

A single-molecule approach to understanding sister chromatid cohesion establishment

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Declaration

I Samson Glaser confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Sister chromatid cohesion is established by the cohesin complex during DNA replication in S phase. This process requires cohesin to transition from binding to one DNA strand to co-entrapping the two newly synthesised sister chromatids. Passage of the eukaryotic replisome through the ring-shaped cohesin complex has been proposed as a fail-safe mechanism to ensure that cohesin entraps the two sister chromatids, enabling their faithful segregation during cell division. However, whether replisomes can indeed pass through cohesin remains unknown, and the details of replisome-cohesin encounters are poorly understood.

Here, I use single molecule fluorescence microscopy to directly visualise encounters between biochemically reconstituted DNA replication forks and cohesin complexes. On a linear forked DNA, I find that the translocating replicative CMG helicase acts as an obstacle to cohesin. The obstacle is overcome by cohesin at low frequencies, which increase in presence of added replisome components with known sister chromatid cohesion establishment functions. When lateral cohesin diffusion is prevented, CMG passage, during which cohesin retains topological DNA entrapment, becomes the predominant outcome. Finally, I find that the likelihood of passage also increases when an active replisome encounters cohesin, resulting in successful cohesion establishment between the two replication products. Interestingly, when cohesin's ability to diffuse on DNA is blocked, replication fork passage is efficiently achieved independently of cohesion establishment factors.

My findings resolve a long-held puzzle by visualising live sister chromatid cohesion establishment as the replisome passes the cohesin ring, providing new insights into the molecular mechanisms of sister chromatid cohesion establishment.

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Abbreviations

a.u.	Arbitrary units
AA	Amino acid
AAA+	ATPases associated with diverse cellular activities
ABC	ATP binding cassette
ACS	ARS consensus sequence
ARS	Autonomously replicating sequence
ATP	adenosine-5'-triphosphate
ATP γ S	Adenosine-5'-(γ -thio)-triphosphate
bp	Base pair
Cdc	Cell division cycle
CDK	Cyclin dependent kinase
Cdt	Chromatin licensing and DNA replication factor
Chl	Cell adhesion molecule L1 like
Chr	Chromosome
CIP	Ctf4 interacting peptide
CMG	Cdc45-Mcm2-7-GINS
cryoEM	Cryogenic electron microscopy
Csm	Chromosome segregation in meiosis
Ctf	Chromosome transmission fidelity
dA	2'- Deoxyadenosine-5'-triphosphate
Dbf	Dumbbell former
dC	2'-Deoxycytidine-5'-triphosphate
dCas9	Dead CRISPR associated protein 9
Dcc	DNA replication and sister chromatid cohesion
DDK	Dbf4-dependent kinase
DH	Double hexamer
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
Dpb	DNA Polymerase B
DSB	Double strand break

dsDNA	Double stranded DNA
dT	2'-Deoxythymidine-5'-triphosphate
Eco	Establishment of cohesion
FACT	Facilitator of chromatin transactions
Fen	Flap structure-specific endonuclease
FtsZ	Filamenting temperature-sensitive mutant Z
G1 phase	Growth 1 phase
G2 phase	Growth 2 phase
GIN5	Go-Ichi-Ni-San
GODCAT	Glucose oxidase and catalase
HEAT	Huntingtin, Elongation factor 3, protein phosphatase 2A, and yeast kinase TOR1)
Hos	Hda One Similar
K-glutamate	Potassium glutamate
kb	Kilo base pairs
kDa	Kilo Dalton
LD	Lumidyne dye
Mcm	Mini-chromosome maintenance
min	Minutes
mM	Millimolar
MO	MCM-ORC
Mrc	Mediator of the Replication Checkpoint
MSD	Mean squared displacement
n	Number of molecules/observations
N	Number of biological repeats
NaCl	Sodium chloride
NHS-ester	N-Hydroxysuccinimide-ester
nm	Nanometer
nM	Nanomolar
nt	Nucleotide
NTP	Nucleoside triphosphate
OCCM	ORC-MCM

ORC	Origin recognition complex
ori	Origin
PCA	Protocatechuic acid
PCD	Protocatechuate-3,4-dioxygenase
PCNA	Proliferating cell nuclear antigen
Pds	Precocious Dissociation of Sisters
PEG	Polyethylene glycol
PIP	PCNA interacting peptide
pN	Piconewton
Pol	Polymerase
Psf	Partner of Sld Five
Rad	Radiation sensitive
Rfc	Replication factor C
RNA	Ribonucleic acid
RPA	Replicaiton factor A
s	Seconds
S phase	Synthesis phase
S.E.M.	Standard error of the mean
S.O.	Sytox orange
Scc	Sister chromatid cohesion
SEC	Size exclusion chromatography
Sfp synthase	4'-Phosphopantetheinyl transferase
Sld	Synthetic Lethality with Dpb11-1
Smc	Structural Maintenance of Chromosome
SNR	Signal-to-noise ratio
ssDNA	Single stranded DNA
TIRF	Total internal reflection fluorescence
Tof	Topoisomerase I-interacting Factor
Topo	Topoisomerase
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
Wpl	Wings apart-like
μM	micromolar

μm

micrometer

Chapter 1. Introduction

Chapter 1. Introduction

1.1 Sister chromatid cohesion

All living organisms are defined by their genetic material, the genome, they carry with them. The accurate duplication of this genome and subsequent segregation of the two copies to daughter cells are fundamental processes required for the continuity of life. Errors in either of these processes can lead to serious disease and even cell death. While inaccurate duplication of the genome may result in local alterations of the genetic material, faulty segregation of the genome among a cell's progeny leads to unequal distribution of the DNA. This results in one daughter cell carrying less genetic material than the other, a phenomenon known as aneuploidy, which is a major hallmark of cancer and must be prevented at all costs.

Eukaryotes have evolved the cell cycle to regulate and ensure that genetic material is copied and segregated accurately. During the cell cycle, the genome is first duplicated by DNA replication in S phase. Once this process is completed successfully, the two copies are divided equally during mitosis. DNA replication occurs once per cell cycle, ensuring there are exactly two copies of the genome, referred to as sister chromatids. During subsequent mitosis, the mitotic spindle connects to the sister chromatids and pulls them towards opposite poles, enabling the distribution into two genetically identical daughter cells.

Eukaryotic cells often carry their genetic material on multiple chromosomes, posing a challenge for equal separation once duplicated. However, cells have developed two advanced mechanisms to address this issue. First, newly synthesised sister chromatids remain connected from S phase until anaphase in mitosis. This physical connection, known as sister chromatid cohesion, is essential for faithful chromosome segregation (Peters & Nishiyama, 2012). Second, chromosomes condense following S phase, preventing their entanglement. Together, these processes ensure the pairwise recognition and equal division of sister chromatids during mitosis. (Figure 1, top)

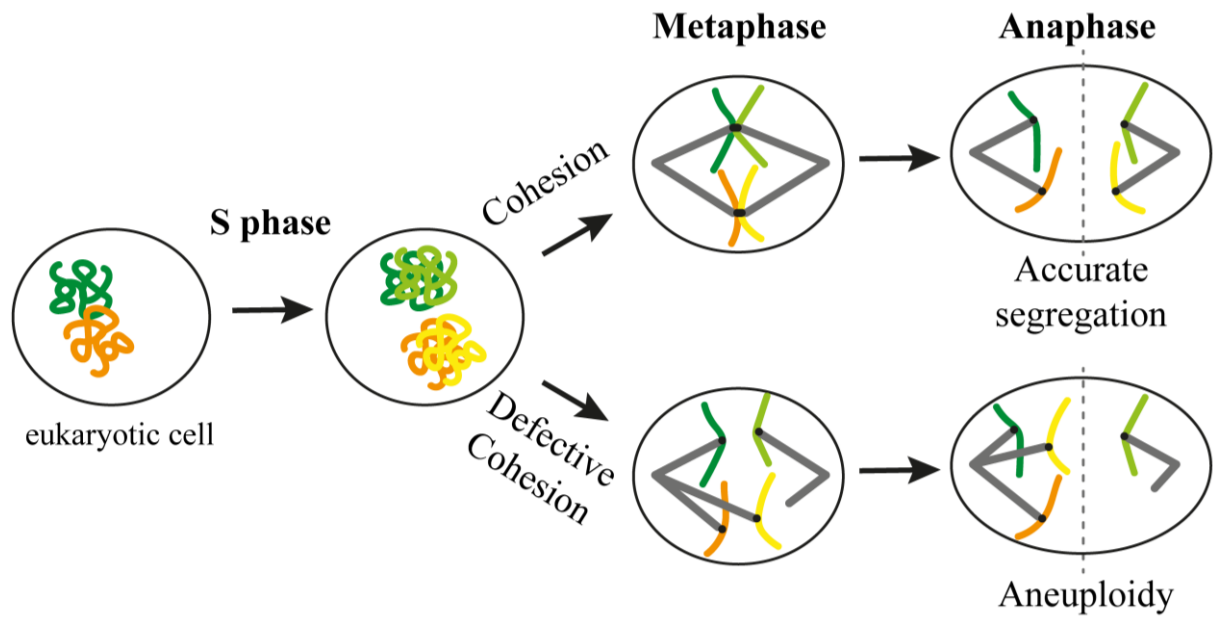


Figure 1: Cohesion is essential for accurate chromosome segregation.

In a eukaryotic cell, the genome carried usually on multiple chromosomes is duplicated during S phase by DNA replication. Sister chromatid cohesion maintains sister chromatids in close proximity allowing for pairwise alignment of chromosomes during metaphase. In subsequent anaphase, sister chromatids are separated equally which leads to accurate chromosome segregation and the creation of two genetically identical daughter cells. If cohesion is defective, sister chromatids are not aligned equally during metaphase leading to faulty chromosome segregation or aneuploidy.

At the onset of mitosis, chromosomes connect to the mitotic spindle apparatus via microtubules, which exert pulling forces to align each pair of chromatids at the metaphase plate. Sister chromatid cohesion is essential to resist these forces, allowing for proper pairwise alignment. Once aligned, cohesion is resolved, leading to the separation of chromosomes towards opposite spindle poles. Loss of cohesion frequently results in both sister chromatids being pulled towards the same pole (K. Tanaka et al., 2001). (Figure 1, bottom)

Early cytological and genetic studies of *Drosophila melanogaster* have described the phenomenon of cohesion and found some genes that lead to the precocious loss of chromosome alignment during anaphase (Davis, 1971; Goldstein, 1980). These findings suggested that proteins might facilitate the cohesion of sister chromatids.

However, the finding that convergence of two replication forks during S phase leads to high tension and catenation during replication termination (Sundin & Varshavsky, 1980), informed a model where DNA catenation between sister chromatids could be responsible for cohesion. In this model, catenation could be resolved by topoisomerases which would be specifically activated at the onset of anaphase.

Alternatively, physical connection of sister DNA molecules by partially replicated centromere DNA could lead to cohesion. In this model, cohesion would be resolved by late replication of centromeric DNA in anaphase. However, these models were not valid for long.

Studies in yeast found that mini-chromosomes were not topologically intertwined with each other in cells arrested before anaphase (Koshland & Hartwell, 1987), suggesting that DNA catenation is unlikely to maintain cohesion until anaphase.

Eventually, genetic screens have identified several proteins that were essential for sister chromatid cohesion, and included Smc1, Smc3 and Scc1 (Mcd1) (Guacci et al., 1997; Michaelis et al., 1997; Tóth et al., 1999). Mutants of either of these proteins led to precocious chromosome segregation, suggesting that they are responsible for holding sister DNA molecules together from S phase onwards. Today we know that these proteins form the core of the conserved protein complex cohesin, involved in many cellular processes beyond maintaining cohesion, such as DNA compaction and DNA repair.

In the following chapters I will provide an overview as well as discuss past and present research on the two fundamental processes that are required for sister chromatid cohesion, first the duplication of the genome by DNA replication, second the establishment of cohesion of replicated chromatids by cohesin.

In a third chapter I will briefly explain why we use single-molecule methods to study cohesion establishment and how total internal reflection fluorescence (TIRF) microscopy can help us to do this.

1.2 Duplicating the genome

1.2.1 DNA replication

In order to maintain life, the DNA genome of a cell needs be accurately duplicated before it is transferred into two progeny cells during subsequent cell division. Faithful duplication of the genome is achieved by DNA replication during S phase, which depends on the concerted action of numerous proteins forming a large dynamic protein machine, called the replisome. The core components of this replisome are a DNA helicase, that unwinds parental DNA, and several DNA polymerases, that accurately synthesis new double stranded DNA. The action of these proteins must be tightly regulated to ensure that the large genome is copied exactly once per cell cycle and that no DNA remains unreplicated.

In bacteria this task is quite trivial. A single circular chromosome is duplicated by two replisomes that are recruited to a single site, the origin of replication (*oriC*), from where they move in opposite directions. For the relatively small genomes of bacteria (< 15 000 kilo bp) this is achievable within less than an hour. (O'Donnell et al., 2013)

However, eukaryotic genomes are much larger with the smallest human chromosome, chr XXI, having a size of 47,000 kilo bp. Hence, eukaryotes evolved an intricate mechanism which allows them to initiate replication from not one but many origins, which are distributed throughout the genome. Development of such a system was crucial for the evolution of eukaryotic life harbouring larger genomes.

To ensure that the eukaryotic genome is replicated exactly once, replisome assembly is highly regulated and directly coupled to the cell cycle. The replisome is powered by a DNA helicase that is a platform for recruitment of DNA polymerases and further accessory proteins, required for rapid and efficient replication. DNA helicases are hetero-hexameric rings that are assembled onto dsDNA at origins of replication as inactive double hexamers (DH) with the help of initiation proteins in a process referred to as helicase loading. Subsequently, DHs are activated during which they are reconfigured to encircle one strand of DNA. Recruitment of additional proteins leads to the formation of an active replicative helicase consisting of Cdc45-Mcm2-7-GINS (CMG).

Helicase loading takes place during G1 phase, ensuring that a large number of origins have a helicase loaded before in subsequent S phase, helicases are activated. In cells, these processes are controlled by cyclin dependent kinase (CDK) which when expressed, abrogate helicase loading while facilitating helicase activation. During G1 phase, low levels of CDK promote the loading of helicases, and a rise in CDK levels at the onset S phase leads to the activation of helicases while further helicase loading is blocked. This dual mechanism ensures that the entire genome is copied exactly once.

In the following chapters I will provide a short summary of the assembly and function of the replicative helicase CMG. Further I will explain how after CMG activation, a replisome is formed and subsequently duplicates DNA.

1.2.2 The CMG helicase

Replicative helicases are ring-shaped protein complexes that use ATP hydrolysis to unwind DNA by translocating on single stranded DNA. The eukaryotic replicative helicase consists of 11 subunits, formed by Cdc45, GINS (Sld5, Psf1, Psf2, Psf3, also called Go-Ichi-Ni-San) and Mcm2-7 (mini-chromosome maintenance) also called CMG (Ilves et al., 2010). The helicase function is provided by the six different, but homologous MCM subunits which forms Mcm2-7, a hetero-hexameric ring complex that encircles one strand of DNA. These MCM subunits are comprised of two globular domains, N terminal domain (N tier) and C terminal domain (C tier), that appear as two stacked donuts (R. Georgescu et al., 2017). The C tier contains ATPase sites which belong to the super family of AAA+ ATPases. CMG formation takes place in two steps, loading of inactive head-to-head double hexamers (helicase loading or licensing) followed by their activation.

During G1 phase of the budding yeast cell cycle, Mcm2-7 are assembled into inactive double hexamers that encircle double stranded DNA. This process requires the origin recognition complex (ORC), Cdc6 and Cdt1 as well as ATP. In a first step, ORC, which is a hetero-hexameric protein complex that contains a N terminal AAA+ ATPase and a C terminal DNA binding domain, recognises and binds to an origin of replication. ORC specifically recognises

non-palindromic ARS consensus sequences (ACS) requiring ATP binding but not hydrolysis (Bell et al., 1993). ACS elements are an essential part of autonomously replicating sequences (ARS) that are the genomic sites of replication initiation. In addition to the essential ACS, ARS contain several flanking sequence elements (B elements) required for proper origin function and are important but not essential (Marahrens York & Stillman, 1992). Single-molecule studies of ORC during origin recognition revealed that ORC diffuses along DNA and halt upon encountering the origin (Sánchez et al., 2021).

Origin bound ORC is bound by the co-loading factor Cdc6 which recruits a first MCM-Cdt1 complex. DNA enters MCM through the Mcm2-5 gate to form a stable OCCM complex (ORC-C-term binding to MCM-C-term). The same ORC complex then acrobatically flips over to bind the N-term side of MCM (forming MO, MCM-ORC) followed by Cdc6 binding and recruitment of a second MCM-Cdt1 complex. This leads to the formation of stable head-to-head double MCM hexamer that encircles duplex DNA which can passively slide along DNA. (Evrin et al., 2009; Gupta et al., 2021; Miller et al., 2019; Remus et al., 2009)

Double hexamers remain catalytically inactive until S phase of the cell cycle. Stepwise assembly of an active helicase requires nine firing factors that convert MCM double hexamers into two active CMG helicases (Yeeles et al., 2015). First, Dbf4-dependent kinase (DDK) phosphorylates MCM at Mcm 4 and Mcm6 followed by recruitment of Sld3 (Deegan et al., 2016). Sld3 forms a complex with Sld7, which when bound to double hexamers, recruit Cdc45 (S. Tanaka et al., 2011). A second kinase, cycline dependent kinase (CDK), phosphorylates Sld3 and Sld2, which promotes the sequential recruitment of Dpb11, Sld2, GINS and Polymerase ϵ (Muramatsu et al., 2010; S. Tanaka et al., 2006; Yeeles et al., 2015; Zegerman & Diffley, 2006). Using *in vitro* reconstitution of this process using purified proteins, it was found that recruitment of all the above factors leads to double hexamer separation into individual hexamers with Cdc45 and GINS stably binding to form a CMG complex (Douglas et al., 2018) while the firing factors Dpb11, Sld2 and Sld3/7 are only required for the activation of helicases (Gambus et al., 2006). During the formation of single CMGs, ATP binding leads to each CMG untwisting 6-7 bp of duplex DNA within its central channel while they move away from one another, only remaining connected through Mcm6 (Douglas et al., 2018; Lewis et al., 2022). Finally, recruitment of Mcm10, a single strand binding protein that binds across MCM (Lööke et al., 2017; Mayle et al., 2019; Warren et al.,

2009), together with ATP hydrolysis induces structural rearrangements that lead to the exclusion of one strand of the duplex DNA from the central channel and disengagement of CMGs (Douglas et al., 2018). The two activated CMG helicases translocate N tier first on ssDNA in 3'-5' direction using ATP hydrolysis allowing the helicases to pass each other.

In addition to promoting CMG activation, CDK inhibits MCM loading during S phase by phosphorylation of ORC, Cdc6 and Mcm3 (Bell & Labib, 2016). Hence, MCM loading can only take place during G1 phase, while helicase activation is confined to S phase. Splitting the initiation of replication into two stages ensures that only correctly assembled replisomes are activated and further minimises the risk of re-replication.

Recent single-molecule studies of *in vitro* activated CMG described that nucleotide binding halts CMG diffusion on duplex DNA, suggesting a mechanism where newly assembled CMG uses nucleotide binding to grip DNA and facilitate DNA melting in its central channel (Ramírez Montero et al., 2023). This is in stark contrast with single-molecule experiments using recombinantly purified CMG that finds that CMG shows diffusive behaviour in presence of ATP (Wasserman et al., 2019). Interestingly, structural studies have found no differences between *in vitro* activated and recombinantly purified CMG (Baretić et al., 2020; Eickhoff et al., 2019; Goswami et al., 2018; Henrikus et al., 2024; Lewis et al., 2022) suggesting that phosphorylation of Mcm2-7 during helicase activation might play a crucial role for helicase-DNA interactions (Lewis et al., 2023).

CMG helicases unwind DNA moving along the leading strand while excluding the lagging strand from its central channel (Kose et al., 2019; Langston et al., 2017). This is consistent with the ability of CMG to bypass lagging strand blocks such as protein-DNA crosslinks (DPC) without stalling (Kose et al., 2019). Interestingly, when using methyltransferase as a roadblock, CMG helicases stall, presumably through direct stabilisation of duplex DNA by the methyltransferase (Kose et al., 2019). In *Xenopus laevis* egg extracts, leading strand roadblocks can be bypassed after significant stalling times, suggesting that additional factors help remove obstacles to facilitate CMG bypass (Sparks et al., 2019).

Using single-molecule magnetic tweezers, *Drosophila melanogaster* CMG complexes were found to unwind DNA via a random walk with individual CMGs exhibiting unwinding and backward movement, as well as the tendency to pause (Burnham et al., 2019). Further, structural studies of translocating CMG suggested a non-symmetric hand over hand rotary mechanism for CMG unwinding, which could explain the asymmetric requirements of ATPase function around the MCM ring of CMG (Eickhoff et al., 2019).

By themselves CMG helicases are rather weak/slow helicases (Ilves et al., 2010; Kose et al., 2020). Their unwinding rates were shown to be stimulated by the single strand binding protein RPA (Kose et al., 2020) as well as Mcm10 (Douglas et al., 2018; Langston et al., 2017; Lööke et al., 2017). Additionally, Mcm10 was shown to facilitate efficient passage past lagging strand roadblocks (Langston et al., 2017). Using DNA replication reactions reconstituted with purified proteins, Mrc1 was found to stimulate the unwinding rate of CMG and phosphorylation of Mrc1 and Mcm10 by the DNA checkpoint protein kinase Rad53 slowed down fork progression (McClure & Diffley, 2021). Taken together, these proteins might facilitate efficient DNA unwinding by coupling DNA translocation with fork unwinding.

1.2.3 The replisome

DNA replication is achieved by the ‘replisome’, which is a large group of interacting proteins that coordinates the efficient and accurate duplication of DNA. The replicative helicase CMG is at the heart of the replisome which recruits many additional proteins to the replication fork to facilitate synthesis of leading and lagging strands. (Figure 2)

DNA unwinding is coupled to DNA synthesis performed by a set of three DNA polymerases with distinct functions. (i) Polymerase ϵ (Pol ϵ) is responsible for most leading strand synthesis. (ii) Polymerase δ (Pol δ) is required for lagging strand synthesis, further it is involved in some leading strand synthesis (R. E. Georgescu et al., 2014; Yeeles et al., 2017), (iii) Polymerase α (Pol α) synthesises RNA-DNA primers required for leading and lagging strand synthesis. Pol ϵ tracks along DNA with the replisome in 3’-5’ direction while synthesising DNA in 5’-3’ direction. This leads to synthesis of a continuous leading strand.

Whereas on the lagging strand Pol δ extends the RNA-DNA primers created by Pol α to create shorter so-called Okazaki fragments. (Bell & Labib, 2016) (Figure 2)

In *in vitro* replication assays, leading strand synthesis can be achieved from a replication fork with CMG in complex with Pol ϵ (Langston et al., 2014). Further, *in vitro* reconstitution of helicase loading, and activation was able to promote regulated DNA replication (Yeeles et al., 2015). Although, both *in vitro* replication systems can support robust leading strand DNA synthesis, *in vivo* rates of replication can only be achieved with addition of fork stabilisation factors Tof1, Csm3 and Mrc1 (TCM) as well as the processivity factor PCNA (Yeeles et al., 2017). With the same replication assay, it was shown that Pol δ and Pol α are required for lagging strand DNA synthesis, and that pol δ can support leading strand synthesis at slower rates.

Tof1, Csm3 and Mrc1 (TCM) are non-essential subunits of the replisome and form a heterotrimeric complex. They are collectively referred to as the fork protection complex, for their role in coupling DNA synthesis to CMG during replication stress (Katou et al., 2003). However, some people refer to them as the fork progression complex for their stimulative role in replication rate (Yeeles et al., 2017). All three proteins travel with the replisome, directly interacting with the CMG helicase (Bando et al., 2009; Gambus et al., 2006). Tof1 and Csm3 form a stable dimeric complex that physically associates with CMG at the N tier (Baretić et al., 2020). If either of them is deleted, the other one does not stably interact with the replisome (Bando et al., 2009). Together, Tof1-Csm3 are required for efficient association of Mrc1 to the replisome. In contrast, absence of Mrc1 does not reduce Tof1-Csm3 levels at the replisome.

Structural studies were able to resolve Tof1-Csm3 engaged with a recombinantly purified CMG (Baretić et al., 2020). Both proteins are placed towards the front of the MCMs where they contact dsDNA before duplex DNA is separated. This puts them at a prime location for facilitating their role in stimulating and pausing replication forks. In contrary, Mrc1 is highly flexible and was not resolved in any structural studies to date. However, protein crosslinking followed by mass spectrometry was used to determine contacts of Mrc1 with CMG (Baretić et al., 2020). Here, contacts across the entire replisome were found, with the N terminus contacting Tof1, the central region contacting Mcm2/6 and the C terminus contacting Cdc45 and Ctf4.

Additionally, Mrc1 was found to interact with the catalytic subunit of pol ϵ , pol2, which is thought to play a regulatory role in polymerase activity (Lou et al., 2008; Z. X. Zhang et al., 2018).

TCM stimulate fork progression *in vivo* (Szyjka et al., 2005) and *in vitro* (Baris et al., 2022; Yeeles et al., 2017). Omission of Mrc1 leads to a more pronounced decrease in replication rate compared to omission of Tof1-Csm3 *in vitro* (Yeeles et al., 2017), suggesting that Mrc1 is the main modulator of replication fork rates, whereas Tof1-Csm3 contribute through stabilising Mrc1 at the replication fork. Moreover, the decrease in fork rate after omission of Tof1-Csm3 could be rescued by adding increased amounts of Mrc1, strongly supporting that they contribute to fork progression by stabilising Mrc1 at the fork.

The role of TCM in fork protection is related to their contribution to the intra S phase checkpoint which responds to DNA damage and stress. Mrc1 (mediator of the replication checkpoint 1) was originally described for its role in DNA replication checkpoint response (K. Tanaka & Russell, 2001). During checkpoint activation, Mrc1 is phosphorylated by the kinase Mec1 leading to recruitment of the kinase Rad53 and further phosphorylation of Mrc1 which results in a slowdown of the CMG helicase (Alcasabas et al., 2001; McClure & Diffley, 2021). By slowing down CMG, TCM couple polymerase action to the CMG helicase, thereby preventing uncoupling of CMG and polymerases during DNA stress.

Independently of the previous roles, the flexible C terminal region of Tof1 is involved in the recruitment of topoisomerase 1 Topo1 and the nucleosome chaperone FACT to the replisome (Safaric et al., 2022).

Finally, all three proteins of the fork protection complex are involved in sister chromatid cohesion establishment (Mayer et al., 2004; Xu et al., 2004). This role is presumably independent from their role in DNA replication and relies on direct contacts with the cohesion mediating complex cohesin (Shrestha et al., 2023).

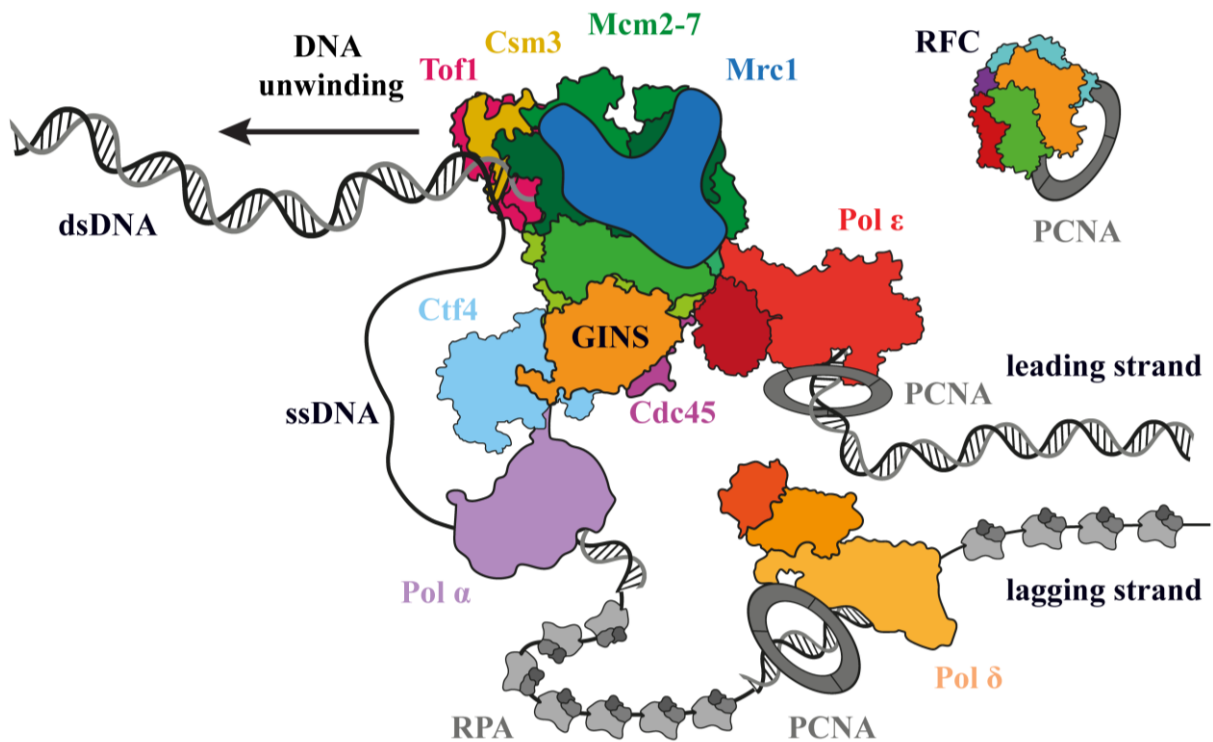


Figure 2: Structural model of the replisome

Cdc45-Mcm2-7-GINS (CMG) forms the core of the replisome and is responsible for unwinding of duplex DNA in 3'-5' direction. CMG recruits polymerases and fork stabilisation factors (Tof1-Csm3-Mrc1) to promote rapid and efficient leading and lagging strand synthesis. Pol ϵ directly interacts with CMG and performs leading strand synthesis in 5'-3' direction. Pol α is recruited to the replisome via the interaction hub Ctf4. Pol δ on the other hand does not directly interact with the replisome and is necessary for lagging strand synthesis and initiation of leading strand synthesis. PCNA is loaded onto DNA by replication factor C (RFC) and acts as a processivity factor for DNA polymerases. This structural model represents a graphical approximation of cryo-EM structures, with the objective of accommodating them into a unified model.

Another replisome associated factor is Ctf4, a homo-trimeric protein that acts as a hub for recruitment of other proteins involved in sister chromatid cohesion, ribosomal DNA maintenance, parental histone transfer and gene silencing, to the replisome (Gan et al., 2018; Samora et al., 2016; Simon et al., 2014; Villa et al., 2016). Ctf4 is recruited to the replisome via Sld5 subunit of GINS (Simon et al., 2014). CMG bound Ctf4 was shown to recruit up to two copies of polymerase α simultaneously (Simon et al., 2014; Yuan et al., 2019). This stoichiometry was recently confirmed using single-molecule experiments of DNA replication with fluorescently polymerase α (Lewis et al., 2020). Nonetheless, Ctf4 is not required for replication establishment and fork progression in vitro (Yeeles et al., 2015). Similar to TCM, deletion of Ctf4 was linked to a marked defect in sister chromatid cohesion (Hanna et al., 2001). The defect in SCC was shown to be related to the role of Ctf4 in recruiting the accessory helicase Chl1 to the replisome (Samora et al., 2016).

In recent years, electron and single-molecule microscopy were used to investigate the overall structure and dynamics of the replisome. The CMG helicase forms the stable core of the replisome, and most proteins are orchestrated around it. Pol ϵ and Pol α are connected to CMG as part of the replisome, however at opposite ends (R. Georgescu et al., 2017; Sun et al., 2015). Whereas Pol ϵ is located at the rear of the replisome where ssDNA is exiting the CMG (Goswami et al., 2018; Yuan et al., 2020; J. C. Zhou et al., 2017), Pol α is connected to CMG via Ctf4, which is conveniently located to the front of the fork where it can interact with the free ssDNA (Yuan et al., 2019). A recent structural study found that Polymerase α can also interact with CMG independently from Ctf4 (Jones et al., 2023). The fork stabilisation factors Csm3 and Tof1 are also positioned at the front of the replisome, where they grip the duplex DNA to facilitate rapid DNA unwinding (Baretić et al., 2020). In contrast to Pol ϵ and Pol α , Pol δ was not found to physically interact with the replisome and lagging strand synthesis is thought to be uncoupled from the CMG helicase (Bell & Labib, 2016). Surprisingly, recent single-molecule observations revealed that all three polymerases can be retained at the replisome to synthesise multiple Okazaki fragments (Lewis et al., 2020). However, when additional polymerases were supplemented in solution, all three polymerases did exchange from solution to the replisome. Interestingly, CMG remains stably associated with the replisome even when challenged with an excess in solution. Additionally, Tof1-Csm3 and Mrc1 were found to bind only transiently to the replisome (Lewis et al., 2017).

Overall, these findings confirm that the replisome is a highly dynamic protein complex that is built around a central hub, the CMG helicase, which orchestrates simultaneous leading and lagging strand DNA synthesis. The plasticity of this complex might be of importance for the establishment of sister chromatid cohesion (see below).

1.3 Sister chromatid cohesion establishment

1.3.1 The cohesin complex

The protein ring to hold sister chromatids together is the cohesin complex. It is recruited to DNA during G1 phase of the eukaryotic cell cycle. Following duplication of the genome in S phase, it holds both sister chromatids together, maintaining their cohesion until anaphase during mitosis.

Cohesin is a member of the structural maintenance of chromosomes (SMC) protein family, large multimeric protein complexes that are characterised through their long arms forming a ring-like structure (Anderson et al., 2002; Haering et al., 2002). It is this ring shape that led to the suggestion that cohesin interacts with DNA by topological embrace (Haering et al., 2002).

The circumference of the cohesin ring is made up of two SMC subunits, Smc1 and Smc3, that form long stretches of alpha helical coils (“coiled coils”) that connect two globular domains at the top (“hinge” domain) and the bottom (“head” domain) of the protein. Individual SMC subunits adopt a rod like shape that possess two globular domains, connected by long anti-parallel coiled coils. The N-terminal region folds back onto the C-terminal region, such that it forms one globular domain, the head, which is connected to a second globular domain lying in the middle of the protein via anti-parallel coiled coils (Melby et al., 1998). Smc1 and Smc3 dimerise through hydrophobic interactions between the hinge domains, forming a stable dimerisation interface that is basis of the characteristic V shaped structure observed in electron microscopy (Haering et al., 2002). (see Figure 3)

On the other end, the heads form ATP binding cassette (ABC) family ATPase domains, that dimerise in presence of ATP and disengage following ATP hydrolysis (Lammens et al., 2004). The coiled coils are rarely rod shaped, owing their flexibility to discontinuities that allow them to adopt various conformations (Haering et al., 2002).

To complete the ring structure, a third protein subunit, Scc1 is needed. This long, mostly unstructured kleisin protein, binds to the coiled coils near the Smc3 head via its N-terminal winged helix domain, and to Smc1 head with its C terminus (Haering et al., 2004). Therefore bridging the two head domains and forming a tripartite ring, that can physically entrap DNA

by topological embrace (Gruber et al., 2003; Srinivasan et al., 2018). Scc1 plays an important regulatory role as its binding to the Smc3 head forms the exit and entry gate for DNA into cohesin (Murayama et al., 2018). Further, Scc1 is the target of proteolytic cleavage during mitosis to remove cohesin from chromosomes (Uhlmann et al., 1999). Finally, Scc1 serves as a binding platform for recruitment for additional accessory factors, such as the fourth and last cohesin subunit, Scc3.

Scc3, is an essential regulatory subunit that is important for both DNA loading and unloading *in vitro* (Collier et al., 2020; Murayama & Uhlmann, 2014, 2015) as well as *in vivo* (Li et al., 2018). *In vivo*, Scc3 was found to always be present together with the other three cohesin subunits (Smc1, Smc3 and Scc1) while its association with chromatin mainly depended on Scc1 (Tóth et al., 1999). Scc3 consists of α -superhelical HEAT (Huntingtin, Elongation factor 3, protein phosphatase 2A, and yeast kinase TOR1) repeats that assemble in a hook-like shape. Scc3 is recruited to cohesin by binding the flexible middle region of Scc1, forming the tetrameric cohesin complex (Li et al., 2018; Tóth et al., 1999).

