labelled with FAM was annealed as described for the DNA oligo duplex. The labelled dsDNA was then added at 0.3μM concentration along with increasing concentrations of protein to a final volume of 10μl. The reaction was incubated for 30 minutes on ice in the dark and visualised at 0.5%TAE - acrylamide native gels. Images were acquired with a Fujifilm FLA7000 scanner using the LD473/Y[520] filter.

Small Angle Xray Scattering

Cohesin trimers were concentrated to 5mg/ml. For the experiment, a series of dilutions were prepared halving the concentration at each step. Imaging and initial analysis was performed at the Diamond facility on the B21 beamline.

Calbibrated Chip-Sequencing

Calibrated Chip-Sequencing experiments were done according to Petela et al., 2018.

E.coli Strains

DH10Bac	F⁻mcrA(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *ga*/U *ga*/K λ⁻*rps*L
Pir+	F⁻Δlac169 rpoS(Am) robA1 creC510 hsdR514 endA recA1 uidA(ΔMlul)::pir-116
TOP10	F⁻mcrA(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *ga*/U *ga*/K *rps*L (StrR) *nup*G

Other strains

Sf9	ThermoFisher Cat# 11496015

Plasmid List

pAceBac1 Smc1 8His-Smc3

pAceBac1 Scc2(139-1493)-2xStrepII

pAceBac1 2xStrepII-Scc3

piDC Scc1-2xStrepII

pAceBac1 Smc1 K201C 8His-Smc3 K198C

pAceBac1 Smc1 R578C 8His-Smc3 V933C

pAceBac1 Smc1 E1158Q / K201C 8His-Smc3 E1155Q / K198C

pAceBac1 Smc1 E1158Q / R578C 8His-Smc3 E1155Q / V933C

pAceBac1 Smc1 E1158Q / N1192C 8His-Smc3 E1155Q / R1222C

pAceBac1 Smc1 E1158Q / S161C 8His-Smc3 E1155Q / K160C

pAceBac1 Smc1 1-410aa / GSGSGSG-linker / 760-1226aa 8His-Smc3 1-430aa / GSGSGSG-linker / 760-1230aa

pAceBac1 Smc1 1-410aa K201C / GSGSGSG-linker / 760-1226aa 8His-Smc3 1-430aa K198C / GSGSGSG-linker / 760-1230aa

pAceBac1 Smc1 1-410aa, SGSGSGSSGSG, ZincHook, SGSGSGSSGSG, 760-1226aa 8His-Smc3 1-429aa, SGSGSGSSGSG, ZincHook, SGSGSGSSGSG, 656-1231aa

pAceBac1 Smc1 1-499aa, SGSGSGSSGSG, 500-696aa, SGSGSGSSGSG, 697-1226aa 8His-Smc3 1-506aa, SGSGSGSSGSG, 507-684aa, SGSGSGSSGSG, 685-1231aa

pAceBac1 Smc1480-710 8His-Smc3 6H-3C-492-720

pAceBac1 Smc1480-710 R507E / K511D / R513E / K514D 8His-Smc3 6H-3C-492-720 K681D / R682E / R684E / K689D

pAceBac1 Smc1480-710 R507A / K511A / R513A / K514A 8His-Smc3 6H-3C-492-720 K681A / R682A / R684A / K689A

pAceBac1 Smc1 R507E / K511D / R513E / K514D 8His-Smc3 K681D / R682E / R684E / K689D

pAceBac1 Smc1 R507A / K511A / R513A / K514A 8His-Smc3 K681A / R682A / R684A / K689A

pAceBac1 Smc1 E1158Q / N1192C / R507E / K511D / R513E, K514D 8His-Smc3 E1155Q / R1222C / K681D / R682E / R684E / K689D

pAceBac1 Smc1 R578C / R507E / K511D / R513E / K514D 8His-Smc3 V933C / K681D / R682E / R684E / K689D

pAceBac1 Smc1 R578C / R507A / K511A / R513A / K514A 8His-Smc3 V933C / K681A / R682A / R684A / K689A

Reagents

Name	Company	Identifier
Cre recombinase	NEB	Cat#M0298S
Supernuclease	SinoBiological	Cat#SSNP01
Enzcheck kit	Invitrogen	Cat#E6646
Gibson Assembly mix	NEB	Cat#E2611L
HiTrap Q HP	GE healthcare Fisher	Cat#GE29-0513-25
StrepTrap HP	Fisher Scientific	Cat#11540654
HiTrap TALON	GE healthcare Fisher	Cat#GE28-9537-67
Superose 6 increase 10/300GL	VWR	Cat#29-0915-96
Superdex 200 Highload 16/60	GE Healthcare	Cat#GE28-9893-35
Carbon support film 300 mesh 3 mm grids	TAB	C267/050
Acetic acid	Sigma	A6283-100ML
ATP	Sigma	Cat#11140965001
BMOE	ThermoFisher	Cat#22323
EDTA free protase inhibitor tablets	Roche	Cat#4693132001
d-desthiobiotin	Fisher Scientific	Cat#12753064
EDTA	ThermoFisher	Cat#17892
FuGeneHD	Promega	Cat#E2311
Glycerol	ThermoFisher	Cat#17904
HEPES	ThermoFisher	Cat#11344041
4xLDS	ThermoFisher	Cat#NP0007
NaCl	Fisher Scientific	Cat#S/3160/60
nuPage 3-8% Tris Acetate Protein Gels	ThermoFisher	Cat#EA0378BOX
PMSF	Fisher Scientific	Cat#36978
Quick Coomassie Stain	Generon	Cat#GEN-QC-STAIN
Sf900SFM	ThermoFisher	Cat#10902104
TCEP	ThermoFisher	Cat#20490
Tris Base	Fisher Scientific	Cat#BP152-500