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Review

In vitro dynamics of DNA loop extrusion by structural maintenance of chromosomes complexes

Marius Rutkauskas and Eugene Kim

Genomic DNA inside the cell's nucleus is highly organized and tightly controlled by the structural maintenance of chromosomes (SMC) protein complexes. These complexes fold genomes by creating and processively enlarging loops, a process called loop extrusion. After more than a decade of accumulating indirect evidence, recent *in vitro* single-molecule studies confirmed loop extrusion as an evolutionarily conserved function among eukaryotic and prokaryotic SMCs. These studies further provided important insights into mechanisms and regulations of these universal molecular machines, which will be discussed in this minireview.

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Introduction

Structural maintenance of chromosomes (SMC) complexes are evolutionarily conserved motor proteins that fold genomes into loops through ATP-powered loop extrusion. Eukaryotic cells contain three types of SMC complexes: cohesin, condensin and Smc5/6 complex (Smc5/6). Cohesin organizes interphase chromatin into functional domains, regulates gene expression [1–3], and is involved in DNA double-strand break repair [4] and immunoglobulin gene recombination [5]. Condensin plays a major role in mitotic chromosome compaction and sister chromatid segregation [6]. Smc5/6 regulates chromosomal replication and repair while functioning as a viral restriction factor [7–9]. In prokaryotes, widespread SMC-ScpAB and MukBEF complexes contribute to chromosome organization and segregation during cell division [10,11], while Wadjet, present in some bacteria and archaea, acts as an anti-plasmid/phage defense system [9].

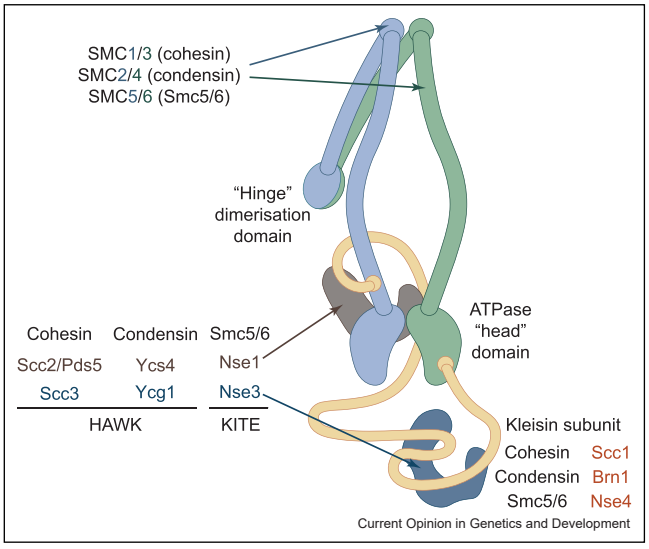
Structurally, all SMC complexes have closely related architecture. The core of these complexes is a tripartite ring composed of a dimer of SMC proteins and a kleisin. SMC protein comprises ~50 nm long coiled-coil arm that separates the 'hinge' dimerization domain from the ATPase 'head' domain, which engages/disengages during the ATPase cycle. The kleisin that bridges SMC dimers at the SMC neck/cap surfaces further recruits additional subunits, namely, HEAT repeat proteins associated with kleisin (HAWK) proteins (for condensin and cohesin) or kleisin-interacting tandem winged-helix element (KITE) proteins (for Smc5/6 and bacterial SMCs; Figure 1).

The proposal that SMC complexes may shape genome by creating and processively enlarging a loop was first put forward more than 20 years ago [12]. Over a decade later, this loop extrusion hypothesis was further supported by series of *in vivo* Hi-C studies [13,14] and *in silico* modeling [15,16], yet a direct confirmation was still lacking. It was until the recent *in vitro* experiments, which directly visualized the process of SMC-driven loop extrusion at the single-molecule level [17–25]. These *in vitro* experiments not only established loop extrusion as an evolutionarily conserved mechanism across kingdoms of life but also revealed crucial insights into the mechanism underlying loop extrusion. Furthermore, more recent experiments including complex designs, for example, the interaction between SMCs [26], the addition of potential roadblocks [21,27–31], combination with different DNA topology [25,32,33], deletion of subunits [23,34,35] or adding regulatory partners [36,37], further advanced our understanding of these molecular motors in a great detail. Here, we therefore mainly focus on these single-molecule studies and discuss the latest understanding of loop extrusion and its regulation mechanisms for different SMC complexes.