DNA entry into the cohesin ring takes place during G1 phase (Ciosk et al., 2000). However, how DNA enters this ring remains a topic of debate. Principally, there are three possible entry paths, the Smc1-Smc3 (hinge) gate, the Smc1-Scc1 gate, Smc3-Scc1 gate, all of which require dissociation of the interface to allow for DNA to pass through. By selectively blocking the different gates, the Smc1-3 hinge domain was identified as a potential entry gate *in vivo* (Gruber et al., 2006). Findings that the hinge domain could interact with dsDNA through a positively charged patch further supported the notion that the Smc1-Smc3 hinge interface forms the DNA entry gate (Srinivasan et al., 2018). However, this model fails to explain why ATP hydrolysis by the ATPase heads is required for loading of cohesin. More recently, *in vitro* reconstitution of topological cohesin loading onto circular plasmid DNA has informed a model where DNA enters and exits through the same gate, the Smc3-Scc1 kleisin gate (Murayama & Uhlmann, 2014, 2015). In this model, ATP hydrolysis fuels both, entry and exit of DNA, suggesting that head engagement and disengagement plays a role in the path the DNA takes. This model was further supported by the cryo-electron microscopy (cryoEM) structure of an intermediate of the fission yeast cohesin loading reaction, the “gripping” state, that finds that Smc3-Scc1 disengagement is required for DNA loading (Higashi et al., 2020).

Other *in vitro* studies of budding yeast cohesin suggested that both, the hinge and the kleisin gate are important for DNA entry (Collier & Nasmyth, 2022). The question whether both or just one gate is responsible for DNA entry into the cohesin ring remains a topic of ongoing investigation.

A major challenge in studying the structure and function of cohesin are its flexible coiled coils. Their folding is very dynamic and can lead to various conformations, that in return fuel different ideas and concepts of cohesin's mode of action (Haering et al., 2002). Additionally, the kleisin subunit Scc1, is very flexible by itself, further complicating the study of cohesin conformation. So far, researchers were able to determine structures of individual globular domains of cohesin, such as the heads and the hinge. However, a complete structure of the full tetrameric cohesin loaded on DNA was not yet reported.

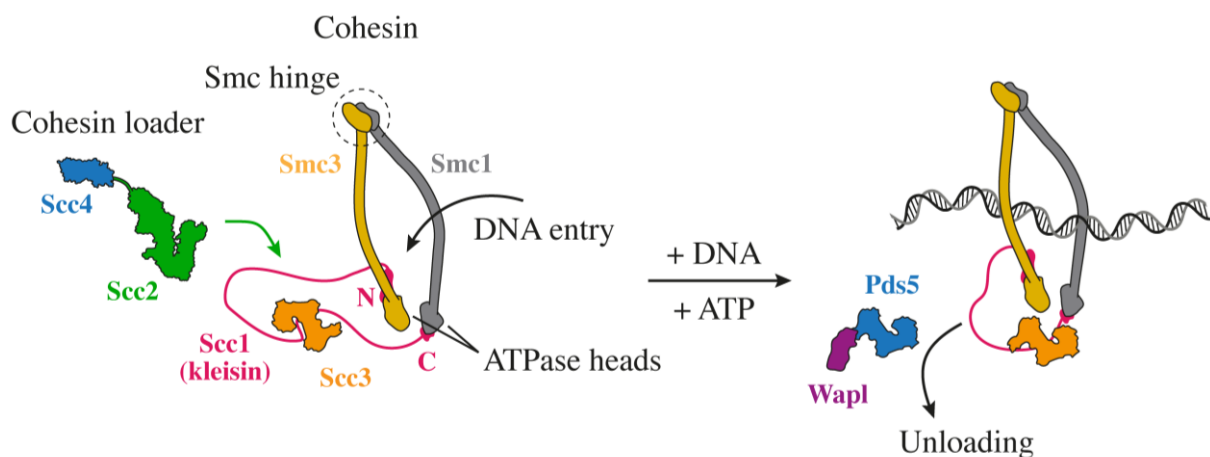


Figure 3: Structural model of the cohesin complex and entrapment of DNA

Smc1 and Smc3 consist of long coiled-coil domains, connecting the dimerisation interface at the top (“hinge”) with ATPase “heads” at the bottom. The ATPase heads engage each other upon ATP binding and are further bridged by a kleisin-subunit Scc1. Scc3 binds to Scc1 and regulates cohesin function and dynamics. Cohesin loading onto DNA takes place during G1, is mediated by the Scc2-Scc4 cohesin loader complex and requires ATP. During loading, DNA entry possibly occurs through the kleisin N-gate, which opens between the kleisin N terminus and the Smc1 ATPase head. Pds5 competes with Scc2 for binding to Scc1 and brings in Wapl which promotes kleisin N-gate opening, resulting in DNA release.

1.3.1.1 *Regulatory subunits of the cohesin ring*

Loading of cohesin onto chromatin depends on the cohesin loader complex, formed by Scc2 and Scc4 (Ciosk et al., 2000). Like Scc3, Scc2 is a hook shaped protein containing multiple HEAT repeats (Chao et al., 2017; Kikuchi et al., 2016) that binds to the flexible region of Scc1. Both, Scc3 and Scc2 can bind to Scc1 at the same time. *In vitro* biochemical studies using purified proteins showed that Scc2 recruitment greatly stimulates the ATPase activity of cohesin which is essential for cohesin loading onto DNA (Murayama & Uhlmann, 2014; Petela et al., 2018). For this activity, the Scc2 C-terminal region containing the HEAT repeats is sufficient. The N-terminus of Scc2 is dispensable for cohesin loading *in vitro* (Murayama 2014) but is needed for chromatin loading of cohesin *in vivo* (Ciosk et al., 2000; Petela et al., 2018). Here, Scc2N is needed for the association with Scc4 to form a Scc2-Scc4 complex (Chao et al., 2017) that is thought to interact with chromatin remodellers, thereby recruiting cohesin to nucleosome free regions on chromosome arms (Muñoz et al., 2019).

Scc2 is assumed to be in constant competition with another protein, Pds5, for binding to Scc1. Precocious dissociation of sisters 5 (Pds5) is a conserved accessory factor that is essential for sister chromatid cohesion (Hartman et al., 2000). It is another hook shaped protein that is very similar in structure with Scc2 and Scc3. Pds5 binds to Scc1 in the N terminal region towards the Smc3-Scc1 kleisin gate, which means that Pds5 and Scc2 might not bind to cohesin at the same time. Indeed, two studies have described that Pds5 replaces Scc2 on Scc1 once cohesin is loaded onto DNA (Murayama & Uhlmann, 2015; Petela et al., 2018). However, the prevailing view of Pds5 as a competitor to Scc2 has been challenged recently. Using AlphaFold predictions, it was demonstrated that Pds5 and Scc2 are capable of simultaneously binding to Scc1. Furthermore, *in vivo* pulldowns revealed that Pds5 and Scc2 colocalise with Scc1 throughout the cell cycle (Unpublished data from Laura Frizzi in our laboratory). The role of Pds5 lies partly in protecting cohesin from removal of DNA by promoting cohesin acetylation (Chan et al., 2013). The phenomenon of cohesin acetylation will be discussed in detail later.

However, Pds5 is also responsible for recruiting the cohesin unloading factor Wpl1, which together facilitate cohesin unloading and thereby counteracting sister chromatid cohesion (Sutani et al., 2009; K. Tanaka et al., 2001). Wpl1 in complex with Pds5 triggers unloading of

cohesin from DNA in a non-proteolytic manner (Sutani et al., 2009). Interestingly, *in vitro*, Wpl1 was found to promote cohesin loading as well as unloading (Murayama & Uhlmann, 2015). It is proposed that Wpl1 recruitment to cohesin is regulating the ATP hydrolysis of the Smc1-Smc3ATP heads leading to either DNA entry or exit (Chatterjee et al., 2013). To date the dual role of Pds5 in stabilising and destabilising cohesin remains incompletely studied.

1.3.1.2 *Functions of cohesin*

Cohesin was originally discovered for being involved in sister chromatid cohesion (Guacci et al., 1997; Michaelis et al., 1997). Since then, cohesin has been found to be involved in other activities, such as DNA organisation and DNA repair (Uhlmann, 2016).

The intact ring structure of cohesin is required for successful sister chromatid cohesion (Gruber et al., 2003; Uhlmann et al., 2000). This directly implies that topological entrapment of DNA by cohesin rather than physical engagement of cohesin with DNA is a strict requirement for cohesion between sister chromatids. This was confirmed first by artificial cleavage of cohesin loaded *in vivo* onto yeast mini-chromosomes (Ivanov & Nasmyth, 2005). However, several models can be envisioned that explain how topologically loaded cohesin molecules maintain cohesion. In a first model, referred to as the “hand-cuff” model, one cohesin is loaded on only one sister chromatid and the dimerization between two cohesin molecules that are loaded on different DNA molecules results in cohesion. Studies in human cell lines have found that Smc1, Smc3 and Scc1 can interact with themselves in a Scc3 dependent manner, suggesting that protein-protein interactions between two cohesin molecules could contribute to cohesion (N. Zhang et al., 2008). A more recent study in yeast investigating cohesin recruitment to sites of DNA double strands (DSB) has found that oligomerisation of cohesin is responsible for holding ends of the DSB together (Phipps et al., 2023). This leads to the suggestion that dimerization could be a more general mechanism of action for cohesin, be it in cohesion or DNA break repair.

Nonetheless, most studies from the last 20 years speak against a model where cohesin molecules can dimerise in order to establish cohesion between sister chromatids. Early evidence for such a model comes from the use of circular mini-chromosomes in yeast cell. When cysteine crosslinking was used to covalently close the three possible exit gates of

cohesin, it was found that one cohesin molecule can indeed accommodate two mini-chromosomes within its central cavity (Haering et al., 2008; Srinivasan et al., 2018). This strongly suggests that cohesion can be established by a single cohesin molecule. Additionally, *in vitro* studies have shown that cohesin can subsequently capture one dsDNA followed by a ssDNA (Murayama et al., 2018). Subsequently, single-molecule studies demonstrated that the sequential capture of DNA can be accomplished by a single cohesin molecule (Richeldi et al., 2023). This scenario might be found *in vivo* right behind an active replication fork with dsDNA on the leading strand and ssDNA on the lagging strand. When ssDNA is subsequently converted to a dsDNA, cohesin continues to maintain cohesion between them. Further live-cell single-molecule spectroscopy has confirmed that cohesin exists mostly as a canonical monomer throughout the cell cycle (W. Liu et al., 2020).

Interestingly, strict topological engagement with DNA is not essential for all of cohesin's functions. Association with chromosomes *in vivo* as well as loop extrusion by mammalian cohesin rings *in vitro* does not require the opening of the tripartite ring (Davidson et al., 2019; Srinivasan et al., 2018). Loop extrusion, an activity where cohesin actively extrudes DNA through its ring into so called loops, was proposed to be responsible for cohesin's ability to compact DNA. Similar to other SMC proteins, cohesin was shown to be directly involved in compacting chromatin in interphase (Rao et al., 2017). However, active loop extrusion was so far only observed *in vitro* (Davidson et al., 2019; Higashi et al., 2021).

An alternative model is based on the observation that cohesin can capture two dsDNA molecules sequentially (Richeldi et al., 2023). In this model, referred to as diffusion capture, cohesin brings together distant locations of the same chromosomal DNA by sequentially entrapping them in its ring, thereby compacting chromosome in the same manner as suggested by loop extrusion. Furthermore, recent *in vivo* studies find that chromatin loops form independent of the loop extrusion activity of cohesin that was described previously *in vitro* (Guérin et al., 2024) and it is proposed that cohesin forms chromatin loops by DNA-DNA capture at sites of active transcription.

Cohesin is also recruited to sites of DNA damage during DNA replication in S phase and G2 phase. Here, cohesin establishes damage induced cohesion which in turn helps to facilitate

DNA resection and repair (Delamarre et al., 2020; Heidinger-Pauli et al., 2008; Phipps et al., 2023). The mechanism of how this cohesion is established as compared to cohesion established in vicinity to the DNA replication fork remains subject of further studies.

1.3.1.3 Cohesin during the cell cycle

Cohesin's association with chromosomes is regulated throughout the cell cycle, which is important to allow for establishing and maintaining cohesion from S phase until anaphase. In yeast, cohesin is starting to be loaded onto DNA soon after Scc1 is expressed in late G1 phase (Ciosk et al., 2000). Before S phase, cohesin association with chromatin is very dynamic, and the turnover of cohesin on DNA is high (Gerlich et al., 2006; Lopez-Serra et al., 2013). This is primarily owed to the continuous cycle of loading and unloading facilitated by Scc2-Scc4 and Pds5-Wpl1, respectively. (Figure 4)

Once loaded onto chromosomes, cohesin molecules translocate from their loading sites to sites of convergent transcription termination (Lengronne et al., 2004). Rather than cohesin actively moving to these sites, it was shown that the transcription machinery is directly pushing cohesin, while cohesin maintains topological contact with chromatin (Ocampo-Hafalla et al., 2016).

During subsequent S phase, topologically loaded cohesin is met by replication forks, and the outcome of these encounters leads to cohesin entrapping the two newly synthesised sister chromatids. How cohesion is established and why it must be established during S phase will be further discussed in the next chapter.

After cohesin entraps sister chromatids, the activity of Pds5-Wpl1 would immediately abrogate cohesion by stimulating cohesin unloading from DNA. To counteract this unlading activity, cohesin is specifically acetylated at the Smc3 head domain by Eco1 (more on this later). Cohesin is acetylated during cohesion establishment in S phase, which leads to a more stable association with DNA ultimately maintaining cohesion from S phase throughout G2 phase until anaphase in mitosis. Acetylated cohesin holds sister chromatids together, resisting the forces applied by the bipolar spindle apparatus allowing for pairwise alignment of

chromosomes at the metaphase plate. In anaphase, segregation of chromosomes is initiated by the proteolytic cleavage of the Scc1 subunit of cohesin by separase (Uhlmann et al., 2000). This removes cohesin from DNA allowing for the sister DNA molecules to be separated into two daughter cells. Following cleavage, Scc1 is degraded, and the Smc3 head domain is deacetylated by the deacetylase Hos1 (Borges et al., 2010). In the next cell cycle, cohesin forms a tetrameric ring upon re-expression of its subunits and cohesin is loaded back onto chromatin during late G1 phase.

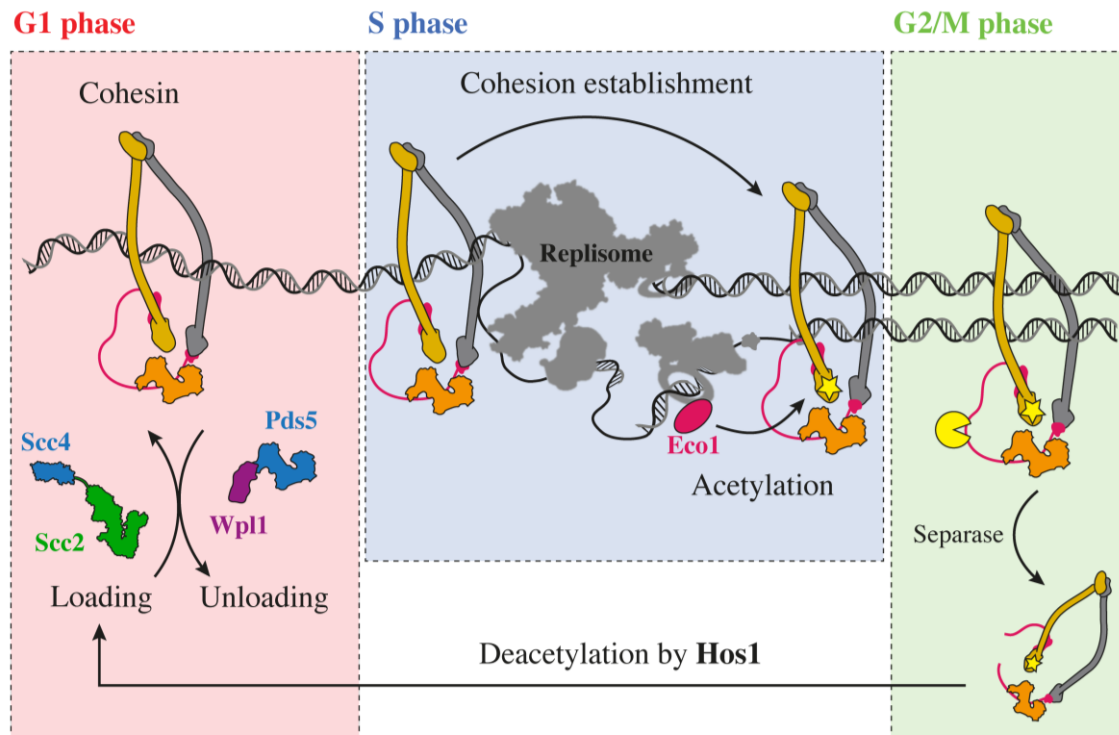


Figure 4: Cohesin during the cell cycle

During G1 phase, cohesin is loaded onto DNA with the help of the Scc2-Scc4 loading complex and ATP. The association of cohesin with chromatin during G1 phase is dynamic, owing to competition of the Scc2-Scc4 with the cohesin unloader Pds5-Wpl1. During S phase, cohesin encounters an active replisome and transitions to physically entrapping the two newly synthesised sister chromatids, thereby establishing cohesion. Replication fork associated Eco1 stabilises cohesive cohesin by acetylating Smc3 (yellow star), rendering it unavailable for further unloading activity by Pds5-Wpl1. Cohesion is maintained throughout G2 phase until M phase. In anaphase during mitosis, the kleisin subunit of cohesin, Scc1, is proteolytically cleaved by separase resulting in the removal of cohesin from DNA. Cohesin is deacetylated, and Scc1 is degraded. Upon Scc1 expression in the following G1 phase, cohesin is loaded onto chromatin.

1.3.2 Chromatin organization by the cohesin complex

Before discussing about how cohesin is involved in its essential function of holding sister chromatids together, I will briefly introduce a second prominent function of the cohesin complex, namely the organisation of chromatin loops.

First described as an insulating factor of interphase chromatin domains (Donze et al., 1999), cohesin has recently been implicated to be involved in several chromatin architectural features, such as topologically associating domains (TADs) and chromatin loops. These domains and loops play an important role in the dynamic structure and function of our genome. Over the years, two popular models have emerged for how cohesin establishes chromatin loops.

In the first model, cohesin acts as a progressive motor, which upon DNA binding generates a small DNA loop that subsequently grows rapidly into a larger energetically favoured loop (Davidson et al., 2019; Higashi & Uhlmann, 2022; Yatskevich et al., 2019). Because of this active “extrusion” of DNA into a loop, this model is called “loop extrusion”. The ability of cohesin to extrude loops is based on *in vitro* experimental evidence where a single cohesin is loaded onto a DNA substrate which is loosely attached to a microscopic flow cell. In the presence of a DNA-intercalating dye and vertical buffer flow, the cohesin binds to the tip of the laterally extended DNA and moves along the DNA, extruding an ever-growing loop (Davidson et al., 2019; Higashi et al., 2021; Kim et al., 2019). Surprisingly, while loop growth is very fast ($\sim 1\text{kb}/\text{min}$), the forces generated by cohesin during loop extrusion remain low $\sim 0.1\text{ pN}$ (Davidson et al., 2023; Kim et al., 2019) which lies in the range of thermal motion (Higashi et al., 2021). The process of loop extrusion requires three things: the cohesin loader, ATP hydrolysis by the cohesin, and the DNA intercalating dye SYTOX orange. Remarkably, several laboratories have attempted to reproduce cohesin loop extrusion in a surface-independent approach. Researchers from different laboratories have tried to visualise loop extrusion of cohesin on a DNA substrate attached between two optically trapped glass beads. However, these efforts have been without success (unpublished data). It is possible that the forces applied to the DNA via the glass beads is too high to allow for the formation of DNA loops by cohesin via loop extrusion.

This prompts the question of how cohesin moves along DNA *in vivo*, where it is faced with a dense chromatin landscape including much stronger molecular machines, such as the transcription machinery, the replisome or chromatin remodellers. *In vitro*, loop extruding cohesin has been demonstrated to bypass large obstacles, including RNA polymerases and large nanoparticles with a diameter of approximately 200 nm (Pradhan et al., 2022). However, *in vivo* RNA polymerases were shown to block loop extruding cohesin (Banigan et al., 2023; Jeppsson et al., 2022). It is evident that these observations can only be explained if cohesin-dependent loop formation *in vivo* employs a distinct mechanism from that of *in vitro* observed loop extrusion.

The second model proposes that cohesin undergoes two sequential DNA loading cycles, resulting in the capture of two distinct DNA molecules. This mechanism, often termed “loop capture,” finds parallel support in *in vitro* experimental evidence, similar to the loop extrusion model. A single cohesin complex, when loaded onto a surface-tethered linear DNA substrate within microscope flow cell was able to capture a second shorter plasmid DNA (Richeldi et al., 2023). The two DNA molecules were held together by cohesin in a topological manner withstanding forces up to 20 pN (Richeldi et al., 2023). The requirement of the DNA intercalating dye SYTOX orange to be able to observe loop extrusion suggests that enhanced DNA bending could be a factor that differentiates the *in vitro* observation of topological loading (required for loop capture) and loop extrusion.

While *in vitro* studies support both models, *in vivo* genetics studies predominantly favour the loop capture mechanism. Recent studies have identified cohesin complex mutants that, while deficient in forming loops *in vitro*, retained the ability for topological DNA entrapment (Bauer et al., 2021; Guérin et al., 2024). Strikingly, budding yeast cells harbouring this mutant cohesin were viable without any growth defects, and chromatin loop formation appeared normal (Guérin et al., 2024). This strongly implies that chromatin loops *in vivo* may form through a distinct mechanism that differs from the loop extrusion observed *in vitro*. Furthermore, a mutant in the Smc1 subunit of cohesin retained *in vitro* loop extrusion activity but displayed compromised *in vivo* loop formation.

The formation of chromatin loops exhibits a tight coupling with transcription. In budding yeast, the disruption of transcription leads to the disappearance of chromatin loops (Guérin et al., 2024; Jeppsson et al., 2022). Moreover, studies in human cells reveal a transient loss of chromatin loops during each cell cycle when the transcriptional machinery is silenced as the cell prepares for its division. The subsequent re-establishment of these chromatin architectural features in the following cell cycle is again dependent on transcriptional activity (S. Zhang et al., 2021). The mechanism of chromatin loop formation by cohesin might be similarly regulated to the *in vitro* process of second strand capture, which displays a preference for single-stranded DNA (ssDNA) over double-stranded DNA (dsDNA). The act of transcription involves the unwinding of DNA, thereby exposing ssDNA in its wake, potentially creating a substrate for cohesin's second strand capture (Guérin et al., 2024). In contrast, loop extrusion appears independently from transcription but is blocked by RNA polymerases *in vivo* (Banigan et al., 2023; Jeppsson et al., 2022) but not *in vitro* (Pradhan et al., 2022).

The loop capture model offers a compelling advantage by suggesting that cohesin employs a unified mechanism for both its initial topological loading onto DNA and the subsequent formation of chromatin loops. In contrast, the loop extrusion model presents a conceptual challenge: how can a single protein complex execute two seemingly disparate and fundamentally different behaviours?

Additionally, recent genetic analyses increasingly challenge the loop extrusion hypothesis. Nevertheless, a comprehensive understanding of cohesin-mediated chromatin organization in the complex *in vivo* environment necessitates further research employing diverse experimental approaches.

1.3.3 Mechanisms of cohesion establishment

Sister chromatid cohesion is established by cohesin topologically encircling the two sister chromatids after DNA replication. Following their synthesis during S phase sister chromatids do not separate for a significant distance and remain paired until segregation in anaphase during mitosis (Guacci et al., 1994). This suggests that cohesion establishment by cohesin takes place in S phase.

To establish cohesion, cohesin has to keep the two sister chromatids in close proximity for an appreciable amount of time, which is achieved by topological embrace (Haering et al., 2008). The ideal moment for achieving such embrace, is during or immediately following replication. This is when the two newly synthesised DNA molecules are in spatial proximity, which would make it easy for cohesin to achieve successful co-entrapment of the right sister DNAs. In contrast, establishment of cohesion during G2 doesn't seem as trivial, given that sister chromatids would have diffused away from each other quickly following their synthesis.

Along this notion, expression of the cohesin kleisin subunit Scc1 only after DNA replication results in defective sister chromatid cohesion (Uhlmann & Nasmyth, 1998). Therefore, cohesion establishment takes place during S phase. Moreover, mini-chromosomes in yeast were used to directly provide evidence that loading onto chromosomes in post-replicative cells does not lead to cohesion, even though cohesin was able to entrap single DNA molecules (Haering et al., 2004; Srinivasan et al., 2019). This brings direct evidence that cohesin can establish cohesion only in timely and spatial proximity of active replication.

Interestingly, several factors of the replication machinery have been identified to be involved in sister chromatid cohesion. These factors, collectively called cohesion establishment factors, have important but non-essential roles in facilitating cohesion. The absence of any of these factors leads to cohesion defects of different severity, such a precocious segregation of chromosomes.

Cohesin is loaded onto DNA already before DNA replication (Ciosk et al., 2000). Together with the observation of replisome bound cohesion factors, invokes the possibility that cohesion is established when a replisome encounters cohesin.

For cohesion to be established at the replication fork, two events must take place.

First, cohesin needs to transition from entrapping one DNA to co-entrapping the newly synthesised sister DNAs. One can envision that the replisome passes pre-loaded cohesin either by direct passage through the cohesin ring, or by transiently dissociating it from DNA.

Alternatively, cohesin is dissociated from DNA upon encountering the replisome, then *de novo* loaded cohesin embraces the two sisters right behind the replisome.

Second, cohesin must be converted into the cohesive state that is protected from Pds5-Wpl1 facilitated release. Cohesin chromatin association before DNA replication is very dynamic (Gerlich et al., 2006), fuelled by the cohesin loader and unloader complexes. For maintenance of cohesion following replication, Wpl1-induced release from DNA must be abrogated. This is achieved through modification of two lysine residues (K112, K113) on the Smc3 head domain by Eco1 acetyltransferase (Ben-Shahar et al., 2008; Ünal et al., 2008). Acetylation of cohesin results in a stable chromosome bound state that is immune to Pds5-Wpl1 mediated release.

Taken together, cohesion is established by cohesin in concert with the replication machinery during S phase. Various models of how their encounters could lead to cohesive cohesin that maintains sister chromatid cohesion can be envisioned. How cohesion is possibly established and how replication bound cohesion factors might contribute will be discussed in more detail in the following chapters.

1.3.3.1 Conversion of pre-loaded cohesin

During S phase the replication machinery unwinds DNA and synthesises new leading and lagging strand of DNA. What happens when this large multi-subunit protein complex encounters cohesin remains largely unknown.

Several models have been proposed, however there is currently not enough scientific evidence to favour any of them.

Generally, one can distinguish between two scenarios, conversion of pre-loaded cohesin to cohesive cohesin, and *de novo* loading of cohesin behind the replication fork (Figure 5).

These models are mutually non-exclusive, as genetic screens and *in vivo* experiments were able to determine two independent pathways responsible for cohesion establishment in yeast (Srinivasan et al., 2020; Xu et al., 2007).

In the first scenario, cohesin topologically loaded onto one DNA in G1 phase, will embrace the two new sister chromatids following replication. One possibility how this can be achieved is that the replication fork might pass through the cohesin ring (Figure 5A). Early electron microscopy studies have informed our view of cohesin as a large ring-shaped protein that has a potential diameter of approximately 40 nm (Anderson et al., 2002; Haering et al., 2002). If cohesin adopts a similar conformation once it is loaded onto DNA, a replisome should be able to fit through. The most complete structure of a replisome to date was accomplished using purified human proteins (names in parentheses) and contains the CMG helicase at a forked DNA together with Tof1-Csm3-Mrc1 (TIMLESS, TIPIN, CLASPIN), Ctf4 (AND-1) and the non-catalytic subunit of polymerase ϵ (Jones et al., 2021). This incomplete replisome has a front facing diameter of approximately 25 nm, theoretically small enough to fit through an open cohesin ring. (Anderson et al., 2002; Haering et al., 2002)

However, single-molecule experiments conducted by three independent labs suggest that cohesin adopts a different conformation once loaded on DNA (Davidson et al., 2016; Dequeker et al., 2022; Stigler et al., 2016). In these studies, cohesin was loaded onto a long linear DNA and its diffusive motion was investigated while being challenged with roadblocks of varying diameter. Fission yeast cohesin was able to pass over smaller obstacles such as dCas9 (10.6 nm) and digoxigenin (6.4 nm), but diffusion was hindered by slightly larger complexes e.g. nucleosomes (>11 nm). Even larger complexes (>19.5 nm) hindered cohesin passage significantly (Stigler et al., 2016). The same study also showed that cohesin can be pushed along DNA by FtsZ translocase (12.5 nm). A second study found that yeast MCM double hexamers hinder cohesin translocation (Dequeker et al., 2022). Similar observations were made for human cohesin, where Halo-tagged T7 RNA polymerase (~12 nm) was able to push cohesin along DNA (Davidson et al., 2016).

Taken together, these findings directly contradicting a model of replisome passage through the cohesin ring and suggest that cohesin adopts a collapsed ring conformation once topologically loaded onto DNA. In a collapsed conformation, cohesin won't be able to pass over a fully assembled replisome. However, one could envision a mechanism in which cohesion establishment factors enable passage of the replisome through the cohesin ring.

A recent study using single-molecule visualisation of DNA replication revealed that the yeast replisome does not stably bind its polymerases, contradicting previous models of a stable association of replisome components (Lewis et al., 2020). Further, the Tof1-Csm3 and Mrc1 were also found to be merely transient binding partners of the replisome (Lewis et al., 2017). This plasticity of the replisome might help to facilitate passage by timely dissociation of replisome components and reassociating after passage through the cohesin ring.

Interestingly, a single-molecule study using *Xenopus laevis* egg extracts has shown that the majority of replication events (32.4%) is able proceed past chromatin loaded cohesin (Kanke et al., 2016). This would fit a model where replisome passage through cohesin is facilitated by additional factors, such as cohesion establishment factors.

Alternatively, upon encountering a replisome, cohesin is dissociated from DNA and retained at the replication fork followed by reloading of the same cohesin behind the fork (Figure 5B). To establish cohesion, cohesin reloading behind the fork could take place sequentially. First, the double stranded leading strand is captured and then the single stranded lagging strand, which is subsequently converted into dsDNA-dsDNA entrapment through DNA synthesis. Evidence for this comes from *in vitro* reconstitution of cohesin second DNA capture, that showed that cohesin can only capture a second strand of DNA if it is single stranded (Murayama et al., 2018).

Similar to recycling of nucleosomes by replisome components that facilitate the transfer to either leading or lagging strand in a conveyor belt like fashion, cohesin transfer could be mediated by cohesion establishment factors. When cohesin encounters the replisome, cohesion establishment factors could facilitate cohesin release, transfer and reloading. In line with this, most cohesion establishment factors directly interact with cohesin (Samora et al., 2016; Shrestha et al., 2023). *In vivo*, the absence of cohesion establishment factors leads to an overall decrease of cohesin association with DNA before and after replication (Srinivasan et al., 2020). Moreover, cohesin is not able to achieve cohesion between replicated mini-chromosomes.

A third model for cohesin conversion proposes that cohesin is merely pushed by replisomes to sites of replication termination where cohesion is established when two forks converge. Given the size of an active replisome (>25 nm), and considering previous single-molecule experiments, pushing of cohesin along DNA is likely. Early *in vivo* experiments observed that cohesin is pushed by the transcription machinery and thereby accumulating at transcription termination sites (Lengronne et al., 2004), whereas DNA replication failed to relocate cohesin during S phase (Lengronne et al., 2006). It is possible that the strong effect of transcription on cohesin's position on DNA completely masks the replisome pushing cohesin. Interestingly, pushing of cohesin by active replication has been observed in a recent single-molecule study using *xenopus laevis* egg extracts (Cameron et al., 2024). Here, convergent replisomes were found to facilitate cohesion establishment in a cohesin dependent manner. However, this study was conducted on linear DNA with only sparse nucleosome occupancy. In cells, the DNA is more crowded, owing to dense nucleosome arrays and other processes such as transcription.

To date, it remains unclear what happens when cohesin encounters an active replisome let alone how cohesin might be converted or transferred from one to two DNA molecules. All of the above models are speculations, mostly based on experiments with obvious drawbacks. To determine how these large protein complexes interact in the context of chromatin, a bottom-up approach might be required. Recent advances were made by determining the requirements for efficient cohesion maintenance provided by Eco1 (Minamino et al., 2023). Building on this, the reconstitution of cohesion establishment *in vitro* might allow us to investigate the mechanisms underlying this fundamental process (ongoing research by Masashi Minamino and myself).

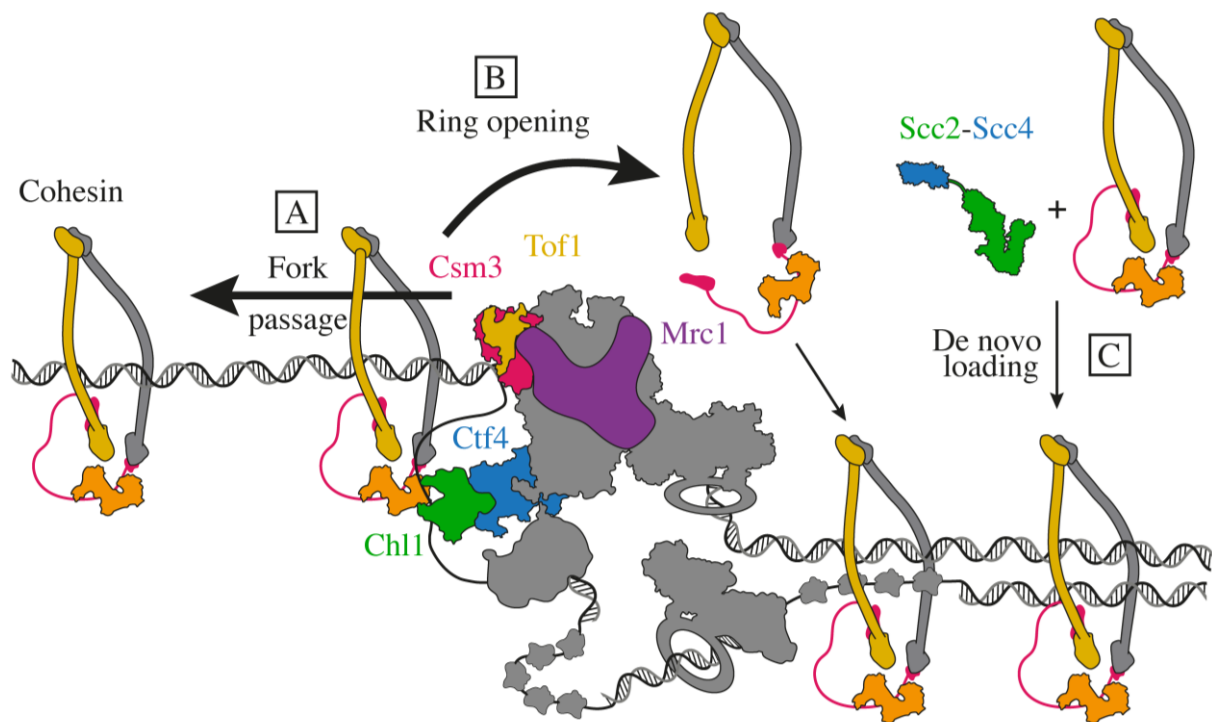


Figure 5: Possible mechanisms for cohesin establishment during DNA replication.

Cohesin loaded onto duplex DNA during G1 phase transitions to two duplicated DNA molecules during S phase. In current literature, two possible mechanisms for how this transition is mediated are discussed, conversion of G1 DNA-loaded cohesin and de novo loading of cohesin during DNA replication.

A. Cohesin conversion by fork passage. Cohesin remains bound to DNA, and the replication fork passes through the cohesin ring.

B. Cohesin conversion by ring opening. The cohesin ring is opened upon encountering a replisome, however cohesin is held at the replisome and is loaded onto the nascent DNA behind the fork. Replication fork associated factors (Chl1, Ctf4, Csm3, Tof1) are suggested to play an essential role during cohesin conversion.

C. De novo loading. Cohesin is dissociated from DNA or pushed along DNA upon meeting a replisome. Subsequently, an unrelated cohesin complex is loaded onto the newly synthesised DNA behind the fork. This pathway might require Scc2-Scc4, Mrc1 and Ctf18-RFC. Genetic studies suggest that more than one pathway might take place in parallel.

1.3.3.2 *De novo loading of cohesin during DNA replication*

In parallel to conversion of pre-loaded cohesin, cohesion establishment was suggested to be achieved by loading of new cohesin right behind the replication fork. Here, cohesin is loaded at the fork in a Scc2-dependent manner (Figure 5C). Similar to transient unloading and reloading of cohesin, sequential dsDNA and ssDNA capture might underly this process (Murayama et al., 2018). Behind the replication machinery, double stranded leading strand and single stranded lagging strand are ideal substrates for this reaction. Subsequent conversion of the lagging strand into dsDNA will complete the cohesion establishment process. *De novo* loading of cohesin requires timely recruitment of Scc2-Scc4 cohesin loader behind the fork to ensure that identical replication products are entrapped by cohesin. It was proposed that Scc2 is recruited to the replisome via its C-terminal PCNA-interacting motif (Psakhye et al., 2023). Furthermore, Masashi Minamino in our lab found that the cohesin loader is interacting with PCNA (unpublished data). However, further experiments are required to fully understand how the loader is recruited to the replisome.

In vivo experiments demonstrated that the absence of the cohesin loader during S phase results in a cohesion defect (Murayama et al., 2018). However, Scc2-Scc4 is dispensable *in vivo* once cohesin is loaded onto DNA in G1 phase (Srinivasan et al., 2019), underlining that there are at least two parallel pathways that allow for cohesion establishment during DNA replication.

Taken together, sister chromatid cohesion can be achieved by conversion of pre-loaded cohesin and *de novo* loading of soluble cohesin. Both processes act in parallel and are mutually non-exclusive. However, the exact mechanisms how either of them achieves successful cohesion remains speculative. In the following, I will investigate the various cohesion establishment factors involved in these processes.

1.3.4 Cohesin acetylation by Eco1 acetyltransferase

Cohesive linkages between cohesin and sister DNA molecules occur during DNA replication and depend on a number of cohesion establishment factors. Among these, Eco1 is the only protein that is essential (Skibbens et al., 1999; Tóth et al., 1999). Eco1 converts dynamic

chromatin association of cohesin during G1 phase into stable cohesive cohesin once it entraps the two sister chromatids (Chan et al., 2012; Gerlich et al., 2006; Lopez-Serra et al., 2013).

Establishment of cohesion 1 (Eco1) is an acetyltransferase, whose expression is upregulated in S phase to acetylate cohesin (Ivanov et al., 2002; Skibbens et al., 1999; Tóth et al., 1999). Overexpression of Eco1 outside S phase does not lead to cohesin acetylation, suggesting it must be directly coupled to DNA replication (Borges et al., 2013). Eco1 is thought to be recruited to the replication fork via its essential PCNA interacting peptide (PIP) box (H. W. Liu et al., 2020; Moldovan et al., 2006). Deletion of this PIP box can be rescued by tethering Eco1 to Fen1, suggesting that Eco1 is recruited to PCNA on the lagging strand (H. W. Liu et al., 2020). Recently, *in vitro* biochemistry studies have shown that cohesin acetylation is greatly stimulated by a nick or a flap on DNA, which both can be found at the lagging strand during lagging maturation (Minamino et al., 2023). Taken together, this suggests that Eco1 is recruited specifically during S phase to the lagging strand, where it acetylates cohesin that entraps the newly synthesised sister chromatids.

However, what effect does this acetylation have on cohesin?

Eco1 acetylates two conserved lysine residues (K112, K113) on the Smc3 head domain (Ben-Shahar et al., 2008; Ünal et al., 2008; J. Zhang et al., 2008). These lysine residues are part of a loop, rich in positively charged amino acids, that engages in interaction with DNA as well as the cohesin loader Scc2 (Collier et al., 2020; Higashi et al., 2020). Acetylation is expected to disrupt these interactions.

And indeed, when these lysine residues are changed to acetyl mimicking glutamine, the formation of a loading intermediate, the gripping state, is inhibited *in vitro* (Higashi et al., 2020). Additionally, cohesin carrying acetyl mimicking amino acids shows reduced DNA loading *in vitro* (Collier et al., 2020; Murayama & Uhlmann, 2015). These observations strongly suggest that the conserved lysine residues are involved in contacting both DNA and Scc2 and by that facilitate cohesin loading onto DNA. Acetylation disables those contacts and renders cohesin unable to load onto DNA.

But how does this fit into the context of stabilising cohesin after it is loaded onto DNA?

In vitro studies suggest a model where cohesin loading and unloading require cohesin's ATPase activity and follow the same path (Murayama & Uhlmann, 2015). The same study found that the cohesin ATPase cannot be stimulated by DNA anymore when the K112 and K113 in the Smc3 head are replaced by acetylation mimicking residues therefore preventing unloading of cohesin from DNA. Interestingly, Scc2 and Pds5 share a similar structure consisting of multiple HEAT repeats, suggesting they might make similar contacts with cohesin. Furthermore, Eco1 becomes dispensable when Pds5 or Wpl1 function is impaired (Ben-Shahar et al., 2008; Sutani et al., 2009). This directly informs a model where acetylation abrogates loading and unloading of cohesin by disrupting the interaction with the accessory factors Scc2 and Pds5.

As Eco1 is directly recruited behind the replication fork via PCNA, it only acts on cohesin that just established cohesion. Acetylation prevents cohesin from forming contact with DNA and presumably with its unloader Pds5-Wpl1. Therefore, Eco1 mediated acetylation serves as a lock that prevents cohesin unloading once it has established cohesion.

Following cohesin removal from chromosomes in anaphase, cohesin is deacetylated by Hos1 (Borges et al., 2010). Deacetylated Smc3 is recycled for usage in the next cell cycle.

1.3.5 Cohesion establishment factors

After cohesin was identified as the essential protein complex to hold sister chromatids together, several other factors have been identified to have a role in sister chromatid cohesion. These are factors that are themselves not part of the cohesin complex, but deletion leads to a pronounced cohesion defect. Strikingly, all these factors are directly or indirectly associated with the replication fork. They are collectively referred to as cohesion establishment factors, as they are not required for cohesin loading during G1 phase but become important for cohesion establishment during DNA replication. Interestingly, none of these factors is required for viability by itself, but epistasis analysis revealed two distinct groups, that were shown to be involved either in the cohesin conversion or de novo loading pathway (Srinivasan et al., 2020; Xu et al., 2007). Furthermore, each of these factors contributes

towards cohesin acetylation suggesting that they act upstream of Eco1 presumably in the initial cohesion establishment or by facilitating acetylation of Smc3 (Borges et al., 2013).

The first factors to be identified as cohesion factors were Ctf18 and Ctf4 (Hanna et al., 2001; Mayer et al., 2001). Deletion of either factor led to sister chromatid cohesion failure and were further found to physically and genetically interact with the replication machinery.

Subsequent genetic screens investigating synthetic growth defects in a Δ Ctf8 background identified additional 55 protein of which Chl1, Ctf4, Tof1 and Csm3 showed a defect in cohesion when arrested in G2/M phase (Mayer et al., 2004). In a separate study, Tof1, Csm3 and Mrc1 were identified to have sister chromatid cohesion defects (Xu et al., 2004). In the following chapters, each factor will be briefly introduced and their known functions in cohesion will be explained.

1.3.5.1 Ctf4-Chl1

Ctf4 is a homo-trimeric protein complex that was originally identified as a DNA polymerase α interaction factor. It plays a significant role as a replisome interaction hub by connecting GINS of the CMG helicase to pol α via a shared interaction motif (Gambus et al., 2006, 2009; Lengronne et al., 2006; Simon et al., 2014; H. Tanaka et al., 2009). Its function in sister chromatid cohesion and recruitment of pol α is conserved in vertebrates (Errico et al., 2009). Ctf4 recruits Chl1 to the replisome via a conserved CIP-box motif, which is thought to be the primary role of Ctf4 in cohesion establishment (Samora et al., 2016).

Chl1 is an accessory helicase that can unwind DNA in 5'-3' direction (Hirota & Lahti, 2000). It was originally identified as one of the first chromosome loss mutant genes (Haber, 1974). Interestingly, it seems that Chl1 recruitment to the replisome via Ctf4 and not its helicase activity is essential for cohesion establishment (Samora et al., 2016). Further, Chl1 was found to directly interact with the Scc1 kleisin subunit of cohesin *in vivo* (Samora et al., 2016), suggesting that its role in cohesion establishment might be to hold cohesin at the replication fork and therefore facilitating conversion of pre-loaded cohesin. In a recent *in vitro* study, Chl1 was found to stimulate cohesin loading at bubble DNA and replication forks, suggesting that Chl1 might act towards cohesion establishment by promoting proper loading of cohesin

in front or behind the replication fork (Murayama et al., 2024). Alternatively, a mechanism could be envisioned where Chl1 contacts cohesin whose coiled coils are in a closed conformation and by opening them up, facilitate passage of replisome through the cohesin ring. This could take place in concert with other replisome bound cohesion establishment factors, such as Tof1-Csm3 and Mrc1.

1.3.5.2 RFC-Ctf18

RFC-Ctf18 is an alternative PCNA clamp loader that preferentially loads PCNA onto the leading strand (H. W. Liu et al., 2020). It is part of the replication factor C (RFC) family, protein complexes that are involved in loading and unloading of the PCNA sliding clamp. RFCs are pentameric AAA+ ATPases, that consist of four common subunits, Rfc2-5, and one specialised subunit that gives the complex its name and identity. RFC-Rfc1 is the only essential RFC complex and is mainly involved in providing PCNA for lagging strand DNA synthesis.

In contrary, RFC-Ctf18 consists of Rfc2-5 as well as Ctf18 and accessory subunits Ctf8 and Dcc1 (Mayer et al., 2001). RFC-Ctf18 is not essential, but its deletion leads to defective sister chromatid cohesion and reduced Smc3 acetylation levels (Hanna et al., 2001; H. W. Liu et al., 2020). *In vitro*, RFC-Ctf18 possesses both PCNA loading and unloading activities (Bermudez et al., 2003). *In vivo*, RFC-Ctf18 enriches and balances PCNA levels at the replication fork, which provides a pool of PCNA that is required to promote sister chromatid cohesion presumably through recruitment of Eco1 (H. W. Liu et al., 2020). Interestingly, RFC-Rfc1 loaded cohesin cannot compensate for a lack of RFC-Ctf18 loaded PCNA (H. W. Liu et al., 2020). Recruitment of RFC-Ctf18 to replication forks depends on Dcc1, which directly interacts with polymerase ϵ but can also bind to ssDNA and dsDNA (Grabarczyk et al., 2018).

1.3.5.3 Tof1-Csm3 and Mrc1

Tof1-Csm3 and Mrc1 (TCM), known as the fork protection complex, form a homo-trimeric complex that is recruited to the CMG helicase and play multiple important roles during DNA replication (Baretić et al., 2020). They are involved in protecting replication forks as a

checkpoint mediator in case of fork stalling upon replication stress but are also essential for replication progression in unperturbed cells. Their role in sister chromatid cohesion establishment is distinct from their replication functions and is suggested to involve direct protein-protein interactions with the cohesin ring (Shrestha et al., 2023). Their positioning towards the front of the replisome (Baretić et al., 2020) puts them into a prime spot to make contact with cohesin upon its encounter with the replisome (Shrestha et al., 2023) possibly facilitating the passage of the replisome through cohesin. Alternatively, TCM could retain transiently unloaded cohesin in close proximity to the replication fork and facilitate re-loading of cohesin onto newly synthesised DNA molecules.

How these interactions contribute to cohesion remains mostly unexplored.

1.3.5.4 Parallel functions of cohesion establishment factors

Apart from Eco1 none of the above-mentioned accessory factors are essential for sister chromatid cohesion, neither are they essential for DNA replication. Efforts have been made to understand their contribution towards cohesion establishment.

One study has investigated the viability of double mutant strains and identified two distinct epistasis groups (Xu et al., 2007) suggesting that there are at least two pathways that these factors act towards. This explains the phenomenon that deleting only one of the factors results in compromised cohesion, but cells are still viable. One epistasis group is made up of Tof1, Csm3, Chl1 and Ctf4, whereas the second group consists of Ctf18 and Mrc1 (Xu et al., 2007). In a recent study, the first group was directly linked to be involved in cohesin conversion, while the second group has been implicated in *de novo* loading in a Scc2-dependent manner (Srinivasan et al., 2020). Both pathways are required for efficient Smc3 acetylation by Eco1 (Borges et al., 2013).

Interestingly, removal of the cohesin unloader Wpl1 can correct for cohesion defects caused by single cohesion mutants, but not in cells lacking Ctf4 or Chl1 (Borges et al., 2013). This suggests that in addition to acetylation, Ctf4 and Chl1, facilitate cohesion by an acetylation independent mechanism. It is possible that part of this mechanism is the previously mentioned role of Chl1 in facilitating topological loading at replication forks (Murayama et al., 2024).

In summary, how sister chromatid cohesion establishment is established by the cohesin ring remains largely unknown. However, the recent years several models have been suggested that to date are all possible and could act at the same time to promote cohesion. The involvement of cohesion establishment factors suggests that cohesin by itself cannot establish cohesion and needs to be transferred or loaded onto DNA nearby the replisome. Two major pathways have been suggested, which is supported by the finding of two epistasis groups *in vivo*. Nonetheless, the molecular mechanisms of how either of these pathways contributes to cohesion remains a black box.

It is essential that sister chromatid cohesion is established in a timely and reliably manner during DNA replication. The most straightforward way for the cell to achieve this would be to allow the replisome to pass through the cohesin ring. This mechanism provides an effective fail-safe mechanism, ensuring that cohesin entraps the sister chromatids after passage. Moreover, passage through the replisome could be more readily regulated in conjunction with other transfer reactions at the replisome, such as nucleosome recycling. It is difficult to envisage a mechanism by which the replisome could facilitate the transfer of nucleosomes while simultaneously transferring cohesin onto newly synthesised sister chromatids. The coordination of these parallel processes necessitates precise regulation, with numerous factors working in concert to guarantee accurate cohesion. The mechanism of replisome passage does not depend on the recruitment of specialised factors, but rather on the already replisome factors (Tof1-Csm3-Mrc1, Ctf4-Chl1). Therefore, it requires minimal energetic effort by the cell. It remains unclear whether circumference of the cohesin ring is sufficiently large to accommodate an entire replisome. While theoretical models suggest that this is possible (Anderson et al., 2002; Haering et al., 2002), in practice, cohesin appears to adopt a folded conformation, which may render it incompetent for replisome passage.

The advances in reconstitution of DNA replication and cohesin loading *in vitro* with purified proteins are enabling us to start studying these complex mechanisms outside of the cell and therefore allow us to narrow down how each of the above proteins contributes to facilitating cohesion establishment during DNA replication.

1.4 Single-molecule fluorescence microscopy

1.4.1 Why single molecules?

Single-molecule experiments monitor the properties of individual molecules in real time. In ensemble experiments, however, a measurement represents the average of an entire population of individual components with different behaviours. The goal of single-molecule experiments therefore is to obtain information of this heterogeneity within a system, that would otherwise be lost in ensemble averaging. Observing molecular processes at this level of detail allows for the detection of sub-populations, rare or short-lived intermediates and extract dynamic behaviour from individual trajectories.

In the recent years, ensemble experiments of complex biological systems have yielded insights of great impact. The reconstitution of DNA replication *in vitro* using purified proteins was a milestone and enabled the study of the molecular mechanisms that drive the duplication of our genome (R. E. Georgescu et al., 2015; Yeeles et al., 2015). However, the dynamics of any of these processes was entirely obscured.

The development of innovative single-molecule approaches together with advances in the biochemistry of complex biological systems have together created many new insights.

Lewis and colleagues have used *in vitro* reconstituted leading strand replication to show the dynamics of individual eukaryotic replisomes for the first time (Lewis et al., 2017).

Surprisingly, they found that the fork protection complex (Tof1-Csm3-Mrc1) not only stimulates the rate of leading-strand replication, but also acts in a dynamic fashion resulting in phases of slow and fast replication within a single trajectory (Lewis et al., 2017). Before these findings were made, the dynamic interaction of Mrc1, was expected but could never be proven.

A few years later, a similar and as surprising finding was made for the three eukaryotic polymerases. Using single-molecule fluorescence microscopy, individual replisomes performing leading and lagging-strand synthesis were visualised (Lewis et al., 2020). The addition of fluorescently labelled polymerases revealed that polymerases within the active replisome exchange with polymerases from solution in a concentration dependent manner. This result beautifully demonstrated the use of single-molecule experiments to study the hidden dynamics of biological systems.

Single-molecule studies were further used to study the behaviour of individual cohesin complexes on DNA and how they encounter a variety of obstacles (Davidson et al., 2016; Dequeker et al., 2022; Kanke et al., 2016; Stigler et al., 2016). In all these studies cohesin exhibited highly dynamic behaviour, which could be limited by obstacles of a certain size. Interestingly, cohesin was not able to surpass obstacles that were larger than 19 nm, which showed for the first time that cohesin might translocate along DNA in a collapsed conformation exhibiting closed coiled-coil arms (Stigler et al., 2016).

All the above-mentioned studies used a total internal reflection fluorescence (TIRF) microscope to study behaviour of proteins on a long linear DNA attached to the coverslip. During my PhD I am combining these established approaches to study encounters of active replisomes and cohesin on DNA. In the following chapters, I will discuss the principals of fluorescence and TIRF microscopy.

1.4.2 Fluorescence microscopy

Fluorescence is the emission of a photon by an optically excited molecule that spontaneously transitions from the excited state back to the electronic ground state. Whenever light strikes a molecule, there is a finite probability that photons are absorbed. Possible electronic transitions are detailed in a Jablonski diagram (Figure 6). If the energy of the photons is sufficient, the molecule will be excited from the singlet ground state S_0 into higher excited states S_1 or S_2 . Each electronic state is subdivided into further vibrational states. Excitation to a vibrational state of S_1 is quickly followed by vibrational relaxation into the lowest vibrational state ν_0 of S_1 denoted as internal conversion. Due to this energy loss, the emission spectrum of a fluorescent dye is always shifted to higher wavelengths (*Stokes Shift*). Further, the emission spectrum is independent of the excitation wavelength (*Kasha's Rule*). From S_1 the molecule has various possibilities to return to the singlet ground state. One being the transition back to a vibrational level of S_0 by spontaneous emission (*Fluorescence*). Another process depopulating S_1 is the transition to a 'forbidden' triplet state T_1 (*Intersystem Crossing*) from where the molecule can return to S_0 by emitting a photon (*Phosphorescence*). Transition to T_1 requires the change in spin of the electron. This is why intersystem crossing and

phosphorescence are ‘forbidden’ and take place on much larger time scales than fluorescence. Additionally, the excited molecule can decay non-radiatively (e.g. relaxation, quenching or resonance energy transfer). Concluding from this, not every absorbed photon directly results into fluorescence.

To depict the ability of a fluorophore to emit photons upon absorption, the fluorescence quantum yield Φ_F was introduced. It is the ratio between the number of emitted photons and the total number of absorbed photons, which can be expressed using the de-excitation rates k .

$$\Phi = \frac{\text{emitted photons}}{\text{absorbed photons}} = \frac{k_{\text{fluorescence}}}{k_{\text{fluorescence}} + k_{\text{non-radiative}}} \quad \text{Equation 1}$$

With k_F being the transition rate for fluorescence and k_{n-r} summarising all other processes depopulating the excited state S_0 .

Further, the ability of a fluorophore to emit light (fluorescence) is characterised by its molecular brightness, which is proportional to the product of the quantum yield Φ_F and the absorption coefficient ε (quantity of light that can be absorbed by the fluorophore).

An additional property of a fluorophore is its fluorescence lifetime τ . The fluorescence lifetime is the time span between excitation and emission of a photon (lifetime of the excited state) describing the fluorescence decay. It is given by:

$$\tau = \frac{1}{k_{\text{fluorescence}} + k_{\text{non-radiative}}} \quad \text{Equation 2}$$

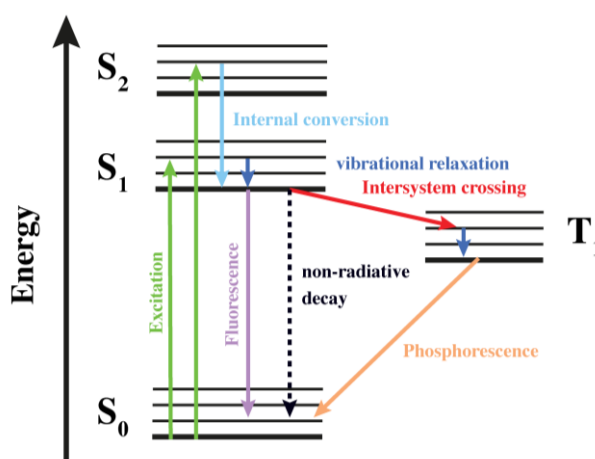


Figure 6: Jablonski diagram

Shown are electronic states (S_0 , S_1 , S_2) and vibrational states of a molecule as well as the possible transitions between them. *Excitation* of a molecule by absorption of photons is followed by rapid relaxation to the lowest vibrational state of S_1 (*Internal conversion*). The molecule can return to a vibrational state of S_0 by emitting a photon (*Fluorescence*). Alternatively, the molecule can transition to a ‘forbidden’ triplet state T_1 (*Intersystem crossing*) from where it returns to the ground state S_0 by emitting a photon (*Phosphorescence*). Excited molecules can also return to the ground state via *non-radiative decay*.

When selecting a suitable fluorophore for fluorescence microscopy, brightness of the dye (high quantum yield and extinction coefficient) is key. Further, photo stability of the fluorophore should be considered. Processes such as *blinking*, *photobleaching* and *quenching* can make it hard to correctly interpret biomolecular dynamic processes, because they lead to an unpredictable loss of fluorescence. Blinking on the one side occurs when an excited molecule transitions into a triplet-state or dark-state. These states usually have a much longer lifetime than fluorescence and will be detected as on-off-on dynamic. Photobleaching describes the irreversible loss of fluorescence due to photon-induced damage or covalent modification of the fluorophore. The loss of fluorescence of a dye due to interactions with other molecules (e.g. formation of a complex) or even with itself, is called quenching. These processes lead to a shorter lifetime of the dye but can also be used to find out how many fluorescent dyes are detected in a trace. For this purpose, it is observed in how many steps bleaching occurs. In general, if there is only one bleaching step, one can assume that only a single dye is present.

A simple way to account for blinking and bleaching is to lower the laser intensity for the duration of the measurement. Furthermore, free oxygen O₂ in the used buffers can stimulate bleaching. Using an oxygen scavenging system such as a combination of the enzymes glucose-oxidase and catalase (GODCAT), will convert added glucose together with free O₂ into gluconic acid and water (Englander et al., 1987). A more recently developed enzymatic system utilises protocatechuate-3,4-dioxygenase (PCD) which oxidise protocatechuic acid (PCA) and thereby removes free oxygen from the system (Aitken et al., 2008; Patil & Ballou, 2000). Removal of free oxygen O₂ contributes to a higher blinking rate as oxygen by itself acts as an efficient triplet-state quencher. Therefore, in most experiments an additional triplet-state quencher such as Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) or β -mercaptoethanol is added (Rasnik et al., 2006).

1.4.3 Total internal reflection fluorescence microscopy

In general, a fluorescence microscope for single-molecule experiments is selected by its ability to separate background noise from actual fluorescent signal. This property is compared using the signal-to-noise ratio (SNR), which is defined as ratio of desired signal (in this case

fluorescence) to background noise. To achieve a good SNR, on the one hand the fluorescent signal must be as high as possible. This can be achieved by choosing fluorescent dyes that have a high extinction coefficient and a good fluorescence quantum yield. On the other hand, the background signal from undesired sources should be reduced. Therefore, selecting an appropriate microscopic method is of great importance.

The main method used to visualise single molecules during my PhD is total internal reflection fluorescence (TIRF) microscopy. In TIRF microscopy wide-field illumination is used which allows one to observe a number of fluorescent particles on the sample surface in a large field of view over a longer time span. Excitation of the fluorophores is achieved through an evanescent wave created by total internal reflection at the border between coverslip and solution (Figure 7). If an incoming light beam strikes the boundary from an optically denser into an optically thinner medium at an incident angle θ_i greater than the critical angle θ_c , it is totally reflected. This critical angle is depending on the refractive indices n of the two media and can be derived from Snell's law:

$$\sin \theta_i n_i = \sin \theta_t n_t \rightarrow \theta_c = \sin^{-1} \frac{n_t}{n_i} \quad \text{Equation 3}$$

With θ_t being the transmitted portion of light and n_t being the refractive index of the optically thinner medium. While the incident light is totally reflected, a small portion of light energy travels as evanescent wave along the border of the two media. Since this evanescent wave decays exponentially across its penetration depth perpendicular to the reflecting surface, only a thin layer above the boundary is significantly illuminated. The depth of penetration d is given as:

$$d = \frac{\lambda}{4\pi \sqrt{n_1^2 \sin^2 \theta - n_2^2}} \quad \text{Equation 4}$$

Where n_1 and n_2 are the refractive indices of the first and second optical media, and θ is the incident angle of the light beam (Axelrod et al., 1983). As given by the above equation, the depth of penetration is depending on refractive indices, wavelength and incident angle of light beam and usually is between 100 and 200 nm. Therefore, only fluorescent dyes which are close to the sample surface are illuminated.

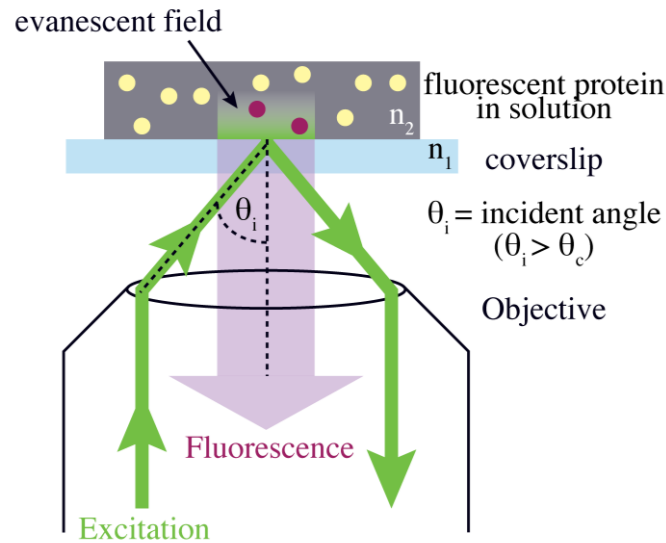


Figure 7: Simplified schematic of an objective-type TIRF setup

The excitation laser is directed through the objective and targeted at the interface of coverslip and sample volume at an incident angle greater than the critical angle ($\theta_i > \theta_c$). This creates an evanescent wave that excites fluorescent proteins close to the coverslip surface (purple). Fluorescence of the sample is collected through the objective.

In general, there are two different types of TIRF setups, which differ in the way the evanescent wave is created. In *prism-type* TIRF the laser beam is guided through a prism before reflection at the border between prism and sample chamber. Using this setup, an incident angle much larger than the critical angle can be created and an evanescent wave with extremely short penetration depth is created. Hence, a higher axial resolution with very little background noise is achieved. In *objective-type* TIRF (Figure 7), an evanescent wave is created at the surface of the coverslip to the sample volume. To create total internal reflection, the laser beam, which is focussed onto the back-focal plane of the objective, is shifted laterally (with respect to the optical axis of the microscope). Hence, the incident laser beam hits the surface plane at an angle which creates an evanescent wavefield if adjusted correctly. Since the excitation light is reflected towards the objective, it is possible to use open sample chambers. However, the signal-to-noise ratio is worse than in prism-type TIRF.

Overall, TIRF microscopy is a great tool to study individual molecules in close proximity to the coverslip surface. For its increased signal-to-noise ratio it became one of the main microscopy methods used to study biological systems at the single-molecule level.

1.5 Scope of this thesis

How sister chromatid cohesion is established during DNA replication remains unknown. Taking advantage of the exciting recent developments in the biochemical reconstitution of both DNA replication and cohesin loading onto DNA. I combined both methods, enabling real-time visualisation of cohesin-replisome encounters. With this approach I investigated the previously unanswered questions of how cohesin interacts with an approaching replication fork and how cohesin-replisome encounters lead to cohesion establishment.

First, I describe step by step how CMG translocation and cohesin loading on DNA is reconstituted with purified proteins *in vitro* and visualised using a TIRF microscope. Using this minimal system of a single CMG helicase and a cohesin molecule, I asked what would happen when they encounter each other on DNA, and how the outcome of encounters would change in the presence of cohesion establishment factors. I found that cohesin is primarily pushed by CMG along the DNA. A small percent of cohesin passing CMG was observed, that significantly increased upon the addition of cohesion establishment factors.

As these experiments are performed on naked DNA, with highly motile cohesin, I next asked how the outcome of cohesin-CMG encounters would change upon abrogating cohesin's ability to diffuse on DNA. These experiments provide strong evidence that CMG can pass cohesin, without disrupting the cohesin ring. Additional side flow experiments showed that cohesin likely does not transiently open during CMG passage, supporting a model where the CMG helicase passes through the cohesin ring circumference.

Next, I describe how DNA replication is reconstituted *in vitro* and visualised at the single-molecule level. Finally, by combining DNA replication with cohesin loading onto DNA, I show for the first time how a single DNA-loaded cohesin encounters an active replisome. These experiments reveal that replisome passage is significantly more likely than CMG passage. Strikingly, most replisome passage events lead to cohesion between sister DNA molecules. This indicates that cohesion can be established by replisome passage through the cohesin ring.

Chapter 2. Materials & Methods

Chapter 2. Materials & Methods

2.1 Strains, plasmids and oligos

All strains are based on a W303 background, sharing the common genotype:

ade2-1, can1-100, leu2-3,112, his3-11,15, ura3, trp1, GAL, psi+

I want to thank Masashi Minamino (MM) and Celine Bouchoux (CB) and the Diffley lab for contributing strains to my study.

Table 1: Strain list

Strain No.	Genotype	Source
y6251	Mcm2-7 MAT α pep4::KanMx4 bar1::Hph-NT1 trp1-1::TRP1pJF3 (MCM5-GAL1-10-MCM4) leu2-3::LEU2pJF4 (MCM7-GAL1-10-MCM6) ura3-1::URA3pRS306/MCM2-GAL1-10-CBP-TEV-MCM3)	Diffley lab stock (yAM22)
y6253	MAT α W303-1b pep4::KanMx4 bar1::Hyg	Diffley lab stock (yJF1 α)
y6281	Psf1, Sld5 MAT α W303-1b pep4::KanMx4 bar1::Hyg trp1-1::TRP1pJCZ1 (PSF1-GAL1-10-SLD5)	This study
y6285	Cdc45-S6, Psf1-3, Sld5 MAT α	This study

	W303-1b pep4::KanMx4 bar1::Hyg trp1-1::TRP1pJCZ1 (PSF1-GAL1-10-SLD5) leu2-3::LEU2pJCZ2 (PSF2-GAL1-10-PSF3) his3-11::HIS3pSMG1 (Cdc45iFLAG2- S6 -GAL1-10-GAL4)	
y6375	CMG-S6 MATa: W303 pep4::KanMx4 bar1::Hph-NT1 trp1-1::TRP1pJF3 (MCM5-GAL1-10-MCM4) leu2-3::LEU2pJF4 (MCM7-GAL1-10-MCM6) ura3-1::URA3pRS306/MCM2-GAL1-10-CBP-TEV-MCM3) MAT α : W303-1b pep4::KanMx4 bar1::Hyg trp1-1::TRP1pJCZ1 (PSF1-GAL1-10-SLD5) leu2-3::LEU2pJCZ2 (PSF2-GAL1-10-PSF3) his3-11::HIS3pSMG1 (Cdc45iFLAG2- S6 -GAL1-10-GAL4)	This study
y5278	Scc2-Scc4 cohesin loader MATa Δ pep4::HIS3 ade2-1::ADE2pGAL-SCC2-2PA trp1-1::TRP1pGAL-SCC4-HA3	MM (Minamino et al., 2018)
y5345	Tetramer Cohesin-SNAP MATa pep4::HIS3	CB (This study)

	Dwpl1::LEU2 Deco1::KANMX6 pGAL-GAL4; ade2-1::ADE2pGAL1-10-SMC1-PK3 trp1-1::TRP1pGAL-OptSCC1--6xHIS-HA-3C-ProtA; pGAL-SMC3-SNAP ura3-1::URA3pGAL1-10-Scc3-Myc	
y5562	Chl1 MATa pep4::HIS3 ade2-1::ADE2pGAL1-10-optimised Chl1-FLAG-2ProtA	MM (Shrestha et al., 2023)
yAE31	RPA MATa bar1::Hyg pep4::KanMX his3-11::HIS3pGAL1-10-CBP-Tev-RFA1+Gal4 ura3-1::URA3pGAL1-10-RFA2+RFA3	Diffley lab stock (Yeeles et al., 2015)
yAE34	Polymerase δ MATa bar1::Hyg pep4::KanMX ura3-1::URA3pGAL1-10 POL31+POL3 his3-11::HIS3pGAL1-10 Pol32-CBP+Gal4	Diffley lab stock (Yeeles et al., 2017)
yAE40	Ctf4 MATa bar1::Hyg pep4::KanMX his3-11::HIS3pGAL1-10-CBP-Tev-Ctf4+Gal4	Diffley lab stock (Yeeles et al., 2015)
yAE41	RFC MATa bar1::Hyg	Diffley lab stock (Yeeles et al., 2017)

	pep4::KanMX ura3-1::URA3pGAL1-10 RFC2+CBP-RFC3 Trp1-1::TRP1pGAL1-10 RFC4 + RFC5 his3-11::HIS3pGAL1-10 RFC1+Gal4	
yAE48	Tof1-Csm3 MATa bar1::Hyg pep4::KanMX ura3-1::URA3pGAL1-10-CBP-Csm3 +Tof1	Diffley lab stock (Yeeles et al., 2017)
yAE71	Mrc1 MATa bar1::Hyg pep4::KanMX his3-11::HIS3pGAL1-10-Mrc1-TEV-3xFLAG-NAT+Gal4	Diffley lab stock (Yeeles et al., 2017)
yAE95	Polymerase α MATa bar1::Hyg pep4::KanMX ura3-1::URA3pGAL1-10 CBP-Tev-Pri1+Pri2 trp1-1:TRP1pGAL1-10 Pol1+Pol12	Diffley lab stock (Yeeles et al., 2015)
yAJ2	Polymerase ϵ MATa bar1::Hyg pep4::KanMX trp1-1::TRP1pGAL1-10 Pol2+Dpb4-CBP ura3-1:URA3pGAL1-10 Dpb2+Dpb3	Diffley lab stock (Yeeles et al., 2015)

The plasmids pJCZ1 and 2 were generous gifts from the lab of Alesandro Costa. pUber was a gift from Lisanne Spenkelink (LS).

Table 2: Plasmid list

Plasmid No.	Genotype	Source
pJCZ1	pRS304-Psf1-GAL1,10-Sld5	Costa Lab
pJCZ2	pRS305-Psf2-GAL1,10-Psf3	Costa Lab
pSMG1	pRS303 Cdc45-iS6-FLAG2-Gal1-10-Gal4	This study
pVP91A-pcaHG	Protocatechuate 3,4- Dioxygenase (PCD) pVP91A T5-pcaH-pcaG	(Knoot et al., 2015) (Addgene #113766)
pUber		(Mueller et al., 2020) (Gift from LS)
pJY19	pET28a-nativePCNA	Diffley lab stock (Yeeles et al., 2017)
pMD132	pET28a-HIS-Mcm10-FLag	Diffley lab stock (McClure & Diffley, 2021)

Oligos for the forked DNA in CMG unwinding assays were adapted from Langston et al. 2017 (50 duplex lead/lag). For the forked DNA used in single-molecule assays, oligos were adapted from Mueller et al. 2020.