Symmetry of DNA loop extrusion by SMC complexes

SMC complexes can reel DNA into loops either bidirectionally or unidirectionally (Figure 2). The bidirectional extrusion can be achieved in two ways: either when two physically linked SMCs perform simultaneous DNA reeling from opposite sides (two-sided extrusion) or in the case of a single one-sided motor that can periodically switch the extrusion direction (Figure 2). The

Figure 1



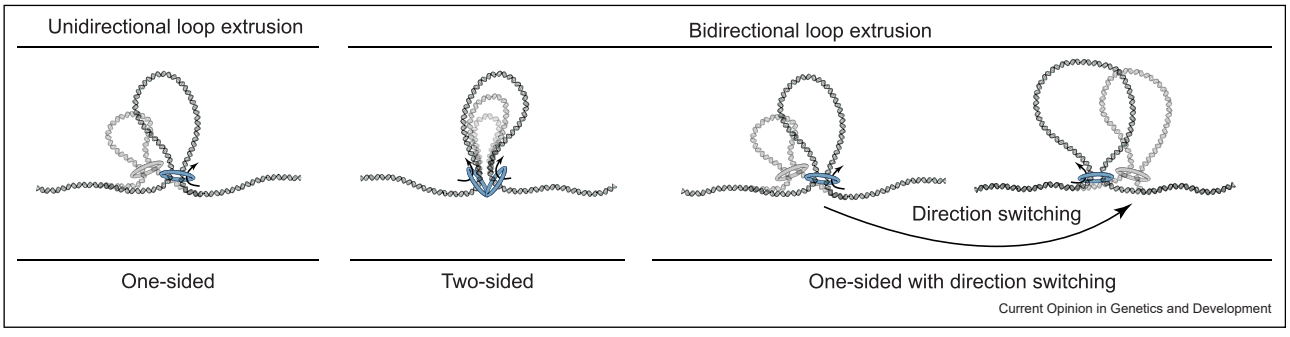
Schematic representation of SMC structure. SMC's ring is composed of two SMC subunits (SMC1/3 for cohesin, SMC2/4 for condensin, SMC5/6 for Smc5/6 complex) that are approximately 50 nm in length on one end forming a 'hinge' dimerization domain and ATPase 'head' domain on the other end. SMC dimer is connected by a kleisin subunit. Cohesin and condensin have additional HAWK subunits, while Smc5/6 and bacterial SMCs contain KITE subunits. Additional subunit names of budding yeast are displayed.

first *in vitro* loop extrusion visualization experiments showed strictly one-sided extrusion by single yeast condensin complex [17], whereas a mixture of one-sided and two-sided extrusion by condensin is seen for human [21] and Xenopus [20]. In the case of cohesin, in contrast to an initial work showing two-sided extrusion by dimers [18], recent work suggests one-sided but bidirectional extrusion by single cohesin [38]. Direction switching of one-sided extrusion seems to depend on HAWK subunits. Deletion of Ycg1-Brn1 enables *Chaetomium thermophilum* condensin to be able to switch extrusion direction [34]. For cohesin, the human analog of the Scc2 subunit (NIBPL) turnover correlates with direction switching, while he human analog of the Scc3 subunit (STAG1) prevents it [38]. In the case of Wadjet complex, two-sided extrusion by dimeric complexes was demonstrated [25]. For Smc5/6, however, conflicting evidence exists showing one-sided and bidirectional extrusion by single complexes [38] and two-sided extrusion by dimeric complexes [23,39]. The discrepancy in the determination of extrusion symmetry observed even for the same proteins (cohesin, Smc5/6) suggests the possible influence of extrinsic factors as well as the exact analysis method employed on the determination of symmetry. In fact, recent work from our group shows that symmetry of loop extrusion is strongly dependent on DNA tension. Two-sided extrusion by dimeric motors like Smc5/6 and Wadjet can turn to one-sided extrusion upon increasing DNA tension [39].