Table 3: Oligonucleotide list

Oligo No.	Sequence
160Ld	/Phos/ACC GAT GTG GTA GGA AGT GAG AAT TGG AGA GTG TGT TTTTTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT GAG GAA AGAATG TTG GTG AGG GTTGGG AAG TGG AAG GAT GGG CTC GAG AGGTTT TTT TTT TTT TTT TTT TTT TTT TTT TTT T*T*T*T
99Lg_biteg	/BITEG/TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTTTTT TTT TTT TTT TTT CAC ACT CTC CAA TTC TCA CTT CCT ACC ACA TCG GTC GAT
Fork primer	CCT CTC GAG CCC ATC CTT CCA CTT CCC AAC CCT CAC C
blockLd_biteg	/BITEG/CTG AAA TGC CAC CTA TAG CTG CGA CTC ATG
blockLg	/Phos/AGT CGC AGC TAT AGG TGG CAT TTC AG

50duplex lead	GAGACCGAACGATCCTGTAATGTCCTAGCAAGCCAGAATTCG GCAGCGTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT*T*T* T*T*T*T
50duplex lag	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGACGCT GCCGAATTCTGGCTTGCTAGGACATTACAGGATCGTTCGGTCT C
50duplex lag Cy5	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGACGCT GCCGAATTCTGGCTTGCTAGGACATTACAGGATCGTTCGGTCT C[3'-Cy5]
50duplex lag FAM	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGACGCT GCCGAATTCTGGCTTGCTAGGACATTACAGGATCGTTCGGTCT C[3'-FAM]

2.2 Biochemistry

2.2.1 Yeast strain construction

All strains used are of W303 background. Strains for CMG overexpression were constructed by lithium acetate transformation. Colonies were tested for overexpression of the inserted protein using western blot probing.

2.2.2 Western blotting

After the transformation of a new overexpression construct, successful integration of the integration plasmid into the yeast genome was confirmed using whole cell protein extraction followed by western blotting. First, cells were grown to OD 1.0-1.5 and overexpression was induced with 2% Galactose for 4 hours (depending on the protein this might change). The equivalent of OD 4.0 of cells were collected by ultracentrifugation and resuspended in 20% TCA. After incubation for at least 20 min, cells were neutralised using 1 M TRIS base and finally resuspended in sample buffer (100 mM TRIS-HCl pH 6.8, 4% SDS, 20% glycerol and 4% β -mercaptoethanol). Cells were broken using cell bead breaking (Fastprep24) and cell debris was removed by ultracentrifugation. 5 μ l of TCA extract was separated on a 4-12% TRIS-glycine polyacrylamide gel at 150V for 40 minutes and then transferred to a nitrocellulose membranes (45 μ m) using a wet transfer tank (100V for 40 min). Membranes were passivated with 5% milk in PBS-T (PBS with 0.1% Tween-20) followed by primary antibody incubation. For CMG strain construction the following primary antibodies at a 1/1000 dilution in PBS-T with 5% milk were used:

Psf1-3: Anti-Psf1-3 (rabbit) – Diffley lab

Sld5: Anti-Sld5 (rabbit) – Diffley lab

Mcm4: Anti-Mcm4 (rabbit) – Diffley lab

Mcm6: Anti-Mcm6 (rabbit) – Diffley lab

Mcm3-CBP: Anti-CBP (rabbit) – clone C16T (04-932, Sigma-Aldrich)

Cdc45-iFlag: Anti-Flag M2 (mouse) – F316, Sigma-Aldrich

After washing with 5% milk in PBS-T, membranes were incubated with secondary antibody (anti-mouse-HRP or anti-rabbit-HRP) at a 1/5000 dilution in PBS-T with 5% milk.

2.2.3 Protein purification and labelling

CMG purification

S. cerevisiae CMG with Cdc45 internally tagged with an S6-tag (y6274) was grown overnight at 30°C in YPA (YP with 4 mg/L adenine) + 2% raffinose. The next day, the pre-culture was diluted to 12 L at OD 0.5. When OD 1.0-1.5 was reached, protein expression was induced by the addition of 2% galactose (v/v) for 6 hours at 30°C. Cells were harvested, washed with water and resuspended in CMG buffer (25 mM HEPES pH 7.5, 300 mM KCl, 2 mM Mg(OAc)₂, 0.02% Tween-20, 0.5 mM TCEP, 10% glycerol) supplemented with protease inhibitors (SIGMAFAST™). This cell suspension was frozen dropwise in liquid nitrogen, crushed by freezer milling and the cell powder was stored at -80°C.

The cell powder was resuspended in CMG buffer supplemented with 0.5 mM AEBSF, protease inhibitors (SIGMAFAST™) and benzonase. After stirring for 30 minutes at 4°C, cell debris was removed by ultracentrifugation in a Ti45 at 40,000 rpm for 60 minutes. The clarified lysate was transferred to anti-FLAG M2 agarose beads (4 mL of beads for 12L of culture) equilibrated in CMG buffer and incubated at 4°C for 4 hours. Flag beads were collected on two 20 ml disposable columns, washed with 50 column volumes (CV) CMG buffer and then incubated with ATP wash (CMG buffer with 1 mM ATP) for 10 minutes. After the final 15 CV CMG buffer wash, proteins were eluted by first incubating for 40 min in 2 CV of CMG buffer supplemented with 0.5 mg/ml 3xFLAG peptide, followed by 20 min in 1 CV of CMG buffer supplemented with 0.25 mg/ml 3xFLAG peptide. The elution fractions were combined and incubated overnight at 4°C with calmodulin affinity resin (2 mL resin for 12L of culture) supplemented with 2 mM CaCl₂. The resin was collected in a 20 mL disposable column, washed with 50 CV calmodulin wash buffer (CMG buffer with 2 mM CaCl₂) and proteins were eluted with calmodulin elution buffer (CMG buffer with 2 mM EDTA, 2 mM EGTA). The calmodulin elution was separated on a MonoQ PC 1.6/5 column with a 300-600 mM KCl gradient over 10 CV. Peak fractions were pooled, snap frozen in liquid nitrogen and stored at -80°C.

CMG labelling

Pooled MonoQ fractions were incubated with phosphopantetheinyl transferase (Sfp synthase), LD655-CoA (1:2:5) and 10 mM MgCl₂ at 23°C for 1 hour. The labelling reaction was

centrifuged for 10 min at maximum speed 4°C followed by removal of Sfp synthase and excess dye using a Superose 6 Increase 3.2/300 equilibrated in CMG buffer. Peak fractions were pooled, snap frozen in liquid nitrogen and stored at -80 °C.

Tetramer cohesin-SNAP purification

S. cerevisiae tetramer cohesin-SNAP was expressed and purified as previously described (Minamino et al., 2018).

Cohesin labelling

Tetramer cohesin was tagged with a SNAP-tag at its Smc3 subunit. The SNAP-tag is a 19 kDa tag that transfers a functional group from benzylguanine onto itself. Following the heparin column step, the pooled peak fractions were incubated with dye-benzylguanine in a ratio of 1:2 in the presence of 1 mM DTT overnight at 4°C. The excess dye was removed on a Superose 6 Increase 10/300 GL equilibrated in gel filtration buffer (50 mM TRIS-HCl pH 7.5, 150 mM NaCl, 0.5 mM TCEP, 10% glycerol). The peak fractions were pooled, snap frozen in liquid nitrogen and stored at -80°C.

Scc2-Scc4

S. cerevisiae Scc2-Scc4 was expressed and purified as previously described (Minamino et al., 2018).

Chl1

S. cerevisiae Chl1 was expressed and purified as previously described (Shrestha et al., 2023).

Protocatechuate 3,4- Dioxygenase (PCD)

T7 express LysY cells carrying PCD (pVP91A-pcaHG) were grown at 37°C overnight in LB supplemented with carbenicillin (Carb). On the following morning, cells were diluted to a ratio of 1/100 in 2x1L of LB+Carb and incubated at 37°C until an OD of 0.5 was reached. Subsequently, cells were transferred to 17°C, and once an OD of 0.7 was reached, the expression of PCD was induced by addition of 0.5 mM IPTG and 10 mg/L Fe(NH₄)₂(SO₄)₂; the cells were then maintained at 17°C for ~18 hours. Next, cells were harvested, resuspended in PCD buffer (50 mM TRIS-HCl pH 7.5, 500 mM NaCl, 10 mM Imidazole, 10% glycerol)

supplemented with protease inhibitors (SIGMAFAST™) and flash frozen in liquid nitrogen. Cells were lysed by subjecting them to three consecutive cycles of freeze-thawing followed by sonication. Cell debris was removed by centrifugation, and the clarified lysate was applied to a HisTrap column that had been equilibrated in PCD buffer. Subsequently, the column was washed with 20 mM imidazole, followed by elution with two consecutive 15 CV steps of 125 mM and 250 mM imidazole, respectively. The peak fractions were then pooled and concentrated using centrifugal filters with a 10 kDa molecular weight cut-off. Finally, the concentrated protein was applied to a Superdex 200 Increase 10/300 size exclusion column equilibrated in buffer containing 50 mM TRIS-HCl pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 10% glycerol. Peak fractions were pooled, flash frozen in liquid nitrogen and stored at -80°C. The activity of purified PCD was verified using an enzymatic activity assay, whereby the decrease in protocatechuic acid (PCA) was monitored over time as a decrease in absorbance at 290 nm.

V5-Antibody functionalisation

V5-antibody (MCA1360) was functionalised with a 20 kDa biotin-PEG-SVA linker through the use of NHS-ester coupling. Biotin-PEG-SVA was dissolved in DMSO to a final concentration of 3 mM. The V5-antibody and the dissolved PEG were mixed in a 1:20 ratio and incubated for two hours at 22°C and 400 rpm. The reaction was quenched by the addition of 2 µl of 1 M TRIS-HCl pH 7.5. The labelling reaction was applied to a Superose 6 Increase 10/300 GL size exclusion column which had been equilibrated in PBS. Typically, three peaks were observed. To ascertain the number of PEG labels per antibody, peak fractions were analysed by mass photometry. The single-PEG labelled antibody fractions were pooled, snap frozen in liquid nitrogen and stored at -80 °C.

Replication proteins

Replication proteins were expressed and purified as previously described (Posse et al., 2021; Yeeles et al., 2015, 2017).

Yeast strains were grown, harvested and lysed by the Fermentation STP (science technology platform) of the Francis Crick Institute. In short, cells were grown in YPA + 2% raffinose at 30°C to $1-3 \times 10^7$ cell/ml. Cells for overexpression of RPA, Pol ε and Ctf4 were arrested in late G1 phase by addition of alpha factor (100 ng/ml) and cells were grown for 2-3 hours until

arrested. Protein expression was induced by addition of 2% galactose and cells were grown for 3 hours at 30°C. Cells were harvested by centrifugation, washed once with buffer (25 mM TRIS-HCl pH 7.2, 0.02% NP40) and resuspended in basic lysis buffer (25 mM TRIS-HCl pH 7.2, 0.01% NP40, 1 mM DTT) supplemented with protease inhibitors (1 mM AEBSF, 10 µg/ml leupeptin and pepstatin A). This cell suspension was frozen dropwise in liquid nitrogen, crushed by freezer milling and the cell powder was stored at -80°C. The cell powder was thawed, and the volume was doubled with cold lysis buffer supplemented with protease inhibitors (see each protein for details). Cell debris was removed by ultracentrifugation in a Ti45 at 40,000 rpm for 45 minutes and the middle layer was subjected affinity chromatography.

See below for subsequent purification steps.

Where specified, an ATP wash step was carried out. 10 ml of buffer of the protein of interest supplemented with 1 mM Mg(OAc)₂ and 1 mM ATP were added to washed beads, incubated for 10 min and the flow through discarded.

Peak elution fractions were analysed by SDS-PAGE using a 4-12% TRIS-glycine gel.

Concentration steps were carried out with the specified centrifugal filter. The filter was equilibrated in buffer of the protein of interest. The protein was concentrated in steps of 5 minutes, after which the volume was checked and gently mixed by pipetting up and down.

To remove precipitates, concentrated proteins were spun down for 5-10 minutes at maximum speed and 4 °C before the supernatant was carefully applied to a chromatography column.

Purified proteins were aliquoted, snap frozen in liquid nitrogen and stored at -80°C.

Ctf4

Ctf4 buffer: 25 mM TRIS-HCl pH7.2, 0.5 mM TCEP, 10% glycerol.

Cleared lysate was added to calmodulin bead slurry (2 ml) equilibrated in Ctf4 buffer (200 mM NaCl) with 2 mM CaCl₂ and incubated for 2 hours at 4°C while rotating. The beads were collected using a 20-ml disposable column, washed with 100 ml Ctf4 buffer (200 mM NaCl)

with 2 mM CaCl_2 and the protein was eluted in three consecutive 5 ml steps of Ctf4 buffer (200 mM NaCl) with 2 mM EDTA and EGTA. Elution fractions were pooled, concentrated (Amicon ultra 10 kDa MWCO) and applied to a MonoQ 5/50 GL column equilibrated in Ctf4 buffer (150 mM NaCl). The column was washed for 20 CV with Ctf4 buffer (150 mM NaCl), and the protein was eluted with gradient elution over 30 CV from 150 mM to 1M NaCl. The peak fractions were pooled, concentrated (Amicon ultra 30 kDa MWCO) and applied to a Superdex 200 Increase 10/300 column equilibrated in Ctf4 buffer (150 mM NaCl, 1 mM EDTA). The protein was eluted in the same buffer. The peak fractions were pooled and concentrated (Amicon ultra 30 kDa MWCO).

Mrc1

Mrc1 buffer: 25 mM TRIS-HCl pH7.2, 0.02 % Igepal, 1 mM EDTA, 0.5 mM TCEP, 10% glycerol.

Cleared lysate was added to FLAG affinity resin (2 ml) equilibrated in Mrc1 buffer (400 mM NaCl) and incubated for 2 hours at 4°C while rotating. The beads were collected using a 20-ml disposable column and subsequently washed in consecutive steps of 40 ml Mrc1 buffer (400 mM NaCl), 10 ml ATP wash (see above) and 40 ml Mrc1 buffer (400 mM NaCl). For elution, 5 ml of Mrc1 buffer (400 mM NaCl) with 0.5 mg/ml 3xFlag peptide were added and collected after 5 min incubation. This process was repeated with 5 ml of Mrc1 buffer (400 mM NaCl) with 0.25 mg/ml 3xFlag peptide and two times 2 ml of Mrc1 buffer (400 mM NaCl). Pooled FLAG elution fractions were diluted with Mrc1 buffer to 200 mM NaCl and then applied to a MonoQ 5/50 GL column equilibrated in Mrc1 buffer (200 mM NaCl). The column was washed for 20 CV with Mrc1 buffer (200 mM NaCl), and the protein was eluted with gradient elution over 10 CV from 200 mM to 1M NaCl. The most concentrated and pure fractions were pooled, aliquoted and snap frozen.

RFC

RFC buffer: 25 mM HEPES pH7.6, 0.5 mM TCEP, 10% glycerol.

Cleared lysate was added to calmodulin bead slurry (2 ml) equilibrated in RFC buffer (400 mM NaCl) with 2 mM CaCl_2 and incubated for 2 hours at 4°C while rotating. The beads were collected in a 20-ml disposable column and subsequently washed in consecutive steps of 60 ml RFC buffer (400 mM NaCl), 10 ml ATP wash (see above) and 60 ml RFC buffer (400 mM

NaCl). The protein was eluted in three consecutive 5 ml steps of RFC buffer (400 mM NaCl) with 2 mM EDTA and EGTA. Pooled calmodulin elution fractions were diluted with RFC buffer to 200 mM NaCl and then applied to a HiTrap SP HP column equilibrated in RFC buffer (200 mM NaCl). The column was washed for 10 CV with Mrc1 buffer (200 mM NaCl), and the protein was eluted with gradient elution over 30 CV from 200 mM to 1M NaCl. Peak fractions were pooled, concentrated (Amicon ultra 30 kDa MWCO) and applied to a Superdex 200 Increase 10/300 column equilibrated in RFC buffer (150 mM NaCl). The protein was eluted in the same buffer. The peak fractions were pooled and concentrated (Amicon ultra 30 kDa MWCO).

Pol α

Pol α buffer: 25 mM TRIS-HCl pH7.2, 0.02% NP40, 0.5 mM TCEP, 10% glycerol.

Cleared lysate was added to calmodulin bead slurry (2 ml) equilibrated in Pol α buffer (400 mM NaCl) with 2 mM CaCl₂ and incubated for 2 hours at 4°C while rotating. The beads were collected using a 20-ml disposable column and subsequently washed in consecutive steps of 50 ml Pol α buffer (400 mM NaCl), 10 ml ATP wash (see above) and 50 ml Pol α buffer (400 mM NaCl). The protein was eluted in three consecutive 5 ml steps of Pol α buffer (400 mM NaCl) with 2 mM EDTA and EGTA. Pooled calmodulin elution fractions were diluted with Pol α buffer to 150 mM NaCl and applied to a MonoQ 5/50 GL column equilibrated in Pol α buffer (150 mM NaCl, 1 mM EDTA). The column was washed for 10 CV with Pol α buffer (150 mM NaCl, 1 mM EDTA), and the protein was eluted with gradient elution over 20 CV from 150 mM to 1M NaCl. The peak fractions were pooled, concentrated (Amicon ultra 30 kDa MWCO) and applied to a Superdex 200 Increase 10/300 column equilibrated in Pol α buffer (150 mM NaCl, 1 mM EDTA). The protein was eluted in the same buffer. The peak fractions were pooled and concentrated (Amicon ultra 30 kDa MWCO).

Pol δ

Pol δ buffer: 25 mM TRIS-HCl pH7.2, 0.02% NP40, 0.5 mM TCEP, 10% glycerol.

Cleared lysate was added to calmodulin bead slurry (2 ml) equilibrated in Pol δ buffer (400 mM NaCl) with 2 mM CaCl₂ and incubated for 2 hours at 4°C while rotating. The beads were collected using a 20-ml disposable column and subsequently washed in consecutive steps of 50 ml Pol δ buffer (400 mM NaCl), 10 ml ATP wash (see above) and 50 ml Pol δ buffer (400

mM NaCl). The protein was eluted in three consecutive 5 ml steps of Pol δ buffer (400 mM NaCl) with 2 mM EDTA and EGTA. Pooled calmodulin elution fractions were diluted with Pol δ buffer to 200 mM NaCl and applied to a HiTrap Heparin column equilibrated in Pol δ buffer (200 mM NaCl, 1 mM EDTA). The column was washed for 10 CV with Pol δ buffer (150 mM NaCl, 1 mM EDTA), and the protein was eluted with gradient elution over 15 CV from 150 mM to 1M NaCl. The peak fractions were pooled, concentrated (Amicon ultra 30 kDa MWCO) and applied to a Superdex 200 Increase 10/300 column equilibrated in Pol δ buffer (150 mM NaCl). The protein was eluted in the same buffer. The peak fractions were pooled and concentrated (Amicon ultra 30 kDa MWCO).

Pol ϵ

Pol ϵ buffer I: 25 mM HEPES-KOH pH7.6, 1 mM TCEP, 10% glycerol.

Pol ϵ buffer II: 25 mM HEPES-KOH pH7.6, 0.5 mM TCEP, 10% glycerol.

Cleared lysate was added to calmodulin bead slurry (2 ml) equilibrated in Pol ϵ buffer I(400 mM KOAc) with 2 mM CaCl₂ and incubated for 2 hours at 4°C while rotating. The beads were collected using a 20-ml disposable column and subsequently washed in consecutive steps of 50 ml Pol ϵ buffer I(400 mM KOAc), 10 ml ATP wash (see above) and 50 ml Pol ϵ buffer I(400 mM KOAc). Pooled calmodulin elution fractions were applied to a HiTrap Heparin column equilibrated in Pol δ buffer II(400 mM KOAc). The column was washed for 25 CV with Pol ϵ buffer II(450 mM KOAc), and the protein was eluted with gradient elution over 30 CV from 450 mM to 1M KOAc. The peak fractions were pooled, concentrated (Amicon ultra 100 kDa MWCO) and applied to a Superdex 200 Increase 10/300 column equilibrated in Pol ϵ buffer II(500 mM KOAc). The protein was eluted in the same buffer. The peak fractions were pooled and concentrated (Amicon ultra 100 kDa MWCO).

RPA

RPA buffer: 25 mM TRIS-HCl pH7.2, 0.5 mM TCEP, 10% glycerol.

Cleared lysate was added to calmodulin bead slurry (2 ml) equilibrated in RPA buffer (500 mM NaCl) with 2 mM CaCl₂ and incubated for 2 hours at 4°C while rotating. The beads were collected using a 20-ml disposable column and subsequently washed in consecutive steps of 50 ml RPA buffer (500 mM NaCl), 10 ml ATP wash (see above) and 50 ml RPA buffer (500 mM NaCl). The protein was eluted in three consecutive 5 ml steps of RPA buffer (500 mM

NaCl) with 2 mM EDTA and EGTA. Pooled calmodulin elution fractions were diluted with RPA buffer to 250 mM NaCl and applied to a HiTrap Heparin column equilibrated in RPA buffer (250 mM NaCl, 1 mM EDTA). The column was washed for 20 CV with RPA buffer (250 mM NaCl, 1 mM EDTA), and the protein was eluted with gradient elution over 20 CV from 250 mM to 1M NaCl. The peak fractions were pooled, concentrated (Amicon ultra 30 kDa MWCO) and applied to a Superdex 200 Increase 10/300 column equilibrated in RPA buffer (150 mM NaCl, 1 mM EDTA). The protein was eluted in the same buffer. The peak fractions were pooled and concentrated (Amicon ultra 10 kDa MWCO).

Tof1-Csm3

TC buffer: 25 mM TRIS-HCl pH7.2, 0.02% Igpal, 0.5 mM TCEP, 10% glycerol.

Cleared lysate was added to calmodulin bead slurry (2 ml) equilibrated in TC buffer (200 mM NaCl) with 2 mM CaCl_2 and incubated for 2 hours at 4°C while rotating. The beads were collected using a 20-ml disposable column and subsequently washed in consecutive steps of 30 ml TC buffer (200 mM NaCl), 10 ml ATP wash (see above) and 70 ml TC buffer (200 mM NaCl). For elution, 1 ml TC buffer (200 mM NaCl) supplemented with 2 mM CaCl_2 and 50-100 $\mu\text{l/ml}$ TEV protease were added and incubated overnight at 4°C. The eluate was collected, and beads were washed 8 times with 1 ml of TC buffer (200 mM NaCl). All fractions were pooled, concentrated (Amicon ultra 30 kDa MWCO) and applied to a Superdex 200 Increase 10/300 column equilibrated in TC buffer (150 mM NaCl, 1 mM EDTA). The protein was eluted in the same buffer. The peak fractions were pooled and concentrated (Amicon ultra 10 kDa MWCO).

Mcm10

Mcm10 buffer: 25 mM HEPES pH 7.6, 0.05% Igpal, 0.5 mM TCEP, 10 % glycerol.

BL21-codonplus-(DE3)-RIL cells carrying His-Mcm10-Flag (pMD132) were grown at 37°C overnight in LB supplemented with kanamycin and chloramphenicol (KAN+CAMP). 2L of LB+KAN+CAMP was inoculated with 25 ml of the overnight pre-culture and incubated at 37°C until an OD of 0.5 was reached. Subsequently, cells were transferred to 25°C and expression of Mcm10 was induced by addition of 1 mM IPTG for 3 hours. Next, cells were harvested, washed with PBS and finally flash frozen in liquid nitrogen. The cell pellet was thawed and resuspended in Mcm10 buffer (500 mM NaCl, 10 mM imidazole) supplemented

with protease inhibitors (SIGMAFAST™) to a final volume of 30 ml. For cell lysis, 0.1 mg/ml lysozyme was added, and cells were incubated for 20 min at 4°C followed by sonication. Cell debris was removed by ultracentrifugation and the cleared lysate was incubated with Ni-NTA beads (2 ml slurry) equilibrated in Mcm10 buffer (500 mM NaCl, 10 mM imidazole) and incubated for 1 hour at 4°C while rotating. The beads were collected using a 20-ml disposable column, washed with 80 ml Mcm10 buffer (500 mM NaCl, 10 mM imidazole) and the protein was eluted in five consecutive 1 ml steps of Mcm10 buffer (500 mM NaCl, 200 mM imidazole). Pooled elution fractions were incubated with FLAG affinity resin (2 ml) equilibrated in Mcm10 buffer (300 mM NaCl) for 2 hours at 4°C while rotating. The beads were collected using a 20-ml disposable column, washed with 100 ml Mcm10 buffer (300 mM NaCl) and the protein was eluted in five consecutive 1 ml steps of Mcm10 buffer (300 mM NaCl) supplemented with 0.5 mg/ml 3xFLAG peptide (10 min incubation each). Elution fractions were pooled, concentrated (Amicon ultra 30 kDa MWCO) and applied to a Superdex 200 Increase 10/300 column equilibrated in Mcm10 buffer (200 mM NaCl, 1 mM EDTA). The protein was eluted in the same buffer. The peak fractions were pooled and concentrated (Amicon ultra 30 kDa MWCO).

PCNA

PCNA buffer: 25 mM TRIS-HCl pH 7.2, 1 mM EDTA, 0.5 mM TCEP, 10 % glycerol. BL21-codonplus-(DE3)-RIL cells carrying native PCNA (pJY19) were grown at 37°C overnight in LB+KAN+CAMP. 5 ml of pre-culture were used to inoculate 750 ml of LB+KAN+CAMP each and incubated at 37°C. Once an OD of 0.5 was reached, 1 mM IPTG was added and PCNA was expressed for 2.5 hours at 37°C. Cells were harvested, washed with PCNA buffer with 100 mg/ml sucrose and resuspended in PCNA buffer 100 mg/ml sucrose and protease inhibitor (SIGMAFAST™+1 mM AEBSF). The cell suspension was frozen in liquid nitrogen.

The cell suspension was thawed, 0.1 mg/ml lysozyme was added, and cells were incubated for 20 min at 4°C followed by sonication. For salt precipitation, 150 mM ammonium sulfate (from 3M stock) and 0.4% polyanion P were slowly added while stirring. The lysate was spun down, the supernatant collected and slowly add 0.23 g/ml ammonium sulphate were added while stirring (add 1/3 of powder at a time and stir for 10 min, then repeat). The lysate was spun down, the supernatant collected and slowly add 0.24 g/ml ammonium sulphate were

added while stirring (add 1/3 of powder at a time and stir for 10 min, then repeat). Finally, the solution was spun down, the supernatant was discarded, and the precipitated material was resuspended in 3 ml PCNA buffer. The conductivity was adjusted to the equivalent of PCNA buffer (150 mM NaCl) by dilution with PCNA buffer. The diluted protein was applied to three with each other connected columns (HiTrap SP FF, HiTrap Heparin, HiTrap DEAE) equilibrated in PCNA buffer (150 mM NaCl). The columns were washed for 10 CV with PCNA buffer (150 mM NaCl), the HiTrap SP FF and Heparin columns were removed and after another 5 CV of washing with PCNA buffer (150 mM NaCl), the protein was eluted with gradient elution over 25 CV from 150 mM to 600 mM NaCl. The peak fractions were pooled and applied to a Capto HiRes Q 5/50 column equilibrated in PCNA buffer (150 mM NaCl). The column was washed for 10 CV with PCNA buffer (150 mM NaCl), and the protein was eluted with gradient elution over 30 CV from 150 mM to 600 mM NaCl. The peak fractions were pooled, concentrated (Amicon ultra 3 kDa MWCO) and applied to a Superdex 200 Increase 10/300 column equilibrated in PCNA buffer (150 mM NaCl). The protein was eluted in the same buffer. The peak fractions were pooled, aliquoted and snap frozen in liquid nitrogen.

2.2.4 *In vitro* ensemble assays

Cohesin ATPase assay

The rate of ATP hydrolysis of cohesin was determined using a previously established assay (Minamino et al., 2018; Murayama & Uhlmann, 2014).

140 nM cohesin, 140 nM Scc2-Scc4 and 10 ng/μl pBluescript KS II DNA were mixed in buffer (50 mM TRIS-HCl pH 7.5, 40 mM NaCl, 1 mM MgCl₂, 13.3% glycerol, 0.003% Tween-20, 0.63 mM TCEP) on ice. Reactions were initiated by the addition of 2 mM ATP spiked with [γ -³²P]-ATP and incubated at 30°C. Samples were collected after 0, 15, 30 and 60 minutes and terminated by addition of 375 mM EDTA. 0.5 μl of a reaction were spotted onto polyethylenimine cellulose F sheets and separated by thin-layer chromatography, in a saturated chamber using 0.4 M LiCl and 1 M formic acid as mobile phase. The chromatograms were exposed to a phosphor screen and imaged using phosphor-imaging. The amount of ATP hydrolysis at a given time point was quantified using ImageJ as the amount of released inorganic phosphate over remaining ATP.

Cohesin DNA loading assay

Cohesin's ability to load onto DNA was determined using a previously established assay (Minamino et al., 2018; Murayama & Uhlmann, 2014).

50 nM cohesin, 100 nM Scc2-Scc4 and 6.7 ng/μl pBluescript KS II DNA were mixed in buffer (50 mM TRIS-HCl pH 7.5, 40 mM NaCl, 0.5 mM MgCl₂, 13.3% glycerol, 0.003% Tween-20, 0.63 mM TCEP) on ice (15 μl final volume). Reactions were initiated by the addition of 0.5 mM ATP and incubated for 120 minutes at 30°C. Reactions were stopped by addition of 500 μl of CP buffer (35 mM TRIS-HCl pH 7.5, 500 mM NaCl, 10 mM EDTA, 0.35% Triton X-100, 0.5 mM TCEP, 5 % glycerol). Anti-PK antibody coated protein A-conjugated magnetic beads equilibrated in CP buffer were added to each reaction and incubated for 15 hours at 4°C while rotating. The beads were consecutively washed with CW buffer 1 (35 mM TRIS-HCl pH 7.5, 750 mM NaCl, 10 mM EDTA, 0.35% Triton X-100, 0.5 mM TCEP), CW buffer 2 (35 mM TRIS-HCl pH 7.5, 500 mM NaCl, 10 mM EDTA, 0.35% Triton X-100, 0.5 mM TCEP) and CW buffer 3 (35 mM TRIS-HCl pH 7.5, 100 mM NaCl, 10 mM EDTA, 0.1% Triton X-100, 0.5 mM TCEP). To retrieve DNA, the beads were resuspended in 15 μl elution buffer (10 mM TRIS-HCl pH 7.5, 50 mM NaCl, 0.75% SDS, 1 mM EDTA, 1 mg/ml protease K) and incubated for 20 min at 50°C. The recovered DNA was analysed by 0.8% agarose gel electrophoresis in TAE buffer. The gel was stained with SyberGold and subsequently imaged.

CMG unwinding assay

CMG unwinding was determined following a previously published protocol with minor changes (Langston et al., 2017).

The forked DNA substrate was created by annealing equal amounts of 50duplex lag FAM/Cy5 with 50duplex lead in 1XTE. The mix was heated to 95°C and allowed to slowly cool down to 20°C. 40 μl of annealed forked DNA were mixed with sucrose (5% final) and run on a 10% TBE gel with 1XTBE buffer. Bands of fully annealed fluorescent forked DNA were cut out and retrieved by electro elution into TE. Finally, forked DNA was dialysed for 2 hours against storage buffer (20 mM TRIS pH 8.0, 20 mM NaCl). The concentration of fluorescently labelled forked DNA was determined by comparison with fluorescently labelled ssDNA of known concentration.

For CMG unwinding assays, 10-25 nM CMG, 0.5-1 nM forked DNA and 0-40 nM Mcm10 were mixed in buffer (20 mM TrisOAc pH 7.6, 10 mM MgSO₄, 30 mM KCl, 2.5 mM TCEP, 0.1 mM EDTA, 40 µg/mk BSA) on ice. The reaction was started by addition of 1 mM ATP and incubated for 5-10 min at 30°C. After 1 min Reactions were quenched by addition of 3XStop buffer (30 mM TRIS-HCl pH 7.5, 60 mM EDTA, 1.5% SDS, 15% Sucrose) and separated on a 10%TBE gel. The gel was imaged using a fluorescence imager and the unwinding activity was quantified as the amount of unwound lag oligo using ImageJ.

2.3 Single-molecule methods

2.3.1 Preparation of long linear forked DNA substrate

The linear forked DNA substrate was prepared following previously published protocols with minor changes (Lewis et al., 2020; Mueller et al., 2020). It is important to obtain the plasmid pUber from Aldevron or equivalent providers, as these plasmid preps are generally cleaner and contain less nicks than compared to in-house bacterial culture and purification methods. During the preparation of the forked DNA, it is advised to pipet carefully, e.g. mix reactions by inverting the tube rather than pipetting. All oligos required for this prep were ordered as PAGE purified to maximise the efficiency of annealing.

First, pUber was digested with BstXI (2.5 Units per μg of DNA) in NEBuffer 3.1 for 8 hours at 37°C. The reaction was quenched by addition of EDTA (12 mM final) and NaCl (300 mM final) and digested DNA was separated by size exclusion chromatography over a home-made Sepharose CL-4B chromatography column (made by Celine Bouchoux) equilibrated in eluent buffer (10 mM TRIS-HCl pH 8.0, 12 mM EDTA, 300 mM NaCl). High concentrations of EDTA and NaCl are necessary to chelate Mg^{2+} and to prevent non-specific binding to the column. Peak fractions were pooled and dialysed two consecutive times for 1 hour against TE buffer (10 mM TRIS-HCl pH 8.0, 1 mM EDTA). Capping oligos were annealed in two separate reactions in 1xTE, blockLd and blockLg were annealed in a 1:6 molar ratio (15 pmol, 90 pmol), 99Lg, 160Ld and fork primer were annealed in a 1:6:60 molar ratio (15 pmol, 90 pmol, 1500pm). Annealing reactions were heated up to 95°C, and after 2 minutes, the temperature was reduced for 1°C every 40 sec until 20°C. Following this, the annealed oligos were ligated to linearised pUber (1.5 pmol, 18 μg) using T4 DNA ligase (100 Units per μg of DNA) in 1XCutSmart supplemented with 1 mM ATP and 1 mM DTT. The ligation reaction was incubated overnight at 16°C and then quenched with 12 mM EDTA and 30 mM NaCl. Excess capping oligos and T4 DNA ligase were removed by size exclusion chromatography over a home-made Sepharose CL-4B chromatography column equilibrated in eluent buffer. Peak fractions were pooled, aliquoted and snap frozen in liquid nitrogen.

2.3.2 Flow cell preparation

Functionalisation of coverslips

Coverslips used for single-molecule fluorescence experiments on a TIRF microscope were cleaned, silanised and PEG functionalised. It is important that the surface used for TIRF experiments is as clean and as homogeneous PEGylated as possible. Therefore, following this protocol in detail is crucial. Better surfaces allow for less unspecific protein and DNA binding, allowing for an overall better signal to noise ratio. The following protocol is adapted from several previous published protocols with some adjustments made (Geertsema et al., 2015; Lewis et al., 2017; Yardimci et al., 2012).

7 coverslips (40x60mm) were placed into a polypropylene staining jar, filled with anhydrous alcohol (ethanol) and sonicated for 20-30 minutes. The ethanol was removed, and coverslips were washed 10 times with Millipore purified water. The jar was filled with 5 M KOH and sonicated for 20-30 minutes. KOH was removed, and coverslips were washed 10 times with Millipore purified water. Ethanol and KOH washes were repeated once.

After removal of KOH and washing with water, coverslips were carefully dried with nitrogen gas and placed into a dry jar. Dried coverslips were plasma cleaned for 3 minutes at max intensity and placed back into the jar. For silanisation, coverslips were first washed several times with acetone to remove any traces of water, followed by incubation in 2% 3-aminopropyltriethoxysilane in acetone while shaking. The silane solution was decanted, washed once with acetone and then sonicated for 30 sec in acetone. Subsequently, coverslips were washed 20 times with Millipore purified water and immediately dried with nitrogen gas. mPEG-SVA and biotin-PEG-SVA (Laysan Bio) were equilibrated to room temperature. For 6 coverslips, 75 mg mPEG-SVA were mixed with 3 mg biotin-PEG-SVA and dissolved in 500 μ l 0.1 M NaCHO_3 . The PEG mix was carefully mixed by inversion (solution is supposed to be cloudy due to the cloud point of PEG which forms micelles) and spun down for 1 min at 3k rpm (to remove air bubbles and to pellet undissolved PEG). 3 coverslips were placed face-up in an empty freezer box. Small coverslips were placed on each end of the coverslips and 150 μ l of PEG solution was added to the middle of each coverslip. A second coverslip was placed on top face-down, sandwiching the PEG solution between the two silanised coverslips. The small coverslips were removed, and the corners of the box were filled with water to prevent drying out of the coverslips. The coverslips are incubated for 3-4 hours. Next, the

PEGylated coverslips were rinsed with water, placed into a clean jar and washed 20 times with Millipore purified water. Subsequently, coverslips are dried with nitrogen gas and the PEGylation process was repeated. After incubation overnight, double PEGylated coverslips were rinsed with water, dried with nitrogen gas and stored under vacuum for up to 2 weeks. Usually, I tried to use coverslips for DNA replication experiments within a week, as the DNA (especially the leading strand coil generated during DNA replication) was generally more susceptible to sticking to the surface if older coverslips were used.

Flow cell assembly

A standard flow cell was comprised of 7 flow channels that were shaped by molten parafilm sandwiched between a coverglass and a PEGylated coverslip.

First, 7 parallel holes were drilled into a coverglass (quartz slide). Subsequently, coverglasses were cleaned with acetone and sonicated for 30 min in ethanol. A piece of parafilm was placed onto the cleaned coverglass and 7 channels connecting the holes of the coverglass were cut into the parafilm. For side flow experiments, a flow channel was comprised of one straight channel connecting the inlet with the main outlet, but with a second channel at 90° connecting the centre of the flow channel to a second outlet. The side flow channels were arranged alternately so that 4 would fit on one coverglass. A coverslip, with its PEGylated side facing downwards, was placed onto the parafilm, and the sandwiched parafilm was molten on a heating block for 3 min at 110°C. To fully seal the coverslip, parafilm and coverglass sandwich, a small amount of epoxy glue was used around the edge of the sandwich. PE60 tubing (0.76/1.22 mm) was cut (9 cm for inlet and 3 cm for outlet), fit into the holes of the coverglass and sealed with epoxy glue.

Once a flow cell was used up, the coverglass could be recovered by thoroughly cleaning with acetone and ethanol.

First, tubing was removed, and the flow cell was placed in an acetone filled jar for at least one day. This way the coverglass could be easier separated from the parafilm and coverslip which was held together by epoxy glue. Next, the flow cell was disassembled, thoroughly cleaned by hand using acetone and sonicated for 30 min in ethanol. The sonicated coverglass is ready to use for a new flow cell.

2.3.3 Single-molecule assays

All buffers used in single-molecule experiments were filtered and degassed for at least 30 min immediately before use.

DNA tethering

Linear forked DNA is tethered to the surface of a PEGylated coverslip via biotin-streptavidin interaction. For this, an assembled flow cell is placed onto the microscope, fixed in place with blue tag and the inlet and outlet tubing of one flow channel is connected to a microfluidics pump system. One flow channel is typically used for one experiment and cannot be recycled. For all washing and DNA tethering steps a flow rate of 100 $\mu\text{l}/\text{min}$ was used. First, the flow channel was flushed with 100 μl blocking buffer (BB: 20 mM TRIS-HCl pH 7.5, 50 mM NaCl, 2 mM EDTA, 0.005% Tween-20) supplemented with neutravidin (0.2 mg/ml final) and incubated for 30 min. This is to allow for enough time that every biotin-PEG on the surface is bound to a neutravidin. Subsequently, the flow channel was washed with 300 μl BB and 10 pM linear forked DNA in BB supplemented with 0.2 μM chloroquine was introduced at 100 $\mu\text{l}/\text{min}$ for 450 μl . Untethered DNA was immediately flushed out with BB. Generally, one can adjust the density of surface-tethered DNA by either increasing the concentration and the buffer volume, or by reducing the flow rate. Before continuing with loading of proteins, new preparations of forked DNA were usually checked for successful surface tethering by flushing the flow channel with 100 μl of BB with 50 nM Sytox Orange (S.O.).

Cohesin loading

Prior to cohesin loading, the surface of the flow channel was additionally passivated by incubating for 20 min with 1 mg/ml Ultrapure BSA in cohesin loading buffer (CLB: 35 mM TRIS-HCl pH 7.5, 25 mM NaCl, 25 mM KCl, 1 mM MgCl_2 , 1 mM DTT, 0.003% Tween-20, 5% glycerol).

For cohesin loading, 1.5 nM fluorescently labelled cohesin and 3 nM Scc-Scc4 were introduced into the flow channel at 20 $\mu\text{l}/\text{min}$ for 150 μl in CLB supplemented with 1 mM ATP and 1 mg/ml BSA. Excess and non-topologically loaded cohesin were immediately removed by washing with CMG buffer (25 mM TRIS-HCl pH 7.5, 250 mM Potassium glutamate, 10 mM $\text{Mg}(\text{OAc})_2$, 0.0025% Tween-20, 1 mM DTT) supplemented with 150 mM

NaCl, followed by CMG buffer. Cohesin was imaged in CMG buffer supplemented with 150 nM S.O. or 5 nM YOYO-1 and an oxygen scavenging system (OSS, see below for details). For the experiment in the presence of a restriction enzyme, XhoI (40 units/ml) in CMG buffer supplemented with 150 nM S.O., 0.1 mg/ml BSA and an OSS was introduced into the flow channel at 50 μ l/min for 900 μ l.

CMG loading and translocation

Prior to CMG loading, the surface of the flow channel was additionally passivated by incubating for 20 min with 1 mg/ml Ultrapure BSA in CMG buffer. For cohesin-CMG encounters, this step was already done before cohesin loading. For CMG loading, 1 nM CMG-LD655 and 10 nM Scc-Scc4 were introduced into the flow channel at 20 μ l/min for 100 μ l in CLB supplemented with 0.33 mM ATP and 1 mg/ml BSA. Excess CMG was immediately removed by washing with CMG buffer supplemented with 150 mM NaCl, followed by CMG buffer. CMG was activated and translocation was imaged in CMG buffer supplemented with 10 nM Mcm10, 3 mM ATP, 1 mg/ml BSA and an OSS (see below for details). For unwinding control experiments, CMG was loaded in presence of 0.33 mM ATP γ S and during imaging 300 nM RPA were added.

Cohesin-CMG encounters

After DNA tethering, cohesin was loaded (cohesin-LD555) and washed as described above. Prior to immobilisation of cohesin, diffusive cohesin was imaged in CMG buffer supplemented with an OSS for 5 min. Subsequently, cohesin was immobilised by introducing 25 nM anti-PK-PEG-biotin in CMG buffer with 0.1 mg/ml BSA for 100 μ l at 20 μ l/min. After incubation for 3 min, the channel was washed with CMG buffer. Next, CMG-LD655 was loaded, and excess CMG was removed as described above. Cohesin-CMG encounters were imaged for 40 min in CMG buffer supplemented with 10 nM Mcm10, 3 mM ATP, 0.1 mg/ml BSA and an OSS. For experiments in presence of cohesion establishment factors, 30 nM TCM and/or Chl1/Ctf4 were added during imaging. For cohesin elution, the flow channel was flushed with CMG buffer supplemented with 50 μ g/ml PK peptide, 0.1 mg/ml BSA and an OSS, and imaged for further 25-30 min. The DNA was post stained with 150 nM S.O. in CMG buffer.

DNA replication

The single-molecule DNA replication assay is highly dependent on the condition of the surface passivation. Efficiency may be significantly reduced from one preparation to another. For this reason, it is recommended that a standard replication assay is performed with the first flow channel of a new coverslip preparation before any other experiments are performed. This will confirm whether the flow cell is suitable for replication experiments.

For additional passivation, the flow channel is incubated with 2% Tween-20 in BB for 10 min prior to DNA tethering. After DNA loading and equilibration in CMG buffer, 10 nM unlabelled CMG in CMG buffer supplemented with 0.33 mM AMPPNP and 0.1 mg/ml BSA was flushed into the flow channel at 20 μ l/min and incubated for 10 min. Replication was initiated by introducing 60 nM Mcm10, 20 nM Ctf4, 30 nM Tof1-Csm3-Mrc1, 200 nM RPA, 20 nM PCNA, 20 nM RFC, 20 nM Pol α , 20 nM Pol δ and 20 nM Pol ϵ in CMG buffer (200 mM potassium glutamate) supplemented with 150 nM S.O., 125 μ M dNTPs, 250 μ M NTPs, 5 mM ATP and 0.1 mg/ml BSA.

Cohesin-replisome encounters

Cohesin-replisome encounter experiments were carried out following the same workflow as cohesin-CMG encounters with minor changes. Importantly, the surface was passivated with 2% Tween-20 and not with 1 mg/ml BSA, as I found that BSA passivation reduced the efficiency of DNA replication, likely due to additional surface interactions of the leading strand DNA coil. Further, all buffers were supplemented with 0.1 mg/ml BSA rather than 1 mg/ml. Cohesin-LD655 was loaded, washed and imaged as described above. Unlabelled CMG was loaded, and replication as initiated as detailed above with the addition of an OSS. For experiments with immobilised cohesin, cohesin was immobilised following diffusive imaging. PK peptide elution as conducted identical to cohesin-CMG encounters.

Side flow experiments

The protein and buffer concentrations used for side flow experiments remain unchanged from normal single-molecule experiments, unless otherwise specified.

Flow cells consisting of 4 channels with one side flow channel each were prepared as described above. PE60 tubing was used to connect the two outlets to an individual microfluidics pump system each. This allowed the main and side channels to be controlled individually. First, neutravidin in BB was flushed through both channels and incubated for 30 min. The flow channel was washed through both channels with 200 μ l BB and linear forked DNA (20 pM in BB with 0.2 μ M chloroquine) was introduced through the main channel for 250 μ l at 50 μ l/min. The unbound DNA was washed sequentially through the main and side channels with BB. Next, the surface tethered DNA was visualised by introducing 2 nM YOYO-1 in BB. While a lateral flow of 50 μ l/min was applied, an ideal area for imaging DNA was determined. This is done at this point during the experiment to avoid photobleaching of cohesin and CMG later in the experiment. Once a suitable area for imaging was found, the flow channels were washed with CLB supplemented with 500 mM NaCl to remove YOYO-1. After incubating both channels with 1 mg/ml BSA in CLB, cohesin was loaded at 20 μ l/min for 150 μ l through the main channel. After sequential washes with CMG buffer supplemented with 150 mM NaCl through the main and side channels, diffusive cohesin was imaged with CMG buffer supplemented with 5 nM YOYO-1, an OSS and 0.1 mg/ml BSA. This was followed by immobilisation of cohesin with 50 nM anti-PK-AB-PEG-biotin in CMG buffer supplemented with 0.1 mg/ml BSA which was introduced into the flow channel under continuous lateral flow of 50 μ l/min for 6 min. Both channels were washed with CMG buffer and CMG was loaded at 20 μ l/min for 100 μ l through the main channel. Excess CMG was immediately removed by washing through both channels with CMG buffer supplemented with 150 mM NaCl, followed by CMG buffer. Finally, cohesin-CMG encounters were imaged in CMG buffer supplemented with 10 nM Mcm10, 5 nM YOYO-1, 3 mM ATP, 1 mg/ml BSA and an OSS while maintaining a constant flow of 5 μ l/min through the main channel. After 40 min, cohesin was eluted with 50 μ g/ml PK-peptide in CMG buffer supplemented with 5 nM YOYO-1, 0.1 mg/ml BSA and an OSS.

2.3.4 Oxygen scavenging systems

Oxygen scavenging systems (OSS) were added to maintain a stable dye fluorescence by preventing the occurrence of photobleaching and long-lived dark states. They function through removal of free oxygen from the system by an oxidising reaction.

The first system contained a mix of PCD, PCA and Trolox, and was generally used for imaging of cohesin, CMG and cohesin-CMG encounters. PCD was purified in-house as described in earlier chapters. PCA was dissolved in DMSO to a final concentration of 650 mM and stored at -20°C. Trolox was dissolved in CMG buffer to a final concentration of 4 mM and stored at -80°C for up to one month. For imaging, 20 nM PCD, 2.5 mM PCA and 1 mM Trolox were added to CMG buffer.

The second OSS system is comprised of glucose oxidase, catalase and glucose and was solely used for cohesin-replisome encounter experiments. Glucose oxidase was dissolved in 50 mM TRIS-HCl pH 7.5, 50 mM NaCl and 50% glycerol to a final concentration of 20 mg/ml. Catalase was dissolved into the same buffer to a final concentration of 3.5 mg/ml. Both solutions were sterile filtered and stored at -20°C. For imaging, final concentrations of 0.2 mg/ml glucose oxidase, 0.035 mg/ml catalase and 0.5% glucose were supplemented.

2.3.5 Data acquisition, processing and analysis

Imaging conditions

All experiments were performed on a Nikon Eclipse Ti2-E inverted TIRF microscope equipped with a 100X TIRF oil immersion objective (CFI SR HP Apo TIRF 100XC 1.49 Oil; Nikon) and a LU-NV-D laser bed (Nikon) providing 5 laser wavelengths (405, 458, 488E, 561E, 647). The temperature was maintained at 30°C by an electrically heated chamber (Okolab). Fast diffusion of cohesin and photobleaching kinetics were visualised every 100 ms for 1-2 min. Other experiments were typically visualised every 20 sec in an area of 4x4 field of views. The fluorescence signal was spectrally separated using a beam splitter (T635lpxr-UF2) and recorded on two Prime 95B sCMOS cameras (Teledyne photometrics) with a pixel area of 10 µm x 10 µm. Images were acquired using the Nikon NIS-Elements software.

Image processing

Field of views (FOV) were first separated into individual files and then saved as tiff files using NIS-Elements (Nikon). Subsequent image processing and image analysis was carried out using ImageJ/Fiji (1.54f). Each FOV was correct for stage drift using a custom written script (provided by Lisanne Spenkelink, Lewis et al., 2020). Channels were aligned manually using an overexposed S.O. stain image as a guide. Individual events occurring on dsDNA

were cropped and saved as individual tiff files for alter analysis. Kymographs were typically created using the plugin KymoResliceWide across a 3 px wide line.

Tracking of CMG translocation and replication

Single fluorescent spots on dsDNA were tracked using the Dog detector and Linear-motion LAP tracker in TrackMate v7.13.2 (Ershov et al., 2021), and the resulting trajectories were exported to MARS (Huisjes et al., 2021). Kinetic change point analysis was used to fit individual rate segments (Duderstadt et al., 2016) and the obtained rates were exported as .csv for further statistical analysis using Matlab 2023b and GraphPad Prism 10.3.0. CMG translocation rates were weighted by the duration of a segment to reflect the amount of time the CMG spends unwinding at this rate.

Tracking of cohesin for photobleaching and MSD analysis

Single fluorescent cohesin on dsDNA was tracked using TrackMate v7.13.2 (Ershov et al., 2021), and the resulting trajectories were exported to MARS (Huisjes et al., 2021). Kinetic change point analysis was used to fit photobleaching steps (Duderstadt et al., 2016) to the mean intensity of cohesin over time.

Mean square displacement (MSD) analysis was performed with the help of the Matlab class msdanalyzer (Tarantino et al., 2014). Cohesin trajectories were imported, and MSD plots were generated using msdanalyzer. The diffusion coefficient of individual cohesin molecules was estimated from the slope of a straight line fit to the first five time delays (0.1-0.5 s). The anomalous diffusion constant was estimated from the slope of a straight line fit to the first 10% of data in the log-log transformed MSD plot. Only sufficiently good fits with a goodness of the fit of $R^2 < 0.8$ were considered.

Quantification and statistical analysis

All plots showing individual data points were made in GraphPad Prism 10.3.0. Errors are reported as the standard error of the mean (SEM), unless otherwise stated. The number of molecules n is indicated in each figure, or its figure description. Experiments were conducted in at least two biological replicates ($N \geq 2$). Statistical significance was evaluated with the unpaired t-test in GraphPad Prism 10.3.0 with a statistical significance defined as $P < 0.05$.

Chapter 3. Visualisation of cohesin-CMG encounters

Chapter 3. Visualisation of cohesin-CMG encounters

In this chapter, I focus on the reconstitution of cohesin-CMG encounters and the characterisation of their individual components, *S. cerevisiae* cohesin and CMG. We adopted this bottom-up approach to study sister chromatid cohesion establishment, aiming to understand each component's contribution to these encounters before progressing to a more complicated system, such as DNA replication.

First, I reconstituted CMG helicase translocation and topological loading of cohesin at the single-molecule level using purified yeast proteins. To achieve this, I purified stoichiometric complexes, characterised their activity, and established an assay for their visualisation at the single-molecule level. Next, I combined CMG translocation with the DNA loading of cohesin to investigate their encounters in real time.

3.1 Visualisation of CMG translocation at the single-molecule level

The initial phase of my PhD was dedicated to purifying a stoichiometric yeast CMG complex. However, the existing strains and purification protocols did not yield stoichiometric CMG in my hands (J. C. Zhou et al., 2017). I decided to reconstruct the CMG strain express equal amounts of each subunit. Additionally, I inserted a 12 amino acid (AA) S6-tag internally into Cdc45 for site-specific labelling. The S6-tag can be modified at its 3rd AA (serine) by Sfp synthase, which transfers a small molecule probe, such as a fluorophore, from a Coenzyme A conjugate to the protein of interest (Z. Zhou et al., 2007). Previously published purification protocols included a single pulldown step (FLAG-Cdc45) followed by two consecutive ion exchange columns (J. C. Zhou et al., 2017). In my hands, this led to sub-stoichiometric CMG complex, with an accumulation of Cdc45 (Figure 8, A1). I adjusted the protocol by replacing the first ion exchange column with a calmodulin affinity step, as described by other labs (Baretić et al., 2020; Deegan et al., 2020). After consecutive FLAG and Calmodulin pulldowns, stoichiometric CMG was obtained (Figure 8, A2). CMG was concentrated using an ion exchange column (Figure 8, A3), followed by site specific fluorescent labelling with Lumidyne dye 655 and a size exclusion step to remove excess dye and Sfp synthase (Figure 8, A4). Purified CMG-LD655 showed minor traces of Mcm10 (66 kDa) as well as a band

around 250 kDa, which we believe might be the large subunit of polymerase ϵ , Pol2 (255 kDa). Mass spectrometry analysis revealed sub-stoichiometric traces of Mcm10 and Ctf4. Fluorescence imaging confirmed that Cdc45 was specifically labelled with LD655 (Figure 8A right).

Next, I investigated the activity of purified fluorescent CMG using an established CMG unwinding assay (Langston et al., 2017). In this assay, the unwinding of a synthetic forked DNA, consisting of 40 nucleotide (nt) poly T tails followed by 50 bp duplex DNA, is analysed via gel electrophoresis. Successful unwinding of template DNA is observed as an accumulation of unwound single-stranded DNA (lower band in gel, Figure 8B). To prevent reannealing of oligos, an excess of unlabelled lagging strand oligo is added.

I found that upon addition of ATP, CMG efficiently unwinds up to 80 % of template DNA within 5 minutes (Figure 8C). Both labelled and unlabelled CMG exhibit similar behaviour, confirming the functionality is not affected by the fluorescent label. Surprisingly, CMG was not stimulated by increasing concentrations of Mcm10, as reported in previous studies (Langston et al., 2017; Mayle et al., 2019). It is possible that traces of Mcm10 in my purified CMG are sufficient to provide efficient unwinding activity. Alternatively, given the stimulation of unwinding by addition of Mcm10 in single-molecule experiments (see below) (Wasserman et al., 2019), the bulk unwinding assay may have reached saturation already after 5 min. A difference might have become detectable at shorter times or lower CMG concentrations.

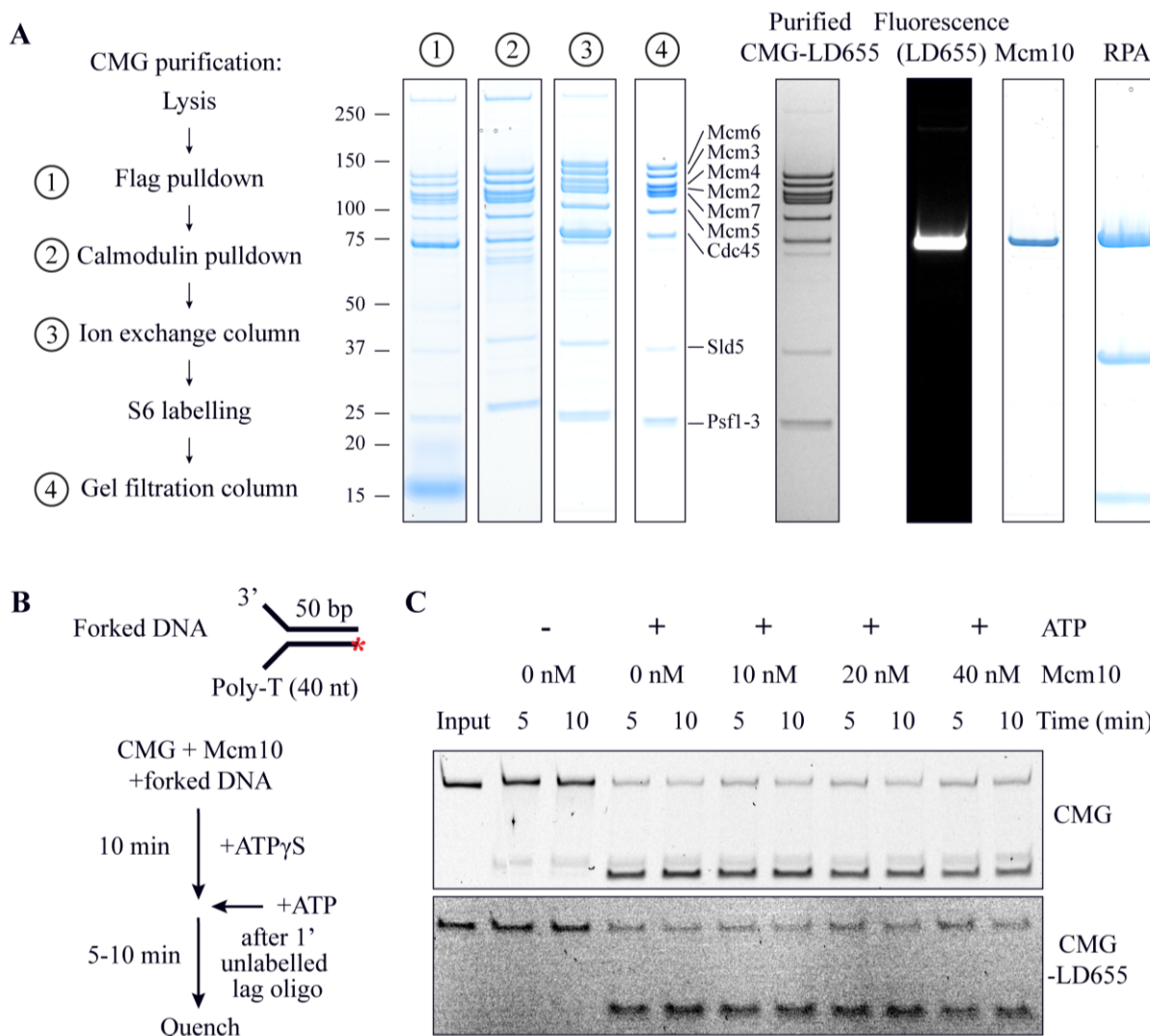


Figure 8: Purification and characterisation of budding yeast CMG.

A. Purification of CMG. Coomassie gels of each purification step and purified CMG with corresponding fluorescence image at 647 nm. Coomassie gels of purified Mcm10 and RPA.

B. Schematic of forked DNA used and experimental procedure for unwinding assays. CMG, Mcm10 and forked DNA are incubated in the presence of ATP γ S for 10 min on ice. Unwinding is initiated by the addition of ATP. After 1 min, unlabelled lagging strand oligo is added. The reaction is quenched after 5 or 10 min and analysed by gel electrophoresis.

C. CMG unwinding assay comparing unlabelled with labelled CMG. Fluorescence image showing fluorescently labelled lagging strand oligo. Reactions were quenched after 5 or 10 min, performed $\pm$ ATP, and with increasing concentrations of Mcm10.

Next, I aimed to visualise individual active CMG helicases in real time. I established a single-molecule translocation assay using a microfluidics flow cell combined with fluorescence imaging on a TIRF microscope (Figure 9A). The flow cell consists of inlet and outlet tubing that allows for stepwise influx of the desired buffer. Prior to experiments, the surface of a flow cell was passivated with polyethylene glycol-5000 (PEG) to minimise nonspecific protein and DNA sticking. Additionally, PEG was mixed with a small percentage of PEG-biotin, allowing molecules of interest to be tethered to the surface via biotin-streptavidin interaction.

First, an 18.3 kb linear forked DNA was tethered to the surface under continuous buffer flow (Mueller et al., 2020). This DNA is labelled with a biotin on each 5' end, ensuring free unrestricted rotation (Figure 9B) while tethered to the surface. The fork comprises a 150 nt leading strand that contains a 3' poly dT for CMG loading, a 37 nt primer binding site followed by a pol ϵ 'idling' sequence (4 nt sequence lacking dC and dA and a 10 nt sequence lacking dC and dT). Finally, a 40 nt poly T spacer is included to accommodate the loaded CMG. (G. Schauer et al., 2017) This fork design allows for threading of CMG onto the fork from the 3' end in the presence of ATP or ATP non-hydrolysable analogs. Under our experimental conditions, we found that flow-stretched DNA has an average length of 4.9 μm (Figure 9C, D). This length corresponds to 79% of the crystallographic length of the forked DNA and results in a tension along the DNA of 0.5 pN (calculated using the worm-like chain model).

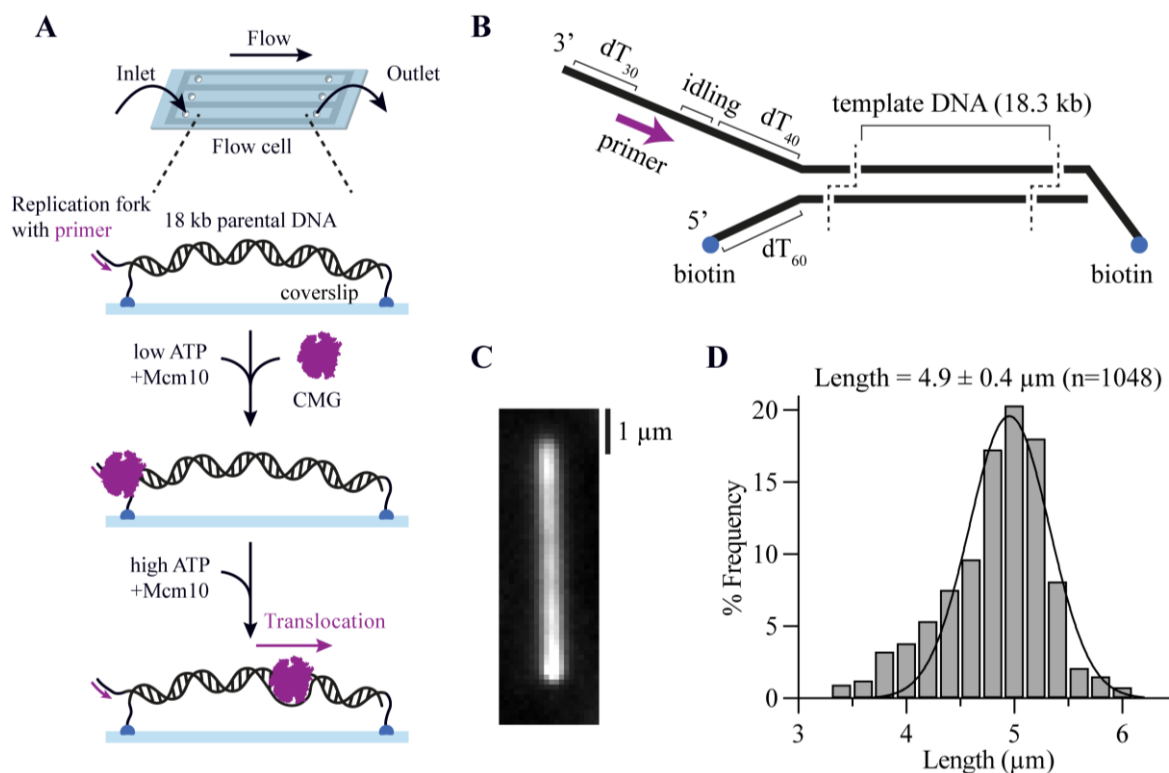


Figure 9: Single-molecule assay to visualise CMG translocation.

- A.** Schematic of the microfluidic flow cell and stepwise overview of the single-molecule CMG translocation assay.
- B.** Schematic of linear forked DNA used for all single-molecule experiments in this study.
- C.** Representative image of doubly tethered DNA in the absence of buffer flow.
- D.** Histogram of DNA length obtained from a standard single-molecule assay. A single Gaussian distribution was fitted. Error represents S.E.M. n = number of molecules.

Following DNA tethering, CMG was loaded onto the forked DNA at low ATP concentrations. After a high salt wash to remove unspecific bound CMG, translocation was imaged in the presence of high ATP concentrations. To stabilise the fluorescent signal during imaging, an oxygen scavenging system (OSS) consisting of either glucose oxidase together with catalase or PCD, PCA and Trolox was used. CMG was observed unidirectionally translocating on template DNA (Figure 10A). Most CMG helicases continuously translocated at a consistent rate along DNA. The rate of individual CMG translocation events was extracted by tracking fluorescent spots over time and fitting segments using kinetic change-point analysis (Duderstadt et al., 2016; Lewis et al., 2020) (Figure 10C). The resulting segment rates were weighted by the segment duration to reflect the time CMG was translocating at this rate. This analysis yielded an average translocation rate of 2.7 ± 0.3 bp/s (mean $\pm$ S.E.M., $n = 80$) (Figure 10D).

Previous studies indicated that addition of Mcm10 increases unwinding rates as well as the loading efficiency of yeast CMG (Langston et al., 2017; Wasserman et al., 2019). Indeed, CMG was significantly faster in presence of Mcm10 (3.9 ± 0.2 bp/s (mean $\pm$ S.E.M., $n = 128$)), consistent with previous single-molecule studies of *in vitro* activated CMG (Ramírez Montero et al., 2023). We cannot confirm if the loading efficiency is drastically changed, as Mcm10 impurities in our CMG generally seem to lead to efficient loading (Figure 8B).

We next questioned whether CMG is truly unwinding the DNA, as in our current assay DNA is reannealing behind CMG and we can merely observe CMG translocation. To this end, an excess of unlabelled RPA was added during CMG imaging. The ssDNA binding protein RPA binds unwound DNA and prevents reannealing, allowing us to visualise DNA unwinding as a loss of DNA stain behind CMG (Figure 10B, top). As expected, most CMG translocation events were accompanied by a loss of DNA stain (89 %, $n = 201$). We analysed CMG rate segments and found that in presence of RPA, CMG rates were significantly increased (6.3 ± 0.5 bp/s (mean $\pm$ S.E.M., $n = 128$)), consistent with a previous report of CMG in presence of RPA (Kose et al., 2020). Interestingly, we observed that some translocation events only temporarily exhibited a loss of DNA stain (Figure 10B, bottom). This rebinding of DNA stain indicates that the DNA is reannealing behind CMG. A possible reason for this observation

could be that CMG unwinding is interrupted by a nick, leading to CMG transitioning to dsDNA followed by translocation without unwinding along dsDNA.

In summary, I purified fluorescently labelled CMG that is functionally active in bulk as well as in single-molecule assays. Additionally, I found that CMG translocation can be stimulated by Mcm10 and RPA.

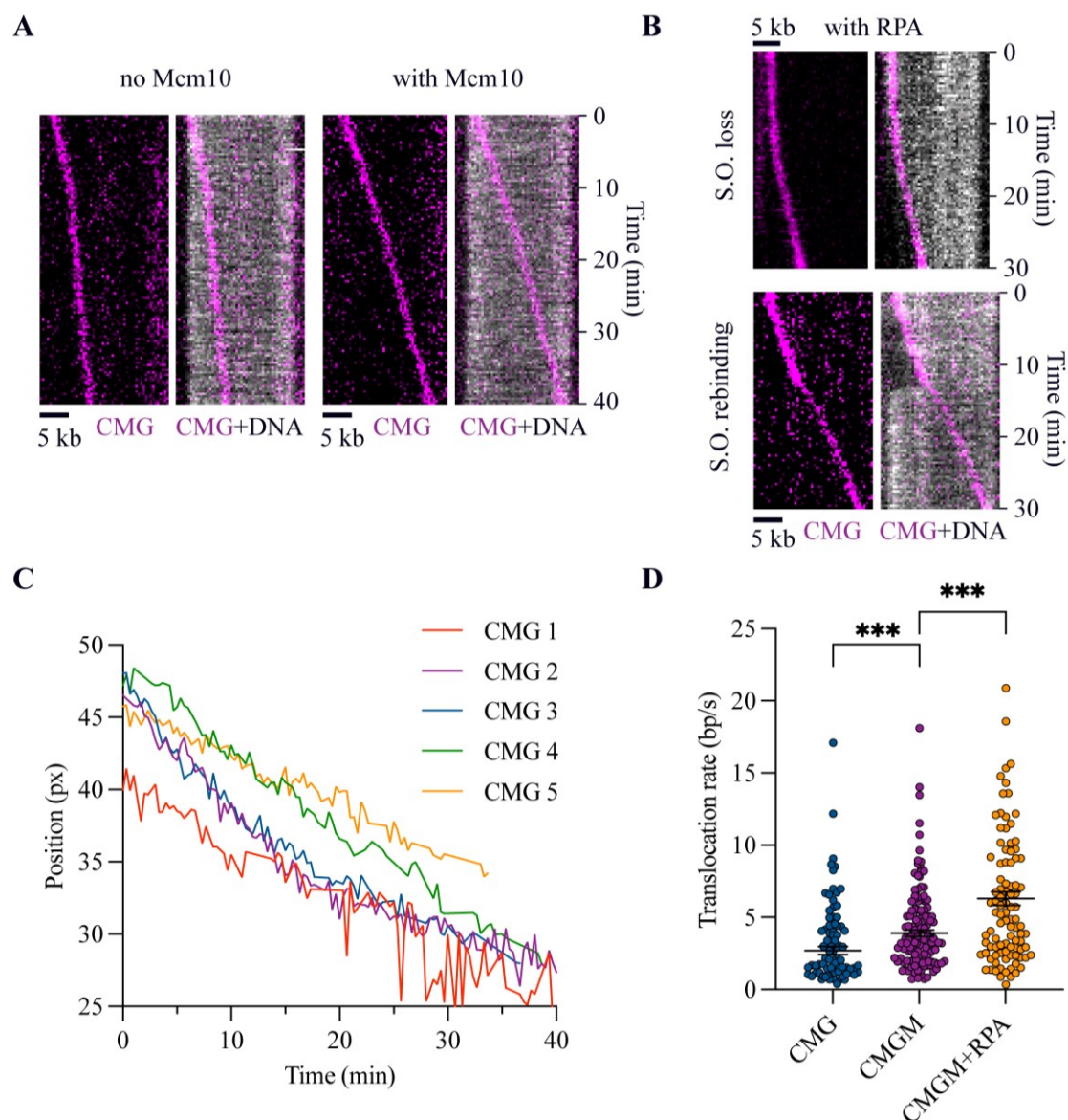


Figure 10: Characterisation of CMG translocation at the single-molecule level.

A. Representative kymographs of CMG translocation in the presence (right) and absence (left) of Mcm10.

B. Representative kymographs of CMG unwinding in presence of RPA. Top: Loss of Sytox Orange (S.O.) indicates DNA unwinding, and RPA binding to ssDNA behind CMG. Bottom: Loss of S.O. is followed by rebinding, possibly indicating a nick on DNA resulting in CMG translocation on dsDNA.

C. Representative CMG trajectories. Position as a function of time for 5 representative CMGs.

D. Distribution of segment translocation rates for CMG (blue), CMG with Mcm10 (purple) and CMG with Mcm10 and RPA (orange). Black lines with error bars represent the mean weighted by the segment duration and S.E.M. ($P < 0.001$, unpaired t-test).

3.2 Visualisation of topologically loaded cohesin at the single-molecule level

Tetrameric cohesin and Scc2-Scc4 cohesin loader were purified following previously established protocols in our lab (Minamino et al., 2018). To visualise cohesin under the microscope, we tagged Smc3 with a SNAP-tag, which could be specifically labelled using a dye-benzylguanine probe. This yielded tetramer cohesin with a specific fluorescent label at Smc3 (Figure 11A). I characterised the ATPase and DNA loading activity of fluorescent cohesin using previously established bulk assays (Minamino et al., 2018; Murayama & Uhlmann, 2014). As expected cohesin by itself shows very little ATPase activity even in presence of DNA (Figure 11B). The addition of the Scc2-Scc4 loader and DNA led to a substantial increase in ATPase activity, consistent with previously published results in budding yeast as well as fission yeast (Minamino et al., 2018; Murayama & Uhlmann, 2014) confirming that the ATPase of tetramer cohesin is unaffected by fluorescent labelling.

DNA entrapment of cohesin was investigated by loading cohesin onto circular plasmid DNA, followed by high salt washes to remove non-topologically loaded cohesin. Cohesin was then immunoprecipitated and bound DNA was analysed via gel electrophoresis. This revealed that cohesin is able to load onto DNA in absence of both, ATP and the loader (Figure 11C). This observation has been previously made for budding yeast, fission yeast and human cohesin (Davidson et al., 2016; Minamino et al., 2018; Murayama & Uhlmann, 2014) and might be linked to a small portion of cohesin that remains ATP bound throughout the purification. This portion of cohesin retains its ability to load on DNA even in absence of ATP. The addition of ATP greatly stimulates DNA loading. However, addition of the loader reduces the amount of loaded cohesin. This phenomenon is specific to budding yeast cohesin and was not observed for fission yeast cohesin (Minamino et al., 2018; Murayama & Uhlmann, 2014). The ATPase of budding yeast cohesin can be activated by Scc2-Scc4 in absence of DNA, which could reduce the pool of ATP bound cohesin therefore leading to a reduction in DNA loading (Petela et al., 2018). Surprisingly, in reactions with both, ATP and Scc2-Scc4, cohesin loading was less efficient as compared to ATP alone. Importantly, fluorescently labelled cohesin showed similar behaviour to unlabelled cohesin, confirming its functionality.

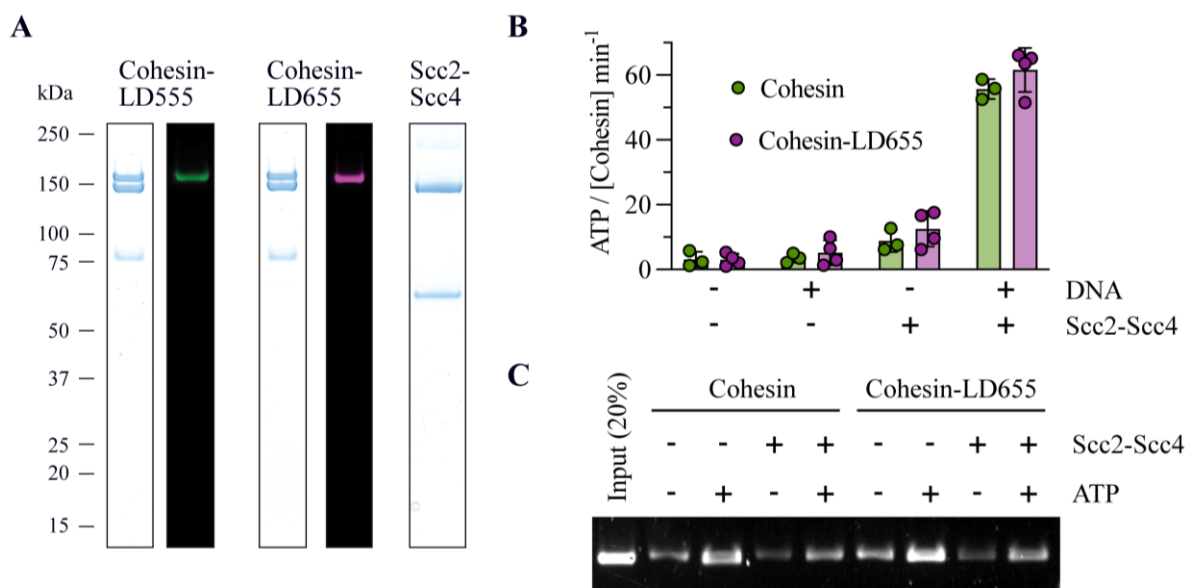


Figure 11: Purification and characterisation of budding yeast cohesin tetramer.

A. Coomassie gel and corresponding fluorescence image of purified fluorescently labelled cohesin tetramer (Smc1, Smc3, Scc1, Scc3) and Scc2-Scc4 cohesin loader.

B. Comparison of ATP hydrolysis rates of unlabelled (green) and labelled cohesin (purple) in the presence or absence of DNA and Scc2-Scc4. Each point represents the result of one biological replicate. Bar graphs show the mean with standard deviation.

C. Gel image of DNA obtained from a cohesin loading assay. Cohesin was loaded onto DNA in the presence or absence of ATP and Scc2-Scc4.

Next, we aimed to visualise topologically bound cohesin on DNA using single-molecule fluorescence microscopy. Similar to CMG translocation, we used a microfluidics flow system to tether a linear forked DNA to a passivated surface (Figure 12A). For DNA loading, cohesin was introduced into the flow cell in a buffer containing ATP and Scc2-Scc4. Non-topologically associated cohesin was washed off by flushing the flow cell with high salt buffer.

I first investigated the nature of DNA binding by the cohesin ring. To do this, Cohesin on DNA was imaged while a restriction enzyme that cleaves the DNA near its centre was flowed in. Upon cutting of the DNA, cohesin previously exhibited diffusive motion slid off the DNA (Figure 12B), strongly suggesting that cohesin was topologically entrapping the DNA rather than merely associating with it.

Next, I conducted photobleaching analysis of diffusive cohesin to investigate the oligomeric state of DNA-bound cohesin under our experimental conditions. Diffusive cohesin was monitored for 2 min in the absence of any oxygen scavenging system. We tracked single fluorescent spots on DNA over time and measured their mean fluorescence intensity (Figure 12C). The number of photobleaching steps reflects the number of cohesin in a fluorescent spot. We found that 90 % of cohesin spots bleached in a single step, indicating a single fluorescently labelled cohesin molecule. Only 10 % of cohesin exhibited two photobleaching steps (Figure 12D).

I was curious whether the loading efficiency in our single-molecule cohesin loading assay is comparable to previous bulk assays. To this end, I counted the percentage of DNA molecules with diffusive cohesin in a given field of view. On average, 10-15% of DNA molecules in a field of view were entrapped by cohesin (Figure 12E). Interestingly, DNA loading in the absence of ATP and Scc2-Scc4 was as efficient as under other conditions, contradicting previous observations in bulk loading assays. This could be explained by some cohesin complexes retaining ATP from the purification, allowing for DNA loading of cohesin in the absence of an excess of ATP. In contrary to bulk loading reactions, there is no excess DNA in solution, which might keep overall efficiencies lower for all conditions.

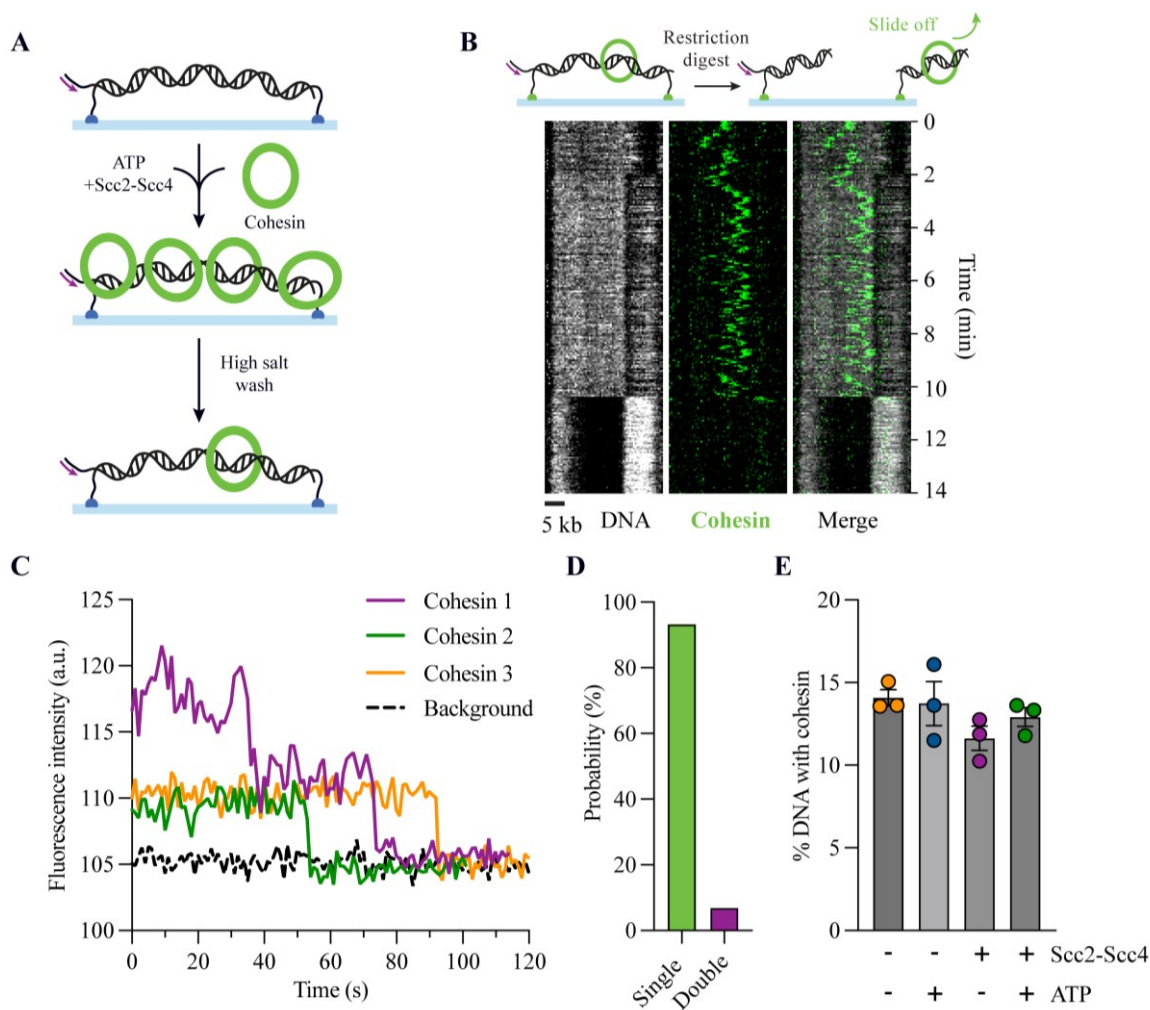


Figure 12: Single-molecule visualisation of topologically loaded cohesin.

A. Schematic of single-molecule cohesin loading assay. Cohesin is loaded onto surface-tethered DNA in the presence of ATP and Scc2-Scc4. Non-specifically bound cohesin is removed by a high salt wash.

B. Schematic and representative kymograph of cohesin^{LD655} sliding off DNA after restriction digestion of surface-tethered DNA.

C. Examples of single- and two-step photobleaching traces of cohesin^{LD655} on DNA.

D. Distribution showing the proportion of photobleaching steps for single cohesin spots on DNA (n = 44).

E. Percentage of DNA with one cohesin molecule after high salt wash. Cohesin^{LD655} was loaded in the presence or absence of ATP and Scc2-Scc4. Each point represents the fraction determined for one field of view. Bar graphs show the mean with S.E.M.

It was previously reported that individual fission yeast and human cohesin complexes move on DNA by diffusion and that diffusion coefficients increase with increasing ionic strength (Davidson et al., 2016; Stigler et al., 2016). I aimed to investigate whether budding yeast cohesin possesses similar capabilities. Cohesin was loaded onto DNA followed by a high salt wash. Next, cohesin was imaged at high temporal resolution in buffers with increasing salt concentrations (Figure 13A). Comparing cohesin behaviour at different ionic strength, it became clear that at lower salt concentrations, cohesin movement is confined and slow. The higher the salt concentration, the faster cohesin was moving along the DNA (Figure 13B). We tracked cohesin and computed mean MSD plots from which we estimated the diffusion coefficient for each condition (Figure 13C, D). As observed for fission yeast cohesin (Stigler et al., 2016) and human cohesin (Kanke et al., 2016), the diffusion coefficient positively correlated with the ionic strength. At 500 mM NaCl, cohesin exhibited fast diffusion along the entire DNA with a diffusion coefficient of $2.65 \pm 0.21 \mu\text{m}^2/\text{s}$ (mean $\pm$ S.E.M., $n = 11$), consistent with previous observations of human ($1.72 \mu\text{m}^2/\text{s}$) and fission yeast ($3.8 \mu\text{m}^2/\text{s}$) cohesin (Davidson et al., 2016; Stigler et al., 2016). PCNA, another ring protein complex that engages DNA by entrapment, was reported to have a diffusion coefficient of $1.16 \mu\text{m}^2/\text{s}$ at 500 mM K-Glutamate, consistent with our observations (Kochaniak et al., 2009). Importantly, the diffusion coefficient above 250 mM NaCl is significantly higher than the upper limit for rotational diffusion along the DNA backbone of approximately $0.03 \mu\text{m}^2/\text{s}$, further confirming that cohesin is not strongly engaging with DNA but rather entraps DNA in its ring (Blainey et al., 2009).

Additionally, we estimated the anomalous diffusion constant (α) by fitting a simple linear regression to the log-log transformed MSD vs time plot. These values indicate whether a molecule's motion is unconfined diffusive ($\alpha = 1$), confined diffusive ($\alpha < 1$), or unidirectional motion ($\alpha > 1$) (Figure 13E). Most cohesin complexes exhibited an α value below 1, indicating that cohesin is diffusive but confined. For lower ionic strength (50 and 100 mM NaCl), α reached values below 0.5, suggesting that at lower salt concentrations, cohesin-DNA interactions more frequently interfere with the diffusive mobility of cohesin. At higher salt concentrations, we estimate α values below 1 as well. Here, cohesin moved at such high frequency between the ends of the DNA that it became confined only by the length of the DNA rather than cohesin-DNA interactions.

In summary, we purified fluorescently labelled tetrameric cohesin and its loader, which showed the same activity as previously reported of budding yeast cohesin. Under our loading conditions, individual cohesin complexes topologically entrapped DNA and rapidly diffused along it in a salt-dependent manner, confined only by the length of the DNA.

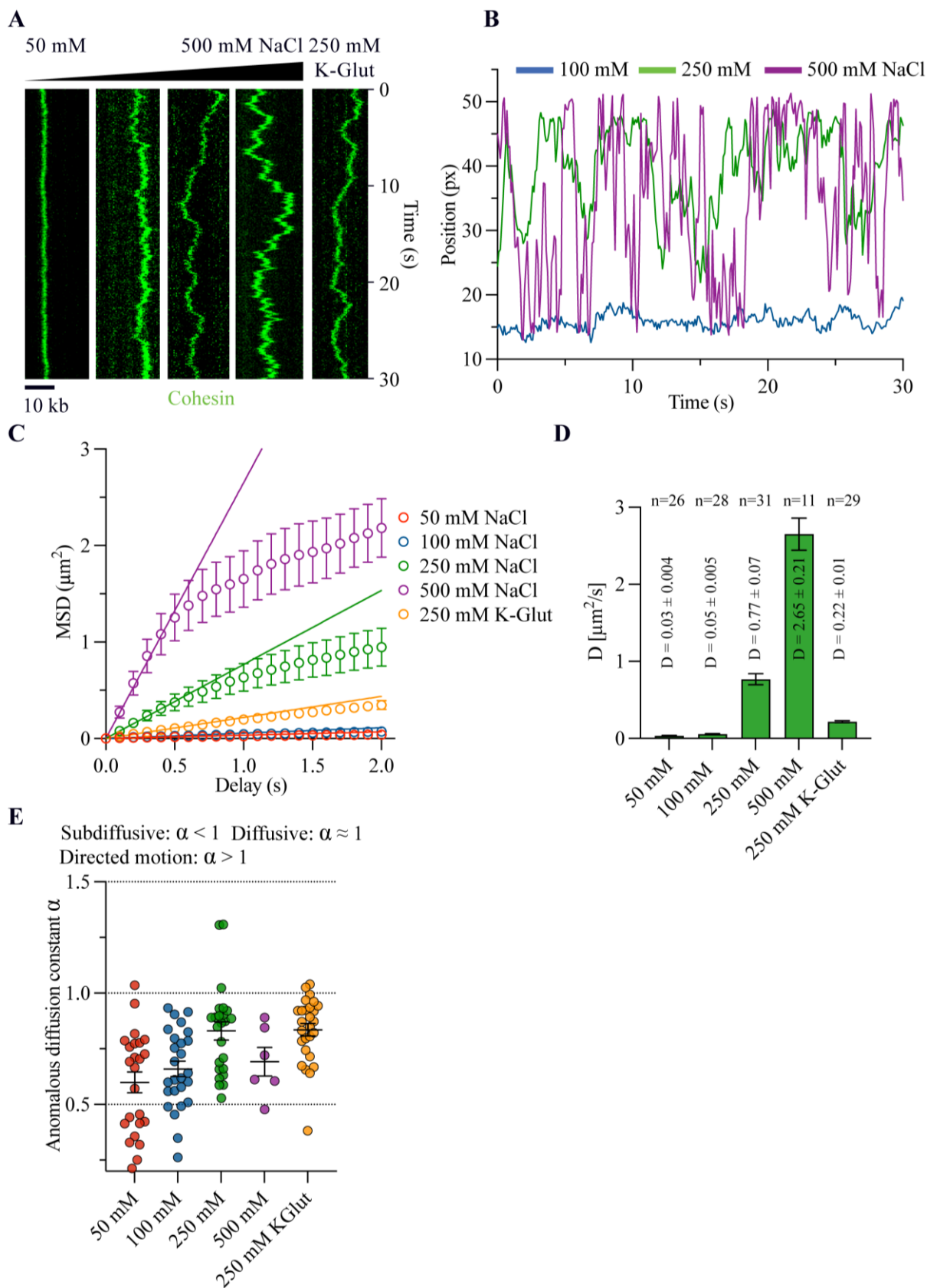


Figure 13: Cohesin diffuses on DNA in a salt dependent manner.

Figure 13: (continued)

- A.** Representative kymographs of cohesin^{LD655} motion on DNA at increasing salt concentrations.
- B.** Representative trajectories of cohesin^{LD655} at 100 mM (blue), 250 mM (green) and 500 mM (purple) sodium chloride.
- C.** Mean square displacement (MSD) as a function of delay time for cohesin^{LD655} at increasing salt concentration. For each time point the mean and S.E.M. are plotted. The first 0.5 seconds are fitted with a simple linear regression.
- D.** Diffusion constants D of cohesin^{LD655} at increasing salt concentrations determined from the slope of the simple linear regression shown in **C**. Values and bars represent the mean $\pm$ S.E.M., n is the number of cohesin molecules.
- E.** Distribution of anomalous diffusion constant α of individual cohesin^{LD655} trajectories with increasing salt concentration. α values were obtained by fitting a simple linear regression to the log-log transformed MSD vs time plot. Black lines indicate the mean with S.E.M.

3.3 The CMG helicase acts as a physical barrier for topologically loaded cohesin

After establishing a single-molecule assay for visualisation of CMG translocation and topologically loaded cohesin, I combined these assays to visualise cohesin-CMG encounters (Figure 14A). First, cohesin was loaded onto surface-tethered DNA, as described above. This was followed by CMG loading and activation. Encounters were imaged in the presence of ATP and Mcm10. We defined four different outcomes for cohesin-CMG encounters:

Pushing: CMG either directly pushes cohesin along DNA, or indirectly confines the space available for cohesin to diffuse on. A mix of both is possible in the same encounter (Figure 14B).

Passage: Cohesin passes active CMG and continues to diffuse behind CMG on the DNA (Figure 14C).

Stalling: CMG stalls upon encountering cohesin (Figure 14D).

Eviction: Cohesin is evicted when encountering CMG. This could occur either immediately at the encounter or after being pushed for some distance. (cannot be distinguished from photobleaching)

In four biological repeats ($n = 555$), we found that most cohesin is pushed along DNA by translocating CMG (Figure 14E). A minority of encounters led to stalling (5 %), passage (4%) and eviction (2 %). Due to the low probability of cohesin eviction, we cannot rule out the possibility that cohesin is undergoing photobleaching rather than being evicted from the DNA.

These observations are consistent with a collapsed conformation of cohesin with closed coiled coils that allow for passage over small obstacles (< 11 nm) but not over larger obstacles (> 21 nm) (Davidson et al., 2016; Stigler et al., 2016). A collapsed conformation of the cohesin ring would be predicted to occasionally allow passage over CMG with a diameter of 18.7 nm (estimated from PDB 6SKL, (Baretić et al., 2020)), exactly as observed in our experiments. Next, we investigated whether cohesion establishment factors would change the outcome of encounters. Strikingly, when Chl1-Ctf4 were added in excess during imaging, the chance of passage significantly increased to 8 %. Addition of Tof1-Csm3-Mrc1 also resulted in an

increase of passage, however, not statistically significant (Figure 14E). This is surprising, as both groups of proteins directly interact with CMG, thereby increasing its diameter. However, a two-fold increase in passage indicates that these factors are actively involved in facilitating the passage of cohesin past the CMG.

Cohesin is predominantly diffusive and does not actively engage with CMG, making passage rare and dependent on the correct conformation of the coiled coils at the right moment.

Cohesion establishment factors interact with cohesin as well as CMG, potentially facilitating passage by maintaining cohesin in close proximity to CMG long enough to allow for conformational changes in cohesin's coiled coils that permit passage over CMG.

Alternatively, cohesion establishment factors may interact with cohesin, thereby facilitating the opening of cohesin's coiled coils and enabling CMG passage.

Our findings suggest that CMG is a physical barrier for cohesin that can occasionally be passed.

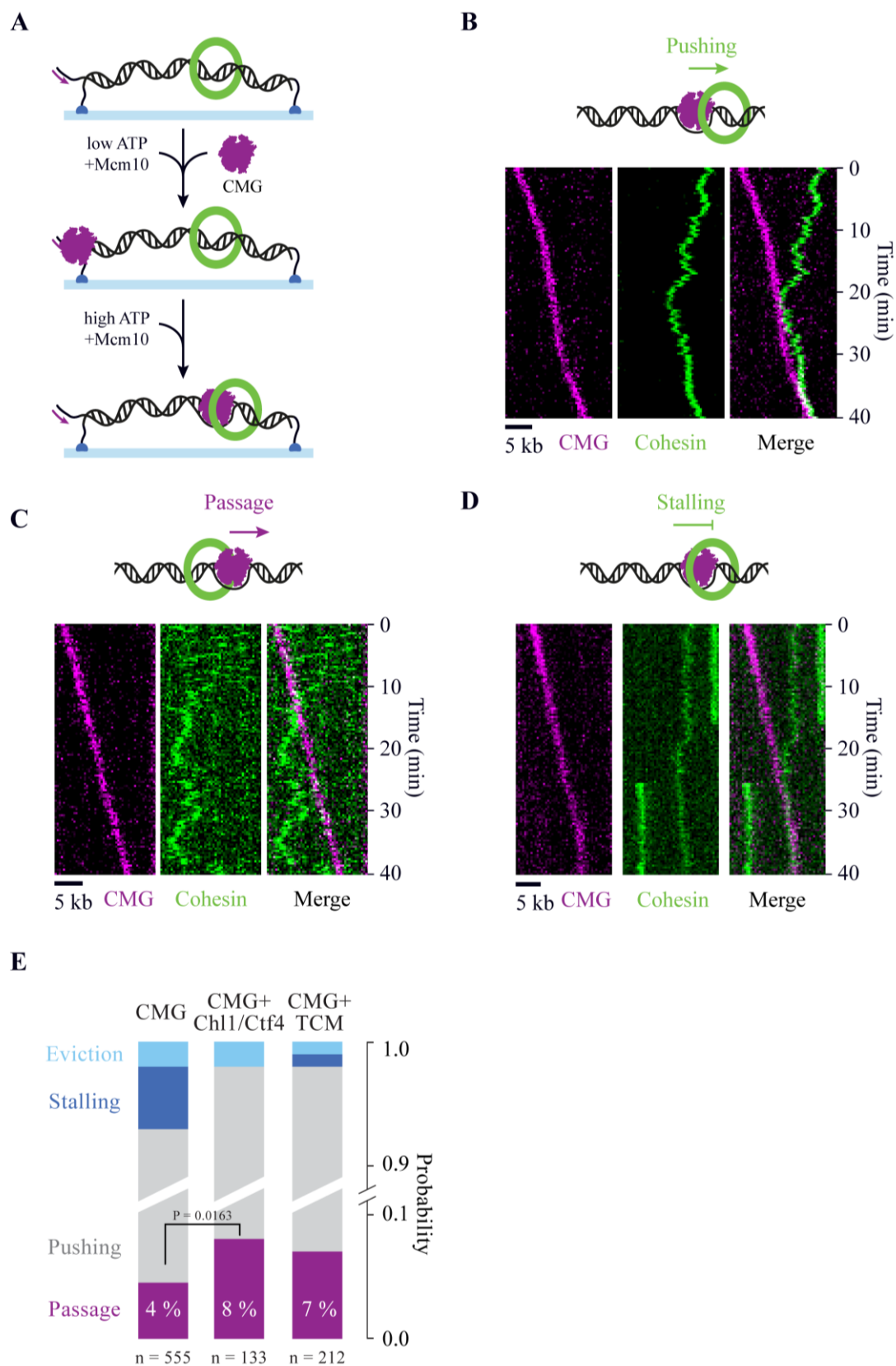


Figure 14: CMG is a physical barrier for diffusive cohesin.

Figure 14: (continued)

- A.** Schematic of the single-molecule cohesin-CMG encounter experiments. After cohesin loading, CMG is loaded in the presence of low ATP and Mcm10. Cohesin-CMG encounters are visualised in the presence of high ATP and Mcm10.
- B.** Schematic and representative kymograph of CMG pushing cohesin along DNA.
- C.** Schematic and representative kymograph of CMG passing cohesin.
- D.** Schematic and representative kymograph of CMG stalling upon encountering cohesin.
- E.** Distribution of outcomes of cohesin-CMG encounters. Values represent the mean of 4 biological replicates (CMG) and 2 biological replicates (CMG+Chl1/Ctf4 and CMG+TCM), n is the total number of encounters observed per condition. The y-axis is truncated to highlight the differences in encounter outcomes between experimental conditions. P (CMG vs. CMG+Chl1) = 0.0163, P (CMG vs. CMG+TCM) = 0.0865, unpaired t-test.

Chapter 4. CMG passage through the cohesin ring

Chapter 4. CMG passage through the cohesin ring

In the previous chapter, I focused on the reconstitution of cohesin-CMG encounters and the characterisation of each individual protein. In yeast, cohesin does not interact with CMG, unlike in higher eukaryotes where several cohesin subunits interact with the CMG helicase (Dequeker et al., 2022; Ryu et al., 2006). This could explain why freely diffusive yeast cohesin rarely engages with CMG complex, let alone passes it. However, *in vivo*, chromatin-bound cohesin faces numerous barriers, such as nucleosomes and the transcription machinery, making it difficult to freely diffuse along DNA. Therefore, we wondered whether we could confine the lateral motion of cohesin to facilitate cohesin-CMG interaction.

In this chapter, I first describe how converging CMG helicases limit cohesin diffusion and how this affects the outcome of cohesin-CMG encounters. We further modify cohesin-CMG encounters by specifically immobilising DNA-loaded cohesin. Thorough analysis of these encounters reveals interesting details about how CMG passes cohesin. Finally, we use side flow experiments to investigate whether cohesin remains associated with DNA during passage.

4.1 Converging CMG helicases pass cohesin

With the goal of limiting lateral diffusion of cohesin during encounters with CMG, I designed a new DNA template that allows for loading of two CMG helicases at opposite ends. To this end, an additional 3' flap was added to the forked DNA, creating a double-forked template DNA. After tethering the DNA to the surface of the flow cell, CMGs were loaded, washed, and imaged as before (Figure 15A). We found that most DNA molecules carried one helicase, but occasionally, two CMG helicases were loaded on opposite ends of the template DNA. When these helicases encountered each other, 56 % of encounters resulted in stalling of CMGs at the site of the encounter. In the other 44 % of encounters, CMG helicases crossed each other and continued translocation unhindered (Figure 15B, C).

CMG has been found to bypass obstacles positioned on the lagging strand (Kose et al., 2019; Langston et al., 2017). Leading strand obstacles, on the other hand, are impassable, requiring

accessory factors such as Pif1 to help CMG bypass (Deegan et al., 2019; Hill et al., 2020; G. D. Schauer et al., 2020). Therefore, the high frequency of crossing events in our single-molecule experiment strongly suggests CMGs are translocating on opposite strands rather than on dsDNA or the same ssDNA.

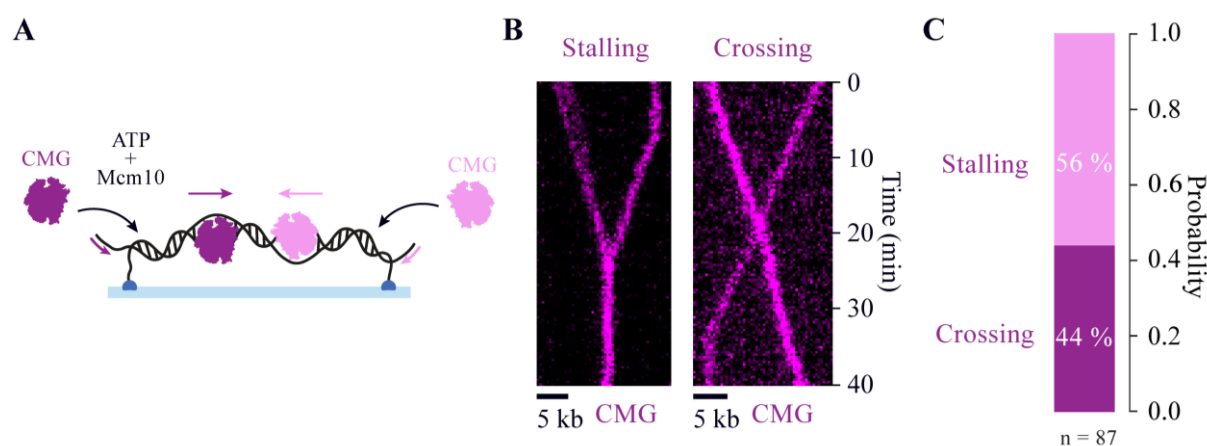


Figure 15: Single-molecule visualisation of converging CMG helicases.

A. Schematic of the single-molecule experiment to visualise converging CMG helicases. CMGs are loaded at opposite ends in the presence of ATP and Mcm10 and translocate towards each other.

B. Representative kymographs of converging CMG encounters. CMGs can either stall (left) or cross (right) each other upon encounter.

C. Distribution of outcomes of converging CMG encounters. Values represent the mean of two biological replicates, n is the total number of encounters.

Next, we investigated what would happen to cohesin when it is caught between converging CMG helicases. I performed cohesin-CMG encounter experiments on double-forked template DNA. Although the frequency of observing cohesin and two converging CMGs was low, I observed enough encounters to categorise the outcomes. We generally distinguished between stalling and crossing CMGs. When cohesin was detected between two stalling helicases, most cohesin remained trapped (84 %) (Figure 16A). Surprisingly, we observed that 16 % of cohesin complexes were able to pass over one of the stalled CMGs (Figure 16B). The chance of passage is significantly greater than our observations for the passage of a single CMG (16 % vs 4 %). Strikingly, when CMG helicases crossed each other, cohesin was always able to pass one of them (Figure 16C), suggesting that cohesin can be pushed over an obstacle by CMG.

Importantly, we did not observe any eviction of cohesin from DNA, suggesting that cohesin remains topologically loaded throughout CMG passage. As cohesin does not directly interact with CMG, our experiment can be viewed as an active motor pushing cohesin into a lagging strand obstacle. Cohesin is trapped between the motor and the obstacle, which might facilitate some conformational changes in the cohesin coiled coils that favour passage. Forces directly acting on cohesin might also play a role in opening the ring for passage, especially in a scenario where two helicases encounter each other.

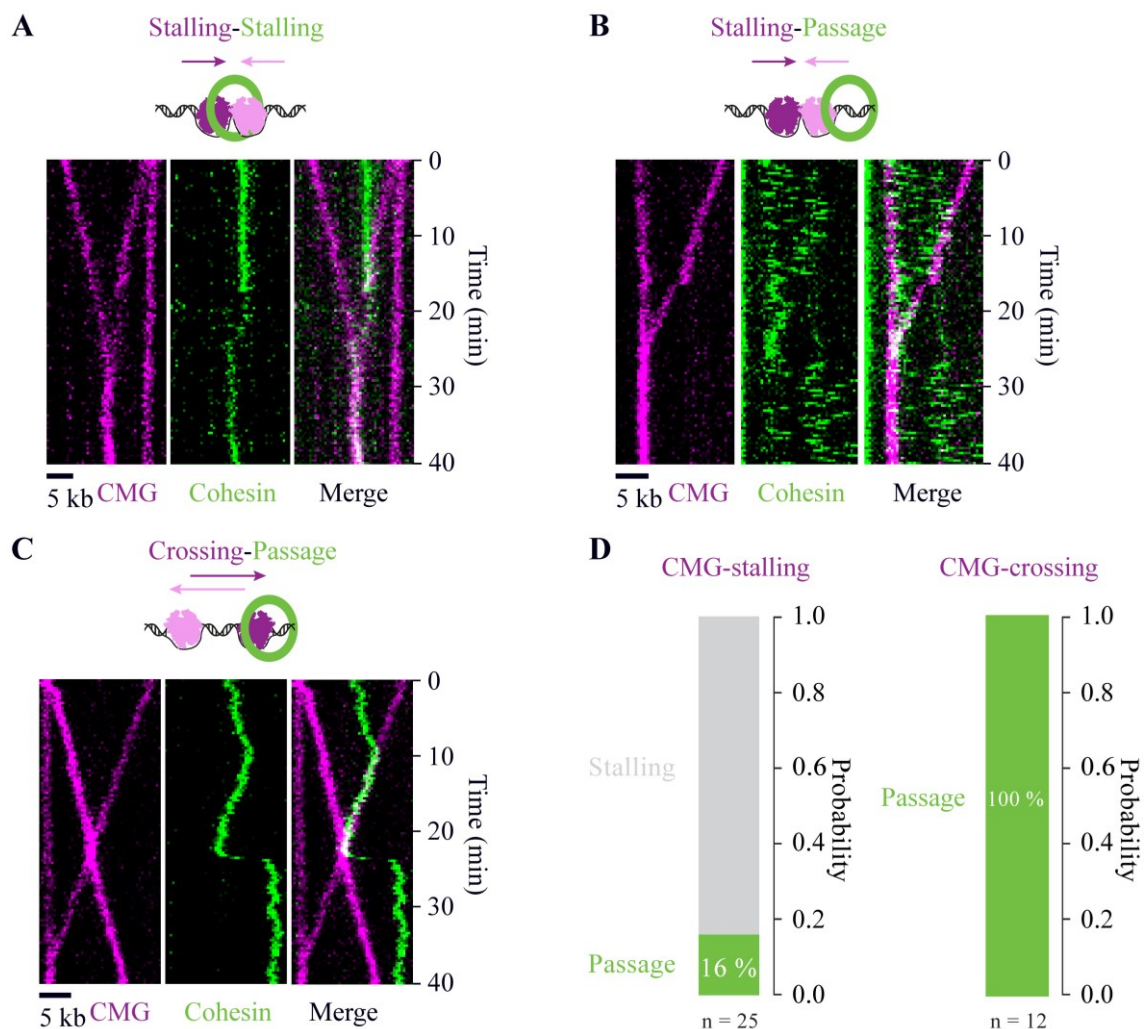


Figure 16: Converging CMG helicases pass cohesin.

- A.** Schematic and representative kymograph of cohesin stalling after stalling of converging CMGs.
- B.** Schematic and representative kymograph of cohesin passage over a CMG after stalling of converging CMGs
- C.** Schematic and representative kymograph of cohesin passage after crossing of converging CMGs.
- D.** Distribution of outcomes of cohesin-CMG encounters. Left: encounters following stalling of converging CMGs. Right: encounters following crossing of converging CMGs. Values represent the mean of two biological replicates, n is the total number of encounters.

4.2 Reversible immobilisation of DNA-loaded cohesin complexes

Decreasing the lateral diffusion of cohesin appears to be a key factor in increasing the likelihood of passage. Therefore, we were curious to see what would happen if the diffusion of DNA-loaded cohesin was prevented in another way. Additionally, we aimed to develop a system that allows for the reversible immobilisation of cohesin. To this end, we choose a different approach by immobilising topologically loaded cohesin to the surface using a functionalised antibody (Figure 17A). The antibody was created by functionalising a commercial PK- antibody with a 20 kDa biotin-PEG-linker via NHS-ester coupling. This way, the antibody binds to the surface via biotin-streptavidin interaction as well as cohesin via its PK-tag. The linker serves as an additional spacer to allow for flexibility and to prevent cohesin from non-specifically binding to the surface.

After coupling to the biotin-PEG-linker, single labelled antibody was separated via size exclusion chromatography (Figure 17B). The labelling stoichiometry was confirmed using mass photometry (Figure 17C). To test the immobilisation of cohesin using our functionalised antibody, we imaged DNA-loaded cohesin while flushing the antibody into the flow cell. Individual mobile cohesin complexes were immobilised within 1-2 min after introducing the antibody (Figure 17D, left). Importantly, neither unspecific biotinylated antibody nor specific antibody without biotin were able to immobilise cohesin (Figure 17D, middle and right), confirming the specificity of our functionalised antibody.

To reverse the antibody -cohesin interaction, an excess of PK-peptide was introduced into the flow cell. We then compared the number of diffusive cohesin detected before immobilisation and after elution with the PK-peptide (Figure 17E). After 20-30 min, most cohesin returned to a diffusive behaviour. We conclude that the functionalised antibody is an effective tool for reversibly immobilising topologically loaded cohesin.

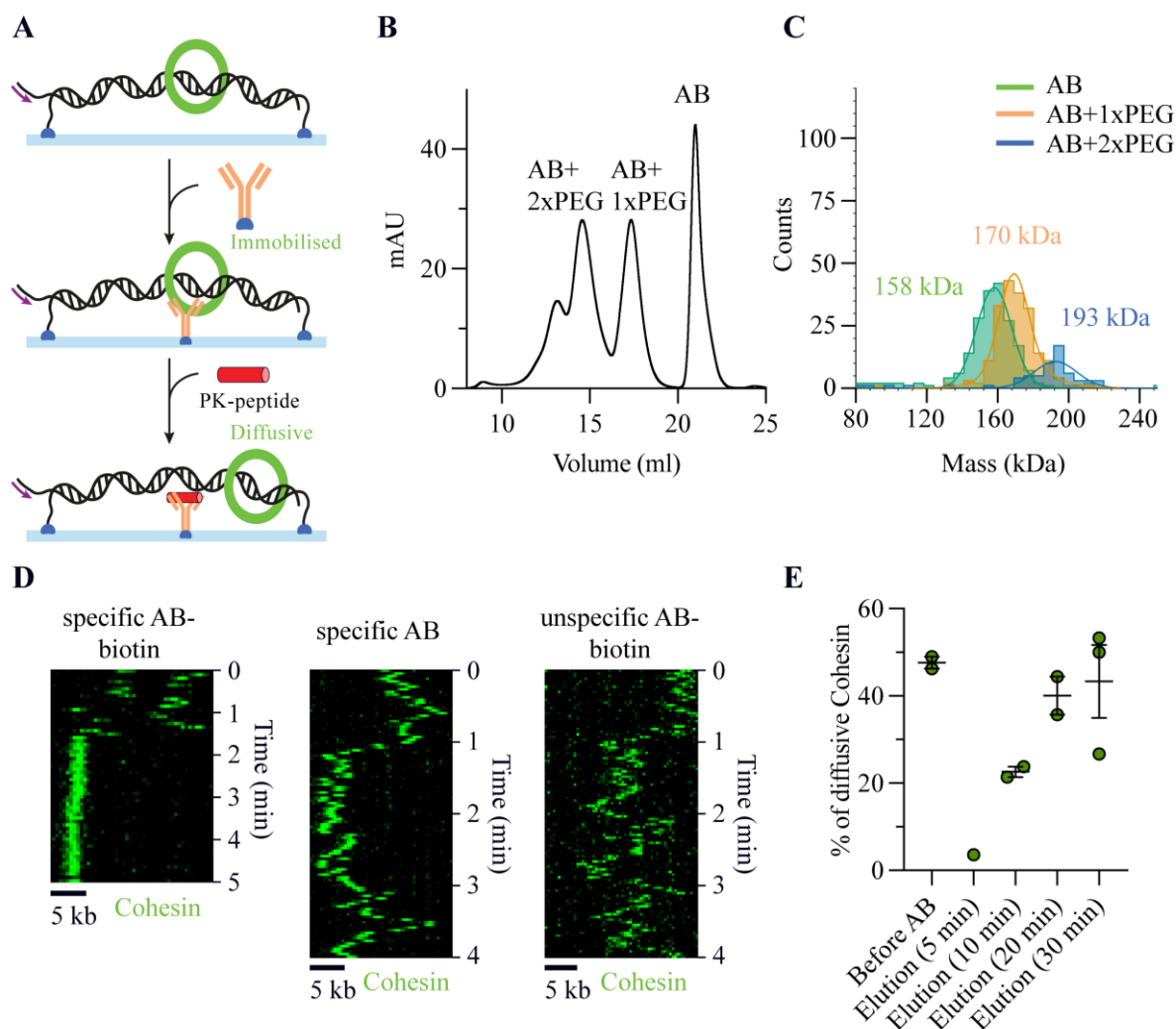


Figure 17: Cohesin immobilisation and release.

A. Schematic of cohesin immobilisation and release. After cohesin loading, functionalised antibody is introduced to immobilise cohesin. Cohesin is eluted from the antibody by peptide elution.

B. Size exclusion chromatography (SEC) profile of antibody after NHS-ester reaction with biotin-PEG-SVA.

C. Mass photometry profile of the peaks from the SEC run shown in B.

D. Representative kymographs of DNA-bound cohesin^{LD555} after flushing the flow channel with functionalised antibody (left), specific non-functionalised antibody (middle) and unspecific functionalised antibody (right).

E. Percentage of diffusive cohesin observed before antibody immobilisation and after peptide elution. Each value represents the fraction of diffusive over static cohesin in one field of view. The black lines indicate the mean with S.E.M.

4.3 CMG passes immobilised cohesin

Using our functionalised antibody, we performed cohesin-CMG encounter experiments while cohesin was immobilised. In these experiments, diffusive cohesin was first imaged for 5 minutes before any antibody was introduced. This serves two purposes: by visually inspecting several fields of view, we ensured that a reasonable amount of cohesin was loaded onto DNA prior to immobilisation. We also confirmed that cohesin was indeed loaded on DNA, which is crucial as immobilised cohesin can be easily mistaken for surface-stuck cohesin or vice versa. Following cohesin immobilisation, CMG was loaded and activated. Encounters were imaged for 40 min before Pk-peptide was introduced into the flow cell to elute antibody bound cohesin. This workflow enabled us to categorise cohesin-CMG encounters not only by their outcomes but also by the behaviour of cohesin prior to immobilisation and whether cohesin remains on DNA after the encounter with CMG. We only considered encounters where cohesin showed diffusive motion before immobilisation.

Strikingly, we find that 40 % of encounters led to the passage of CMG past immobilised cohesin (Figure 18A, D), underlining our previous observation that reducing lateral motion of cohesin drastically increases the chance of passage. Interestingly, even though cohesin was immobilised, we observed 36 % of pushing events (Figure 18B, D). Some of these instances coincided with cohesin detaching from the antibody before encountering CMG. It is possible that some antibody-cohesin interactions are disrupted in some cases, causing them to spontaneously dissociate or allowing CMG to dislodge them. Stalling of CMG at cohesin was observed for 24 % of encounters (Figure 18C, D).

Next, we specifically investigated the fate of cohesin after CMG passage, categorising cohesin behaviour during peptide elution into three groups: diffusive, static and dissociated (Figure 18E, F). Most cohesin remained on DNA after passage (85 %), with the majority reverting to their original diffusive behaviour (60 %). A minority of cohesin complexes were dissociated (15 %). To determine the difference between spontaneous dissociation of cohesin and dissociation resulting from CMG passage, we repeated the experiment without CMG. Interestingly, 10 % of cohesin dissociated from DNA (Figure 18E, green). It is possible that a fraction of this cohesin was photobleached rather than dissociated. We conclude that cohesin

retains DNA entrapment after CMG passage, with only a small fraction (approximately 5 %) dissociating.

Since cohesion establishment factors increased the chance of passage in the previous chapter, we repeated our experiments in presence of an excess of either Chl1/Ctf4 or Chl1/Ctf4+TCM. The addition of Chl1/Ctf4 increased the chance of passage from 40 to 57 %, while the addition of Chl1/Ctf4+TCM resulted in an increase to 48 % (Figure 18D). These results consistent with the idea that these factors contribute to cohesion establishment by facilitating CMG passage.

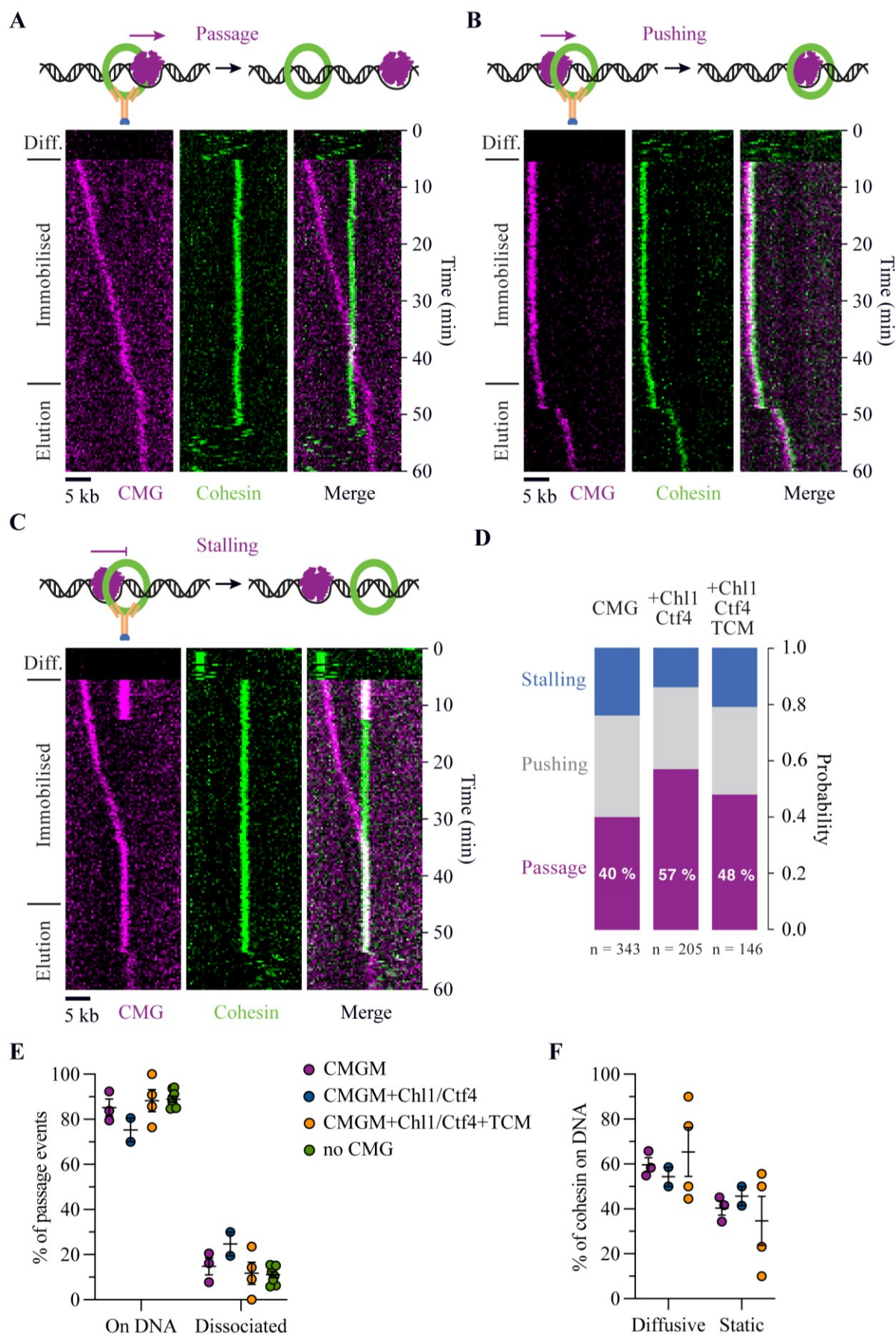


Figure 18: Cohesin remains on DNA after CMG passage.

- A.** Schematic and representative kymographs of CMG passing immobilised cohesin. Diffusive cohesin is imaged for 5 min. After immobilisation and CMG loading, cohesin-CMG encounters are imaged for 40 min, followed by peptide elution of cohesin.
- B.** Schematic and representative kymographs of CMG pushing immobilised cohesin.
- C.** Schematic and representative kymographs of CMG stalling at immobilised cohesin.
- D.** Distribution of outcomes of cohesin-CMG encounters. Values represent the mean of 3 biological replicates (CMG) and 2 biological replicates (CMG+Chl1/Ctf4) and 4 biological replicates (CMG+Chl1/Ctf4+TCM), n is the total number of encounters observed per condition. $P(\text{CMG vs. CMG+Chl1}) = 0.0926$, $P(\text{CMG vs CMG+Chl1+TCM}) = 0.4599$, unpaired t-test.
- E.** Comparison of cohesin remaining on or dissociated from DNA after CMG passage for different experimental conditions. Each data point represents an independent experiment. Black lines represent the mean with S.E.M.
- F.** Comparison of cohesin behaviour after CMG passage. Cohesin remaining on DNA is categorised as either diffusive or static. Each data point represents an independent experiment. Black lines represent the mean with S.E.M.

While watching CMG passage of cohesin events, we noticed two behaviours by which CMG passes cohesin. In the first mechanism, CMG translocation is continuous, with only brief pauses upon encountering cohesin. In the second mechanism, CMG stalls at cohesin, then, after passing it, leaps forward along DNA and resumes translocating (Figure 19A).

Our hypothesis is that CMG continues to translocate along DNA during stalling at immobilised cohesin. Specifically, CMG reels in the DNA through the cohesin ring, stretching the DNA and building up a force that acts against cohesin. This force gradually increases until CMG eventually bypasses cohesin. Subsequently, the DNA relaxes, resulting in what we observe as CMG jumping forward along the DNA.

To test our hypothesis, we conducted three-colour TIRF experiments, simultaneously visualising DNA, cohesin and CMG. If the CMG complex indeed continues translocating along the DNA during stalling, we would expect an increase in DNA stain ahead of CMG. This is because the DNA stain we use, Sytox orange, a bis-intercalator, more easily intercalates into slightly stretched or unwound DNA. Our observations during prolonged CMG stalling at cohesin support our hypothesis – the DNA signal ahead of CMG increases (Figure 19A, right).

Importantly, most cohesin remains associated with DNA even after passage events, suggesting that CMG does not break cohesin interfaces during passage. Instead, it passes through the cohesin ring, likely involving conformational rearrangements from collapsed to open coiled coils, which require force and time.

Although cohesin does not appear to dissociate from DNA, we investigated whether the force acting against immobilised cohesin could disengage any interfaces. We used the worm-like chain model, we calculated the force from individual CMG jump events by considering their location and length on the DNA (Marko & Siggia, 1995) (Calculations were performed with the help of Maxim Molodstov). We found that most passage events exhibit a force of 5 pN or lower, regardless of experimental condition (Figure 19C). Previous research by Richeldi et al. (Richeldi et al., 2023) demonstrated that cohesin can withstand a pulling force of 20 pN before its hinge interface ruptures to release entrapped DNA. Consequently, we conclude that the force generated during CMG stalling before passage is insufficient to dissociate cohesin

from DNA. Considering our hypothesis that cohesion establishment factors facilitate passage, we compared the percentages of passage events with and without a CMG jump (Figure 19B). Surprisingly, we observed no significant changes. Additionally, we explored whether cohesion establishment factors affect stalling times during passage (Figure 19D). Although we found a small decrease in stalling times, the broad distribution of observed stalling times renders none of the differences between conditions statistically significant. These findings indicate that cohesion establishment factors do not contribute to the passage of CMG when cohesin's ability to laterally diffuse is abrogated. This leads to the speculation that cohesion establishment factors may not be involved in accelerating or facilitating the passage of CMG, but rather in maintaining cohesin at CMG. This model could explain why, in encounters with immobilised cohesin, cohesion establishment factors do not alter the observations, as cohesin is already maintained at CMG.

Overall, we found that reducing lateral diffusion of cohesin significantly increase the likelihood of CMG passage. After passage cohesin remains associated with DNA, implying that CMG either passes through the cohesin ring or cohesin transiently opens its interfaces. While cohesion establishment factors slightly increase the chance of passage, they do not alter the mechanism of passage.

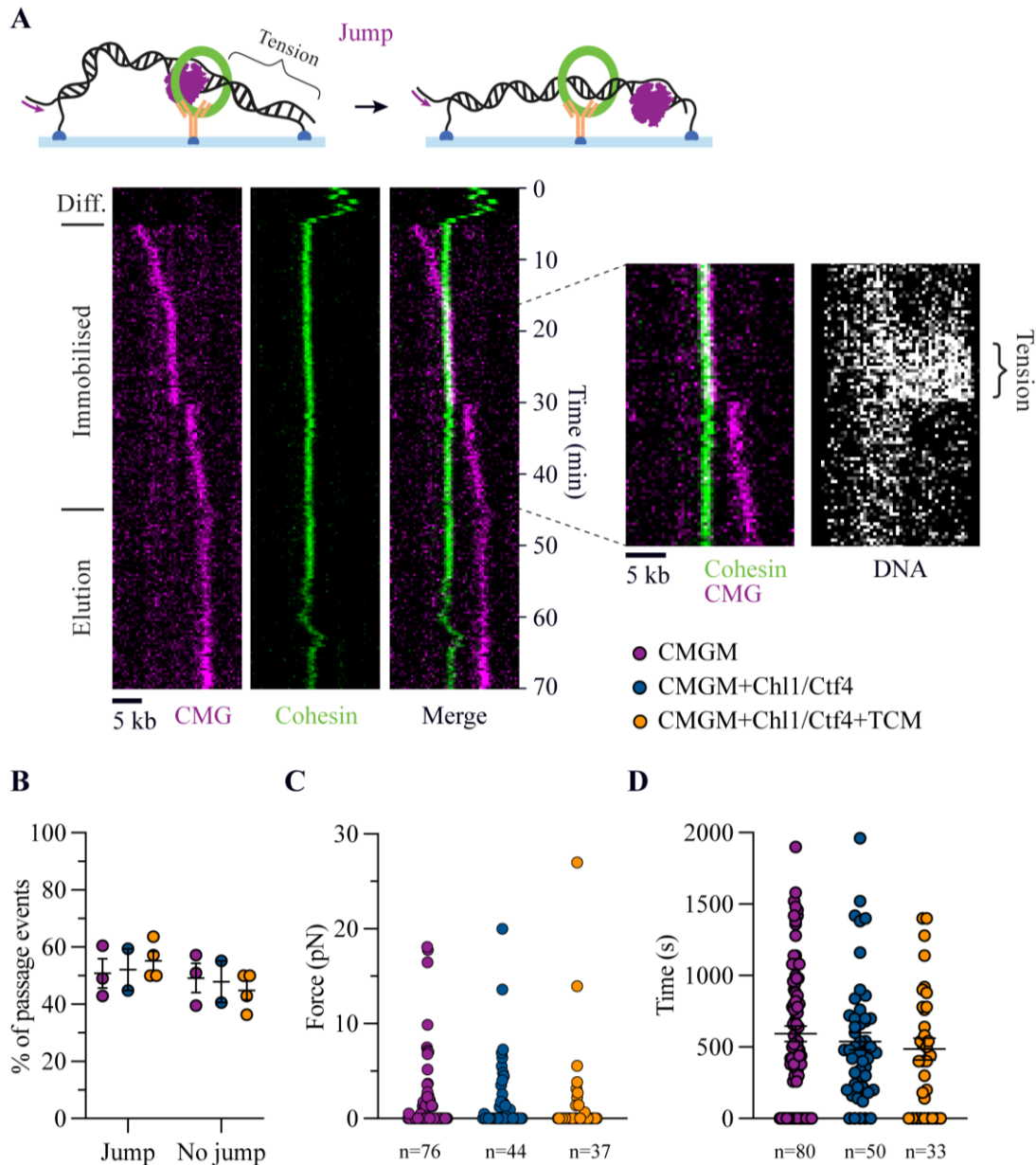


Figure 19: Cohesin withstands force created during CMG passage.

A. Schematic and representative kymograph of CMG stalling and jumping past immobilised cohesin. While stalling at cohesin, CMG continues to translocate on DNA, stretching it and creating tension. After passing cohesin, CMG is seen jumping forward as a result of DNA relaxation. Right: Enlarged view of CMG stalling and passage with corresponding DNA channel.

B. Comparison of CMG passage for different experimental conditions. CMG can pass cohesin with or without a jumping. Each data point represents an independent experiment. Black lines represent the mean with S.E.M.

C. Comparison of forces generated during CMG stalling at cohesin. Each data point represents a single passage event, n is the total number of passage events.

D. Comparison of the duration of CMG stalling before passing cohesin. Each data point represents a single passage event. Black lines represent the mean with S.E.M., n is the total number of passage events.

4.4 Cohesin remains on DNA during CMG passage

Although, we have confirmed that cohesin maintains topological contact after passage, there remains a gap of our understanding of what happens during passage. To differentiate between passage through the cohesin ring and transient disengagement of cohesin, we turned to side flow experiments. Here, cohesin is first loaded onto DNA, and after thorough washes cohesin is immobilised with the functionalised antibody in presence of a strong lateral flow. This resulted in cohesin being immobilised while the DNA adopts a laterally extended state (Figure 20A). Subsequent imaging takes place without any lateral flow, such that upon cohesin elution, both the DNA and cohesin are released back to their relaxed state. In control experiments conducted without CMG, we initially observe immobilised cohesin and DNA in a laterally extended state. As anticipated, upon cohesin elution, the DNA relaxes, and cohesin resumes diffusive motion along DNA (Figure 20B).

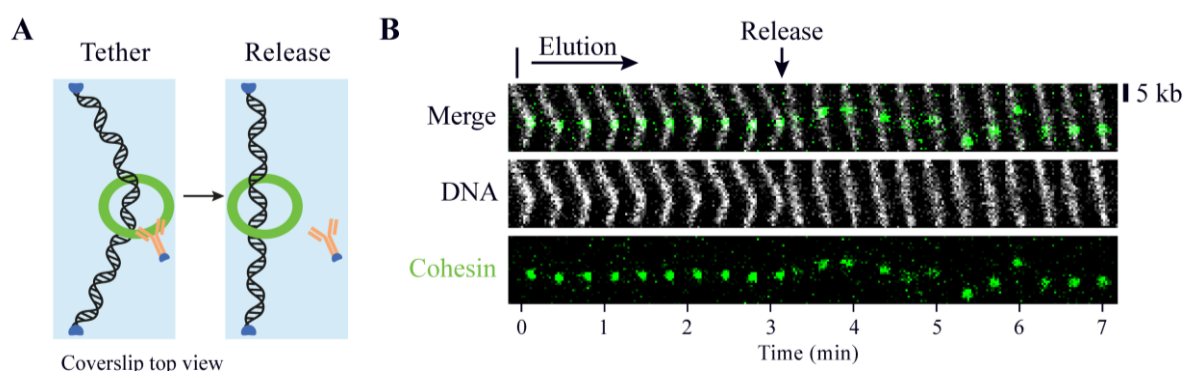


Figure 20: Reversible lateral tethering of cohesin and DNA.

A. Schematic of cohesin immobilisation during the application of side flow. As a result, the DNA, tethered by cohesin and the antibody, appears in a laterally extended state. Upon peptide elution, DNA and cohesin are released from the antibody.

B. Representative montage of immobilised cohesin tethering the DNA in a laterally extended state. After peptide elution of cohesin, the DNA and cohesin are released and return to their relaxed state (black arrow).

Our assay was designed to detect any transient opening of cohesin interfaces during CMG passage. If cohesin briefly opened an interface to allow CMG passage, the DNA would escape the cohesin ring, returning to its relaxed state while cohesin remained bound to the antibody. However, no such observations occurred. In all four passage events we observed, the DNA remained entrapped by cohesin (Figure 21B), strongly suggesting the model that CMG passes through the cohesin ring (Figure 21A). Interestingly, we did notice instances where the DNA was spontaneously released while cohesin remained bound to the antibody (Figure 21C, D), although these events did not coincide with CMG translocation. These are likely events where cohesin spontaneously unloads from the DNA, resulting in DNA release and are consistent with the ~10% of cohesin that was previously found to dissociate in the absence of CMG over the course of an experiment (Figure 18E).

In summary, our experiments provide compelling evidence for CMG passes through the cohesin ring. Nonetheless, we acknowledge the possibility that cohesin may retain non-topological contact despite interface opening during passage.

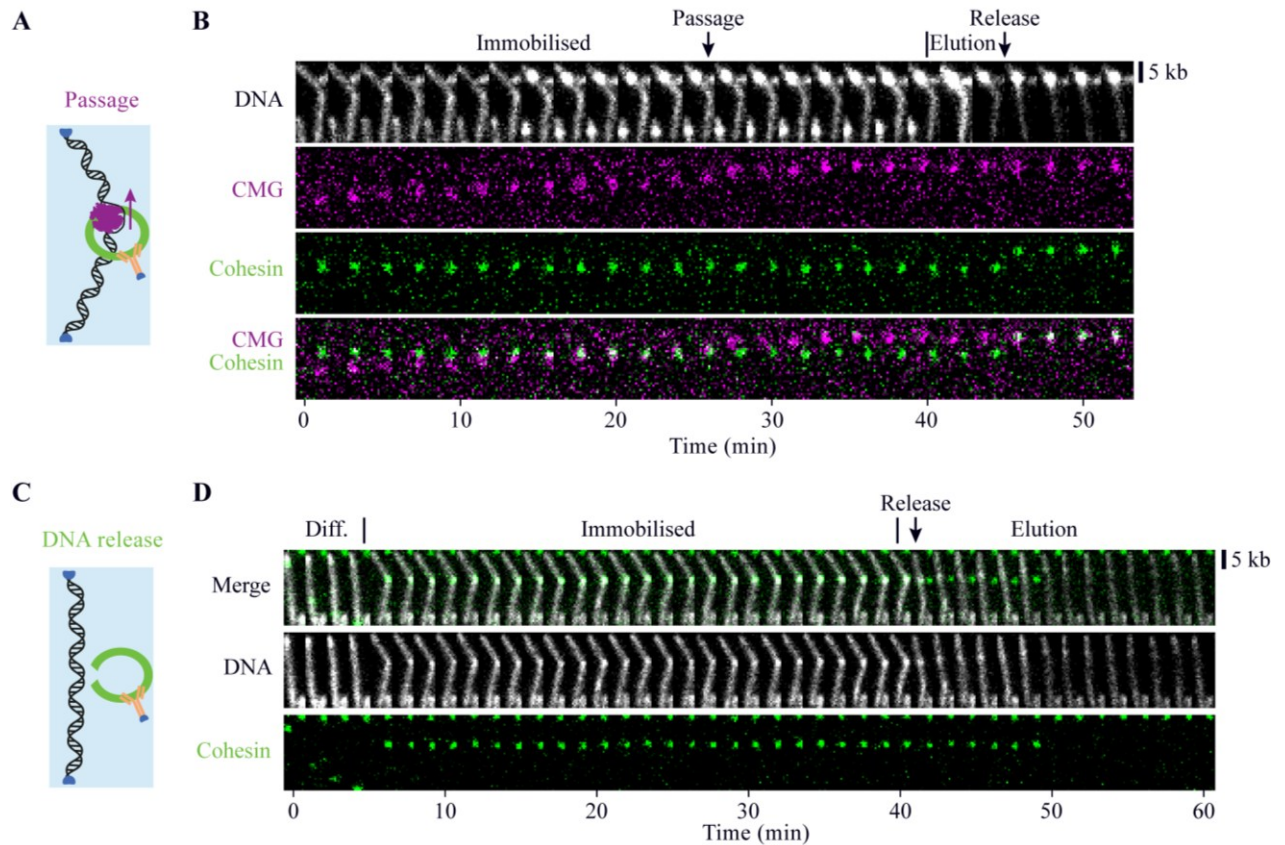


Figure 21: Cohesin remains on DNA during CMG passage.

A. Schematic of CMG passage through immobilised cohesin ring.

B. Representative montage of CMG passage through immobilised cohesin. The DNA is not released during CMG passage (Passage, black arrow). After passage, elution of cohesin from the antibody results in relaxation of the DNA (Release, black arrow).

C. Schematic of spontaneous DNA release from the cohesin ring.

D. Representative montage of spontaneous DNA release from the cohesin ring (black arrow) prior to cohesin elution. Cohesin and DNA are tethered in a laterally stretched state. DNA relaxes as it escapes the cohesin ring and cohesin disappears from the surface after peptide elution (Release, black arrow).

Chapter 5. Visualisation of cohesin-replisome encounters

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Expanding upon the minimal cohesin-CMG system in the previous chapters, our study aimed to investigate encounters between cohesin and an active replisome. During S phase, cohesin encounters CMG, which recruits additional proteins to facilitate fast and efficient DNA duplication. Given its bigger size and distinct dynamics compared to CMG alone, we were curious to see whether the outcomes change if a full-size replisome encounters cohesin. In this chapter, I describe our visualisation of *in vitro* reconstituted DNA replication using purified proteins. Additionally, we established an assay to visualise cohesin-replisome encounters, revealing significant distinctions from cohesin's encounter with the CMG helicase alone. Finally, we provide evidence that cohesion can be established by the replisome's passage through the cohesin ring.

5.1 Real-time observation of DNA replication

We initially purified additional budding yeast proteins known to be essential for fast and efficient DNA replication (Yeeles et al., 2015, 2017). These proteins include the polymerase alpha, polymerase delta and polymerase epsilon, PCNA and its loader RFC as well as the previously purified Tof1-Csm3-Mrc1 and Ctf4 (Figure 22B). To visualise DNA replication at the single-molecule level, we adopted a previously described protocol (Lewis et al., 2020). In this protocol, unlabelled CMG was loaded onto surface-tethered DNA in presence of a non-hydrolysable ATP analog. Subsequently, DNA replication was initiated by adding replication factors (TCM, Ctf4, RPA, Polymerase α , δ , ϵ , PCNA, RFC), ribonucleotides (NTP), and deoxyribonucleotides (dNTP) (Figure 22A). Without buffer flow, we visualised DNA replication using the DNA stain Sytox orange. During active DNA replication, leading strand synthesis appeared as a spot of DNA intensity that unidirectionally translocated along the DNA template (Figure 22C). This is because the leading product is not tethered to the surface and instead accumulates into a collapsed globular coil of DNA. As DNA synthesis progressed, the intensity of the leading strand spot increased. In contrast, the lagging product remained tethered to the surface and appeared as stretched-out DNA stain behind the leading strand spot.

We detected active DNA replication using fluorescently labelled CMG, confirming its presence of CMG and functionality in comparison with unlabelled CMG (Figure 22D). To benchmark our replication system against prior research, we extracted replication rates by tracking the position of the leading strand spot over time. Interestingly, replication occurred with varying rates between individual replisomes and sometimes even within a single event (Figure 22E). To account for these rate changes, we employed kinetic change point analysis, as described before (Duderstadt et al., 2016; Lewis et al., 2020). Our analysis revealed a broad distribution of replication segment rates, with an average rate of 19.9 ± 0.6 bp/s (mean $\pm$ S.E.M., $n = 376$) (Figure 22F). Notably, this average rate aligns with previously published *in vitro* single-molecule observations (Lewis et al., 2020). We also observed faster replisomes with a rate of 30 bp/s. This finding is consistent with both *in vitro* ensemble studies and *in vivo* estimates of replication (Yeeles et al., 2017) (24 bp/s), (Conti et al., 2007) (24.3 bp/s), (Hodgson et al., 2007) (31.7 bp/s) and (Sekedat et al., 2010) (26.7 bp/s)). Intriguingly, earlier studies reported a distinct bimodal distribution of replication rates, attributed to the dynamic interaction of the fork protection complex TCMN with active replisomes (Lewis et al., 2017, 2020). However, our data reveals a broader distribution of rates, without clear evidence for two distinct peaks (Figure 22F).

To further confirm the proper functioning of our replication system, we conducted experiments omitting either Mcm10, Tof1-Csm3 or Mrc1 (Figure 22G). As expected, reducing or excluding Mcm10 led to only a slight decrease in replication rate. However, omitting Tof1-Csm3 or Mrc1 resulted in a significant defect, with markedly slower rates and lower efficiencies observed. These findings align with previous *in vitro* ensemble experiments that emphasised the essential role of the Tof1-Csm3-Mrc1 complex in establishing *in vivo* rates of replication (Lewis et al., 2017; Yeeles et al., 2017). In summary, our successful reconstitution and visualisation of DNA replication *in vitro* demonstrate replication rates consistent with both *in vitro* and *in vivo* reports.

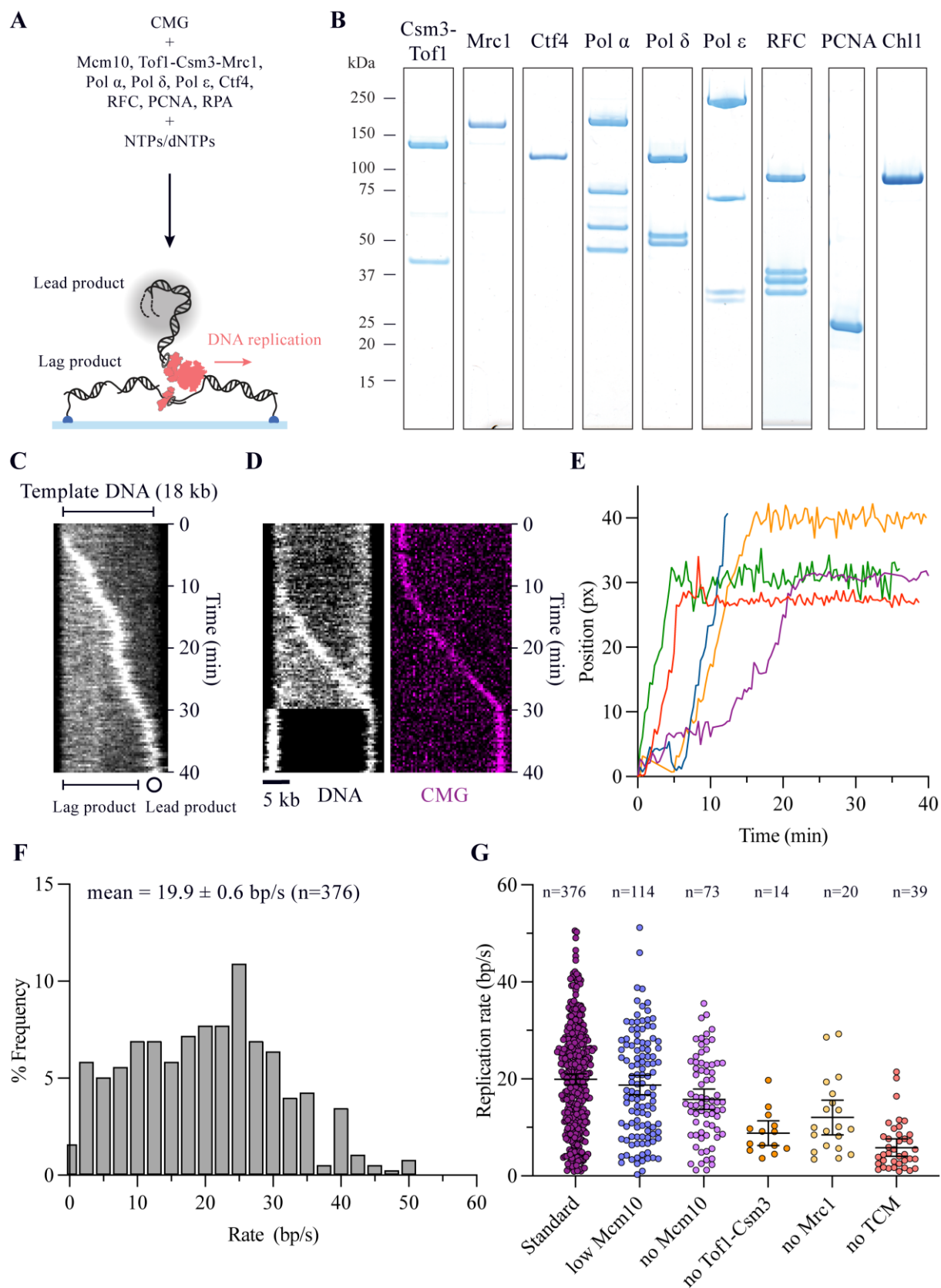


Figure 22: Single-molecule visualisation of DNA replication.

Figure 22: (continued)

- A.** Schematic of single-molecule DNA replication assay. After CMG loading onto surface-tethered DNA, DNA replication is initiated by the addition of replication factors (Mcm10, Tof1-Csm3, Mrc1, Pol α , Pol δ , Pol ϵ , Ctf4, RFC, PCNA, RPA) in the presence of NTPs and dNTPs.
- B.** Coomassie gels of purified replication factors.
- C.** Representative kymograph of DNA replication. The leading strand globular coil increases in intensity as it moves along DNA, indicating active DNA synthesis.
- D.** Representative kymograph of DNA replication in the presence of fluorescently labelled CMG. Once the replication fork reaches the end of the DNA, leading and lagging strand relax and snap back to opposite ends.
- E.** Representative trajectories of DNA replication. Position as a function of time for 5 replication events.
- F.** Histogram of single-molecule DNA replication rates. Error represents the S.E.M., n is the total number of segments.
- G.** Comparison of DNA replication rates. Experiments were performed as described in **A** (standard) or in the absence of the indicated proteins. Each data point represents a single DNA replication segment. Black lines represent the mean with S.E.M., n is the total number of segments.

5.2 Replisome passage through the cohesin ring

In our subsequent experiments we focused on visualising cohesin-replisome encounters. To achieve this, we initially loaded cohesin onto surface-tethered DNA and imaged its diffusive behaviour for 5 min in imaging buffer. This step was crucial to confirm that cohesin is indeed topologically loaded on DNA, as we have observed an increased number of static cohesin during replication reactions which were challenging to distinguish from surface-bound cohesin. The increase in static cohesin is likely due to the increased number of DNA-binding proteins present in the replication reactions, which impede the lateral diffusion of cohesin. Following cohesin loading, we proceeded to load CMG and initiated replication.

In line with our prior cohesin-CMG experiments, we consistently observed that the most cohesin-replisome encounters led to pushing of cohesin along the DNA (73 %) (Figure 23A). However, a challenge arises from the leading strand spot accumulating in a globular coil behind the replisome. This accumulation makes it difficult to distinguish between two scenarios: direct pushing of cohesin and cohesin passing the replisome but subsequently becoming ensnared at the leading strand coil, which then drags cohesin along the DNA. Eviction of cohesin from the DNA (3 %) and stalling of the replisome at cohesin (6 %) remain rarely observed events (Figure 23B).

Remarkably, we find a substantial increase in the likelihood of passage to 18 %, in contrast to 4 % detected for cohesin-CMG encounters (Figure 23D). As the replisome passes, cohesin remains behind, while the DNA intensity between cohesin and the moving replisome doubles in intensity, indicating synthesis of leading and lagging strand products.

Notably, most of these passage events coincide with cohesion between leading and lagging strand replication products (Figure 23C). During these instances, the leading strand spot encounters cohesin and pushes it for some distance along DNA. Subsequent stalling occurs, while DNA replication continues, resulting in increased stretching force along the DNA and an immediate increase in DNA signal ahead of the replisome (similar to observations with stalled CMG). Once the replisome passes, the force dissipates instantly. Interestingly, after passage, we observe a gradual increase of DNA intensity moving towards the far end of the DNA, while the leading strand spot remains trapped at cohesin.

These findings provide evidence that cohesin maintains cohesion between leading and lagging strand products, while the replisome continues DNA synthesis and progresses along DNA. Our data indicate that passage of the replisome results in capture of leading and lagging strand products within the cohesin ring, thereby initiating cohesion. The observed increase in likelihood of passage, relative to prior experiments involving only the CMG helicase, suggests that components of the replisome beyond the previously identified cohesion establishment factors may play a role in facilitating this process. Importantly, approximately 75% of replisome passage events are accompanied by cohesion establishment (Figure 23E).

We repeated these experiments in the presence of Chl1. Surprisingly, we did not observe any significant changes in the likelihood of replisome passage (Figure 23D, right). This finding suggest that Chl1 may not be essential for establishing cohesion directly but rather plays a role in processes leading up to cohesin-replisome encounters. For instance, Chl1 might be involved in ensuring proper cohesin loading (Murayama et al., 2024). Alternatively, Chl1 could play a role during cohesion establishment in a more complex chromatin context.

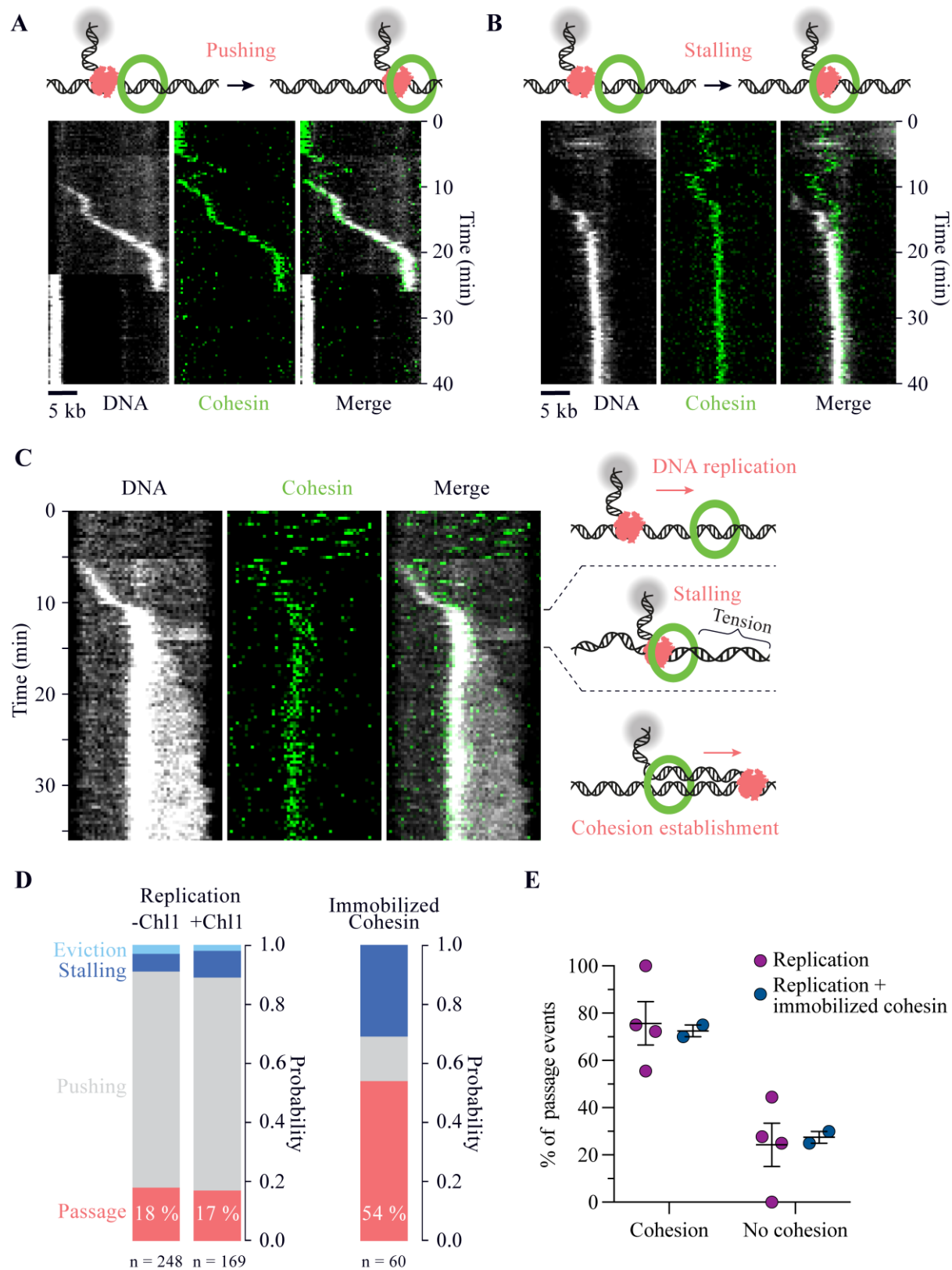


Figure 23: Replisome passage through the cohesin ring establishes cohesion.

Figure 23: (continued)

A. Schematic and representative kymograph of DNA replication pushing DNA-bound cohesin.

B. Schematic and representative kymograph of DNA replication stalling at DNA-bound cohesin.

C. Representative kymograph of DNA replication passing through cohesin (left). Schematic of cohesin-replisome encounter (right). An active replisome approaches cohesin. As it stalls, at cohesin, ongoing DNA synthesis generates a force that stretches the DNA in front of cohesin. After the replisome has passed through the cohesin ring, the newly synthesised leading and lagging strands are held together by cohesin, indicating the establishment of cohesion.

D. Distribution of outcomes of cohesin-replisome encounters. Values represent the mean of 4 biological replicates (-Chl1) and 2 biological replicates (+Chl1) and 2 biological replicates (immobilised cohesin), n is the total number of encounters observed per condition.

E. Comparison of replisome passage events. Replisome passage can either result in cohesion between replication products or no cohesion is established. Each data point represents an independent experiment. Black lines represent the mean with S.E.M.

We lastly investigated the impact of lateral diffusion of cohesin on its encounters with the replisome. Consistent with previous findings, our experiments demonstrated that immobilising cohesin significantly increased the frequency of passage events to 54 % (Figure 23D). Notably, replisome passage resulted in cohesion between replication products. Specifically, upon encountering immobilised cohesin, we observed transient stalling of the replisome (Figure 24). This stalling occasionally preceded an immediate rise in DNA staining ahead of the replication fork – indicative of stretching force along the DNA. Subsequent to stalling, 68 % of passage events resulted in successful cohesion establishment (Figure 23E). Furthermore, when the replisome reached the end of the DNA, we occasionally noted that leading and lagging strand products retracted to the site of cohesin attachment, suggesting continuous entrapment within the cohesin ring (Figure 24). We hypothesise that this results from the replisome-induced dislodgment of the biotin-streptavidin binding causing DNA detachment of the 3' end from the flow cell surface; thus, both ends of the leading strand product are free to retract towards cohesin. Introduction of PK-peptide released cohesin from antibody binding; consequently, allowing both cohesin and entrapped DNA to retract towards the DNA end that remains attached to the surface. These observations lend further support to our hypothesis that cohesin can establish cohesion between sister DNA molecules during replisome passage.

In this study, we provide the first real-time observations of reconstituted cohesin-replisome encounters *in vitro*. Our findings suggest that the replisome can indeed pass through the cohesin ring, shedding new light on the mechanisms underlying sister chromatid cohesion establishment *in vivo*.

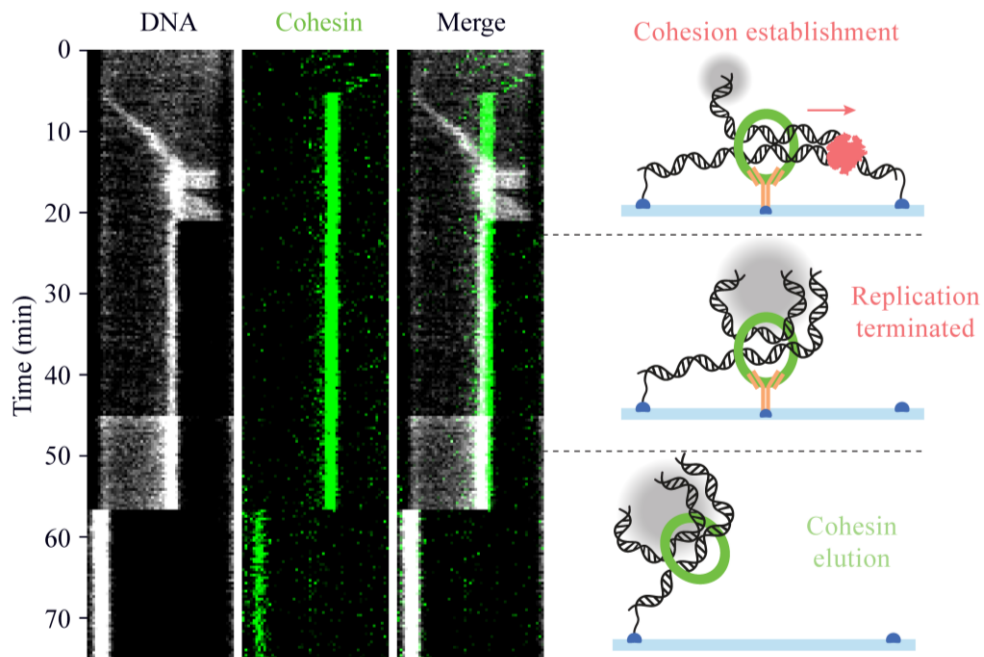


Figure 24: Replisome passage through immobilised cohesin.

Representative kymograph of DNA replication encountering immobilised cohesin (left). Schematic of cohesion establishment after replisome passage through the cohesin ring (right). Upon completion of DNA replication, the leading and lagging strands are released from the terminal tether and remain trapped within the cohesin ring. After cohesin elution, cohesin and entrapped DNA snap back to the tethered end of the DNA.

Chapter 6. Discussion

Chapter 6. Discussion

In this study, I demonstrate for the first time how the eukaryotic replicative helicase or an active replisome encounters a topologically loaded cohesin, providing valuable insights into how sister chromatid cohesion is established at replication forks.

While mobile cohesin is primarily pushed along DNA by the CMG helicase, reducing cohesin's ability to diffuse freely on DNA results in a significant increase in CMG passage. I further found that cohesin remained intact and associated with DNA throughout and after CMG passage, suggesting that the helicase passed through the cohesin ring. Visualisation of cohesin-replisome encounters revealed an increased likelihood of passage compared to cohesin-CMG encounters. Remarkably, replisome passage was frequently accompanied by cohesion establishment, providing the first evidence that a replisome passing a single cohesin can establish cohesion between replication products.

6.1 Factors influencing cohesin-CMG encounters

I reconstituted cohesin loading onto linear surface-tethered DNA and demonstrated that individual cohesin molecules diffuse rapidly along DNA. Cohesin's rate of diffusion positively correlates with the ionic strength. These findings are consistent with previous reports of human and fission yeast cohesin (Kanke et al., 2016; Stigler et al., 2016).

When this diffusive cohesin encounters a translocating CMG helicase, it rarely passes CMG and instead continues to diffuse in front of it. Our observations that cohesin rarely passes CMG are consistent with previous single-molecule experiments of fission yeast cohesin, which found that cohesin can only pass over smaller obstacles (<12 nm) and is significantly hindered by larger obstacles (>19.5 nm) (Stigler et al., 2016). Based on the size of the coiled coils in Smc1 and Smc3, the cohesin ring was estimated to have a diameter of ~40 nm (Anderson et al., 2002; Haering et al., 2002), which should easily allow for the passage of a CMG helicase with an estimated size of approximately 21 nm (Baretić et al., 2020).

Instead, it was observed that cohesin rarely passes CMG, which suggests that cohesin is not in a complete O-shaped conformation. This conformation may be similar to a gripping state of

cohesin, wherein the two coiled coils of the Smc1/3 are aligned and bent such that the hinge domain is brought down towards the heads (Higashi et al., 2020).

Structural rearrangements would be required to open up this ring in order to allow for CMG passage. It is known that cohesin's long coiled coil domains are highly flexible and can adopt a variety of different conformations (Anderson et al., 2002; Haering et al., 2002). Therefore, it can be hypothesised that cohesin might behave like a flexible rubber ring that allows for the passage of smaller proteins, but that it might require some time and additional proteins to facilitate the passage of larger proteins such as the CMG helicase.

However, cohesin is rarely seen to directly interact with CMG and rather diffuses in front of CMG, while CMG reduces the space available for cohesin to diffuse on. This indicates that cohesin is not directly interacting with the CMG helicase. Consistent with our findings, a recent single-molecule study observed that yeast MCM double hexamers are barriers for cohesin translocation, and cohesin rarely paused or interacted with MCM (Dequeker et al., 2022). Interestingly, when a chimeric MCM complex containing a humanised Mcm3 subunit was used, the probability of cohesin pausing at MCM double hexamers was significantly increased. This suggests that human MCM has sequence motifs that enable direct protein-protein interactions, whereas in yeast these sequences are lacking.

Interestingly, when I performed cohesin-CMG encounters in the presence of cohesion establishment factors (TCM or Chl1/Ctf4), a significant increase in cohesin passage past CMG was observed. These factors were previously found to interact directly with cohesin as well as with the CMG helicase (Shrestha et al., 2023). Additionally, TCM as well as Ctf4 and Chl1 are positioned at the N-tier of CMG (Baretić et al., 2020), placing them at the front of the advancing fork. This would allow them to contact cohesin in front of CMG, possibly facilitating passage.

Genetic studies have identified Tof1-Csm3 and Chl1/Ctf4 are part of one pathway for establishing cohesion *in vivo* (Xu et al., 2007). This pathway was later suggested to be involved in converting pre-loaded cohesin into cohesive cohesin during DNA replication (Srinivasan et al., 2020). These findings align with my observations that TCM and Chl1/Ctf4 increase the likelihood of CMG passage past cohesin.

Cohesin's coiled coils are very flexible and can undergo large conformational rearrangements. Cohesion establishment factors could facilitate these rearrangements, such that upon encountering CMG, cohesin's coiled coils are opened to pass over the CMG helicase. The low percentage of passage might be explained by the significant difference in velocities of CMG and cohesin, which might not allow for establishment of proper contacts between them. Under standard experimental conditions used for cohesin-CMG encounters (250 mM potassium glutamate), cohesin exhibits a diffusion coefficient of $0.22 \mu\text{m}^2/\text{s}$, which can be approximated to an average velocity of $0.66 \mu\text{m}/\text{s}$. Given an average DNA length in our single-molecule experiments of $4.9 \mu\text{m}$, cohesin moves along DNA at $>2 \text{ kbp}$ per second. Translocating CMG exhibited a velocity of $4 \text{ bp}/\text{s}$ in presence of Mcm10. Given the absence of binding interfaces for budding yeast cohesin and CMG, the considerable difference in velocity may result in a short contact time between the two upon encountering each other on DNA. Consequently, the time frame for cohesion establishment factors to contact cohesin for facilitating passage may be limited.

Overall, the low percentage of cohesin passage over CMG suggests that it is a stochastic event that might require the right conformational change at the right moment. Therefore, the time of contact between cohesin and CMG might be an important factor determining the outcome of encounters. This would be directly dictated by cohesin's diffusive behaviour as well as cohesion establishment factors bridging the interaction between cohesin and CMG.

Next, I found that reducing the lateral motion of cohesin, either by two converging CMG helicases or by direct immobilisation of cohesin, increased the probability of passage. In these encounters, cohesin is unable to diffuse away from CMG and is 'enforced' to interact with it. Therefore, the time of contact is maximised, which increases the likelihood of passage.

I further found that addition of cohesion establishment factors to encounters of CMG with immobilised cohesin only slightly increased the chance of passage but not enough to be significant. This is in contrast with encounters between CMG and diffusive cohesin, where cohesion establishment factors increased the chance of passage two-fold while the overall likelihood of passage was very low. These observations suggest that cohesion establishment

factors primarily act by facilitating passage possibly by increasing the contact between CMG and cohesin. This is no longer necessary when cohesin is immobilised.

Prior to passage, small forces (<5 pN) were detected that might facilitate passage by promoting the opening of the otherwise folded coiled coils. I initially speculated whether cohesion establishment factors would facilitate passage. However, addition of these factors did not significantly reduce the measured force prior to passage. Furthermore, they did not affect the manner in which CMG passes immobilised cohesin (Jump vs No jump). On one hand, the force measurements are likely to be inaccurate and limited by the resolution of the microscope. Even an error of 1 pixel ($0.11\text{ }\mu\text{m}$) could significantly alter the calculated force. On the other hand, it is possible that cohesion establishment factors become obsolete if cohesin is ‘enforced’ to pass over CMG, as seems to be the case for immobilised cohesin.

In vivo, nucleosomes are the most frequent obstacles of cohesin. Single-molecule data showed that the diffusive motion of cohesin was greatly restricted by an array of nucleosomes *in vitro*, strongly suggesting that nucleosomes act as a barrier for cohesin *in vivo* as well (Davidson et al., 2016; Stigler et al., 2016). We speculate that pushing cohesin into an array of nucleosomes leads to an increase in passage through the cohesin ring, similar to what was observed with converging CMGs. Thus, chromatin might indirectly contribute to the passage of cohesin past replisomes *in vivo*. Consistent with this, single-molecule experiments using *Xenopus laevis* egg extracts revealed that replisomes mostly pushed cohesin when naked DNA was used (Cameron et al., 2024); however, when chromatinised DNA was used, replisome passage became the most prominent observation (Kanke et al., 2016). Additionally, yeast ChIP-seq data does not reveal any major re-localisation of cohesin upon DNA replication, suggesting that pushing is not the favoured outcome in yeast cells (Lengronne et al., 2006). The question remains whether obstacles on DNA contribute to cohesion establishment *in vivo*, and we will be actively pursuing this question in the future.

Taken together, the effect of lateral diffusion and cohesion establishment factors on the passage of cohesin past translocating CMG was investigated. I found that lateral diffusion has a negative effect on the probability of cohesin passage, whereas cohesion establishment factors facilitate passage through bridging interactions between the two protein complexes.

This leads to an increased contact time between cohesin and CMG, allowing for conformational rearrangements in the cohesin ring and an increased probability of passage.

6.2 CMG passage through the cohesin ring

Immobilising cohesin prior to cohesin-CMG encounters allowed me to investigate how CMG passes cohesin. I found that cohesin remains associated to DNA following CMG passage. Additionally, side flow experiments revealed that cohesin does not detach from DNA during CMG passage. Combined, these observations suggest that, *in vitro*, CMG passes through the cohesin ring.

Transient opening of cohesin ring interfaces during passage would be possible if the DNA is retained by strong electrostatic interactions within the cohesin ring. The two evolutionary conserved lysine residues, K112 and K113, were shown to be involved in contacting DNA during cohesin loading (Collier et al., 2020; Higashi et al., 2020). In my experiments, cohesin remains unacetylated, making it possible for cohesin to contact and retain DNA during CMG passage. A possible control experiment could involve using mutant cohesin with acetyl mimicking residues to see if the behaviour during passage events changes. However, it was shown that cohesin with mutated lysin residues exhibit reduced loading onto DNA (Murayama & Uhlmann, 2015), likely due to inhibition of an important loading intermediate (Higashi et al., 2020). Therefore, experiments using mutated cohesin might be challenging. Alternatively, it is possible to utilise a cohesin molecule that can close its interfaces. Such constructs have been used before to study how DNA enters and exits the cohesin ring (Collier & Nasmyth, 2022; Richeldi et al., 2023).

In some cases, CMG stalls significantly before passing through immobilised cohesin. This suggests that only a certain orientation or conformation of cohesin allows for passage. The force building up over time might facilitate passage by forcing cohesin into the correct conformation. Cohesion establishment factors do not affect this force, similar to how they do not significantly affect the probability of passage when cohesin is immobilised prior to encounters. Moreover, the forces are not strong enough to disengage any cohesin interfaces. Previous work using optical tweezers paired with TIRF microscopy found that cohesin can resist forces up to approximately 20 pN before its hinge disengages and DNA exits (Richeldi et al., 2023). In my experiments I find forces below 20 pN, suggesting that ring interfaces are not forcefully disengaged.

Overall, my observations suggest that CMG passage occurs through the cohesin ring rather than transient disengagement of cohesin from DNA. However, additional experiments might be needed to rule out alternative models.

6.3 Cohesion establishment during DNA replication

I found that cohesin is more likely to pass an active replisome than a translocating CMG. This was surprising, because the replisome is overall larger than the CMG helicase alone. Based on their sizes, cohesin should be more likely to pass CMG rather than the replisome. However, the opposite is the case. How can this be explained?

One first possibility is that additional proteins bound to the replisome might facilitate passage. Beyond the known factors TCM and Chl1-Ctf4, polymerase epsilon, emerges as a plausible candidate involved in cohesion establishment. Mutants of polymerase epsilon led to defective sister chromatid cohesion (Edwards et al., 2003). Additionally, it was shown that pol2, the catalytic subunit of pol ϵ , interacts with SMC1 as well as ECO1 (Edwards et al., 2003).

Remarkably, pol2 is dispensable for viability as pol δ can take over leading strand synthesis (Kesti et al., 1999). This raises the more general question of whether other replisome factors essential for viability are involved in facilitating cohesin transfer. Unfortunately, this remains difficult to investigate *in vivo*. However, using my *in vitro* approach, it would be possible to test for this. One possibility would be to conduct cohesin-replisome encounters in absence of any of the polymerases.

Alternatively, the replisome might pass cohesin relatively easily due to its inherent plasticity. Single-molecule fluorescence microscopy has shown that polymerases as well as the fork protection complex, TCM, associate dynamically with the active replisome (Lewis et al., 2017, 2020). This could allow the replisome to disassemble when encountering cohesin, at reduced size, the replisome then pass, and then afterwards, polymerases could reassociate. Additionally, CMG was shown to not exchange even when challenged with excess CMG in solution. During my experiments, all replisome components, except the CMG helicase, are supplemented in excess in solution. However, even if disassembly of the replisome prior to passage takes place, additional factors are needed to increase the chance of passage from 4-8% observed for cohesin-CMG encounters to the 18% observed for cohesin-replisome encounters. Using my single-molecule approach, I could perform an experiment using a pre-assembled replisome. This is a replisome that is assembled onto the forked DNA and then initiated without additional polymerases in excess. Nonetheless, it was shown that pre-assembled replisomes replicate similarly to the replisomes that can exchange with proteins

from solution (Lewis et al., 2020). This approach would allow us to investigate whether exchange of proteins is needed for passage past cohesion or not.

In addition to showing an increased possibility of passage, most of these passage events led to cohesion between leading and lagging strand products. This observation could only be explained by the replisome passing through the cohesin ring or transient opening and closing of the cohesin ring. Furthermore, when immobilising cohesin prior to initiating replication, more than 50 % of encounters resulted in replisome passage and most of these were exhibiting cohesion establishment. Taken together, these observations strongly suggest that conversion of cohesin loaded prior to replication occurs via replisome passage through the cohesin ring.

Previous single-molecule studies investigating cohesin-replisome encounters using *Xenopus laevis* egg extracts have observed conflicting results. One study found that cohesin is pushed by the replisome to sites of replication termination, where after disassembly, cohesin holds sister chromatids together without needing to pass the replisome. Surprisingly, this study reports only 5% of cohesin passage (Cameron et al., 2024). In contrast, other experiments found that the majority of cohesin was integrated into replicated DNA, but also found that cohesin could be pushed or dissociate by the replisome (Kanke et al., 2016). In yeast, pushing of cohesin was only reported for the transcription machinery (Lengronne et al., 2004), in contrast, replication was not able to relocate cohesin on DNA (Lengronne et al., 2006). However, transcription is constantly active, and the possibility remains that transcription masks any effects that the replication machinery has on cohesin. More up to date experiments will be required to elucidate how transcription and replication relocate cohesin during S phase.

Interestingly, when performing cohesin-replisome encounter experiments in presence of Chl1, no changes in outcomes were observed, even though I observed an increased chance of passage for cohesin-CMG encounters. Why is this?

Is it possible that Chl1 is not necessary for transfer of cohesin? Previous genetic and biochemical studies clearly implicated that Chl1 is involved in facilitating the transfer of pre-loaded cohesin behind the replisome to establish sister chromatid cohesion (Srinivasan et al.,

2020). Additionally, deletion of Chl1 was shown to drastically reduce cohesin levels across an entire chromosome (Samora et al., 2016; Srinivasan et al., 2020). However, this does not necessarily mean that Chl1 is directly facilitating cohesion establishment. A more recent study investigated the coordination of cohesin and DNA replication in an ensemble biochemical assay *in vitro* (Murayama et al., 2024). Here, Chl1 was shown to be required to promote the topological loading of cohesin at physiological salt concentrations. If this would similarly occur in cells, Chl1 would become obsolete in my single-molecule experiments, as cohesin is already topologically loaded prior to initiating DNA replication.

Overall, my experiments provide first evidence that replisome passage through the cohesin ring is a possible mechanism for cohesion establishment. In comparison to CMG helicase alone, DNA replication appears to facilitate passage through direct interactions between replication fork-associated components and cohesin.

Future studies are essential to elucidate how cohesin and the replisome work together to establish cohesion, especially in the context of a chromatinised template.

6.4 Contribution of cohesion establishment factors

During the course of my study, I was able to identify evidence regarding the role of cohesion establishment factors in the transfer of pre-loaded cohesin. Initially, I observed that the addition of Tof1-Csm3-Mrc1 or Chl1-Ctf4 increased the chance of CMG passage past cohesin. This was true for both freely diffusive cohesin and to a lesser extent for immobilised cohesin. In contrast, addition of Chl1 had no effect on cohesin-replisome encounters.

It was shown that deletion of any of the cohesion establishment proteins results in a cohesion defect. This indicates that the addition of Chl1 to cohesin-replisome encounters, where Tof1-Csm3 and Mrc1 are already included, should increase the likelihood of passage if this is how Chl1 contributes to cohesion. However, in my single-molecule experiments, cohesion is established independent from Chl1. What is the difference between CMG and replication?

I generally observed more immobile cohesin during DNA replication, even though they showed diffusive behaviour before initiating replication. This might be caused by all the DNA-binding proteins in my replication mix, which could hinder cohesin diffusion. As observed during experiments with immobilised cohesin, reducing the lateral diffusion of cohesin favours passage. In other words, any DNA binding proteins could reduce the freedom of cohesin on DNA, which could indirectly lead to an increase in passage.

Therefore, the increased chance of passage during DNA replication *in vitro* could be an artifact of having additional DNA-binding proteins in excess solution. However, even if this were the case, the number of obstacles inhibiting cohesin diffusion *in vivo* would be even greater, such as the transcription machinery and nucleosome.

Additionally, DNA topology, such as positive or negative supercoiling, could be a challenge faced by cohesin *in vivo*. In my single-molecule experiments, DNA ends are tethered to the surface via biotin. This biotin is covalently linked to one single strand on the one end and the other single strand on the other end. This results in a DNA that is free to rotate, without accumulating supercoiling during DNA replication.

Considering these observations, I want to propose a model in which reducing lateral diffusion of cohesin in a chromatin context *in vivo*, together with cohesion establishment factors, guarantees efficient passage of CMG to establish sister chromatid cohesion.

Genetic studies have clearly shown that Tof1-Csm3 and Chl1-Ctf4 act within a single pathway for cohesion establishment (Xu et al., 2007). In contrast, Mrc1 was identified as participating in a second pathway, alongside Ctf18-RFC and the cohesin loader. Given the current understanding of the replisome architecture, it is somewhat unexpected that Mrc1 is placed within a different pathway from Tof1-Csm3. It is possible that the extended structure of Mrc1, which binds to Tof1 but also reaches across the MCM helicase to contact Ctf4, Cdc45 and polymerase ϵ , may contribute to its distinct role (Baretić et al., 2020). Both, Ctf18-RFC and Mrc1, are observed to interact with the catalytic subunit of pol ϵ (pol2) at the back of the replisome. This positioning enables them to facilitate the loading of cohesin behind the fork. In contrast, the proteins belonging to the first pathway, Tof1-Csm3 and Chl1-Ctf4, are positioned towards the front face of the advancing replisome. The distinct binding locations may be the reason why the cohesion establishment factors act in different pathways.

However, an alternative model could propose that all proteins act together in a conveyor belt-like mechanism. Firstly, the replisome makes contact with cohesin via Tof1-Csm3 and Chl1-Ctf4. This either facilitates the passage of the replisome through the ring, as demonstrated in this study, or the transient unloading of cohesin. Subsequently, Mrc1 makes contact with and transfers cohesin across the replisome. Ctf18-RFC has been observed to load PCNA preferentially on the leading strand, which could facilitate the recruitment of the cohesin loader and the subsequent reloading of transferred cohesin onto the newly synthesised sister DNA strands.

The recycling of nucleosomes is achieved via a similar mechanism that involves transfer of histones via multiple contacts across the replisome. It is an intriguing question as to how the replisome is able to navigate both these fundamental processes in parallel.

At this time, it remains unclear how these proteins act together to facilitate cohesion establishment. However, the data presented here suggests that Chl1-Ctf4, and to a lesser extent Tof1-Csm3-Mrc1, facilitate CMG passage past diffusive and immobilised cohesin. Further work is needed to clarify the individual roles of each of these proteins.

6.5 Limitations of this study

It is an intriguing model that active replisomes merrily must pass through the cohesin ring, and that replication associated factors facilitate the conformational rearrangements needed for this passage. Here, I provide evidence that this can truly take place. However, how often this happens in cells remains to be tested.

In vivo, two genetic pathways have been identified. My PhD focused on how pre-loaded cohesin is transferred to establish cohesion. Another possibility, namely *de novo* loading behind the replisome was not addressed. In this second pathway, cohesin from solution is loaded onto DNA right behind the replisome. This pathway could involve Mrc1, Scc2 and Ctf18. Nevertheless, direct experimental evidence of *de novo* loading of cohesin behind the replisome is lacking.

The reason why I focused on the conversion pathway was that *de novo* loading is experimentally difficult. Loading of fluorescently labelled cohesin during single-molecule imaging would result in a large background signal. Therefore, new experimental approaches might be needed to investigate how this second pathway contribute to cohesion establishment.

My research has focused on the combined impact of Tof1-Csm3 and Mrc1 on contacting cohesin and their function in facilitating CMG and replisome passage. It would be interesting to ascertain how they act individually during these encounters. Furthermore, it was challenging to test the contribution of Tof1-Csm3 and Mrc1 towards cohesin-replisome encounters, as they are essential for promoting fast and efficient DNA replication. Therefore, I was unable to exclude them from any of my cohesin-replisome experiments. However, it may be possible to exclude one of the three proteins while increasing the concentration of the other two proteins. This will be subject of future investigation.

In my experiments I am building on the previously established minimal system to established DNA replication *in vitro* as well as the known cohesion establishment factors. I do not exclude the possibility that important factors facilitating the conversion of cohesin *in vivo* are missing. The advantage of my approach is that I exactly know which proteins are present, however, this is also the disadvantage, as I do not know if any important factors are missing.

This raises the general question, if we have found all essential cohesion establishment factors, or if there are more factors that are needed. This remains for future studies to be figured out.

Chapter 7. Concluding remarks

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Since the discovery of the first sister chromatid cohesion loss mutant more than 50 years ago (Davis 1971), a myriad of techniques, ranging from yeast genetics to structural biochemistry, have significantly advanced our understanding of sister chromatid cohesion establishment. The cohesin ring complex was identified for its primary function in holding two sister DNA molecules together (Guacci et al., 1997; Michaelis et al., 1997; Tóth et al., 1999). Additional proteins assist cohesin in establishing and maintaining this cohesion from DNA replication until chromosome segregation in anaphase (Mayer et al., 2001, 2004; Skibbens et al., 1999; Tóth et al., 1999; Xu et al., 2004). However, the precise mechanism of sister chromatid cohesion establishment during S phase remains elusive.

In this study, I visualised how an active replication fork can pass a single cohesin molecule, thereby establishing cohesion between the two newly synthesised sister DNA molecules. This provides first evidence that replisome passage is indeed possible. This finding not only sheds light on the mechanics of cohesion establishment but also opens new avenues for exploring the dynamic interactions between the replication machinery and cohesin complexes.

I hope that my findings will contribute to future insights into how the fundamental process of cohesion establishment is mediated in cells. Understanding these mechanisms at a molecular level is crucial, as defects in cohesion and its regulatory subunits can lead to chromosomal instability, which is a hallmark of many cancers and other genetic disorders.

Finally, my single-molecule work demonstrates the remarkable advancements in today's science, enabling the visualisation of complex biological processes *in vitro*. This underscores the power of modern biochemical and biophysical techniques in unravelling the intricacies of cellular mechanisms. I sincerely hope that this work will inspire future scientists to undertake challenging projects, even if they are 'just' basic biology.

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