The topology of DNA and SMC complexes

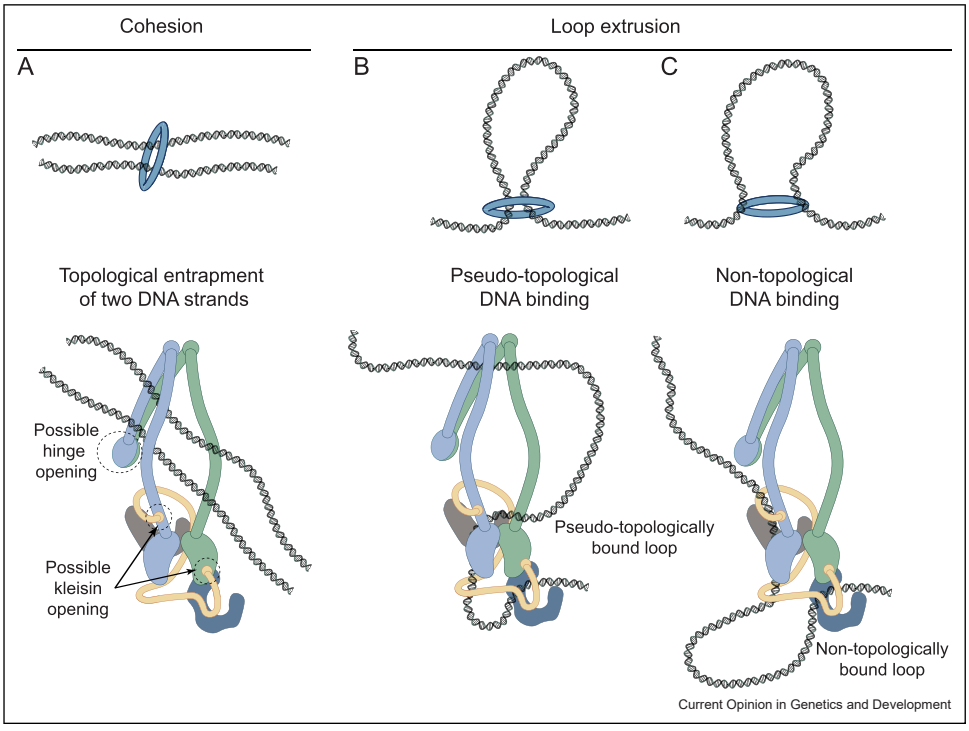
It is well known that SMC complexes can entrap DNA topologically, in other words, encircle DNA within the trimeric SMC-kleisin ring (Figure 3A). Cohesin [40], Condensin [41], Smc5/6 [42] and SMC-ScpAB [43] can topologically load onto chromosomal DNA *in vivo*. Cohesin can also encircle two unreplicated and replicated DNA molecules [44]. Topological loading requires SMCs to open their trimeric rings. Through which gate (s) DNA can enter (and exit) has been therefore one of the central questions. In the case of cohesin, although both SMC3/kleisin and SMC1/SMC3 hinge can open, stabilization of SMC1/SMC3 hinge interactions [45] reduces the entry of DNA into cohesin, and mutation of hinge leads to defects in cohesion *in vivo* [46], suggesting that hinge may serve as an entry gate for DNA into the ring. Interestingly, recent single-molecule experiments [47] identify the hinge as the weakest interface within the cohesin ring, which can release DNA at DNA pulling force of ~20 pN. This value is significantly

Figure 2



Symmetricity of loop extrusion. DNA loops can be extruded unidirectionally or bidirectionally. Bidirectional loop extrusion can take place when a dimeric SMC reels DNA simultaneously from both sides of a loop or when a one-sided monomeric SMC switches extrusion direction periodically.

Figure 3



Possible topology of SMC complexes during cohesion and loop extrusion in simple (top) and detailed (bottom) schematics. Single cohesins, which bind two sister chromatids, can entrap two DNA strands topologically (A). To do that, cohesin ring must open, and this can be done either through hinge dimerization domain opening or by reversible kleisin detachment. Loop extruding SMCs may bind DNA pseudo-topologically (B), where a loop is inserted within the SMC ring, or non-topologically, where DNA is externally bound by electrostatic interaction **(C)**.
The detailed schematics of loop extrusion model are adapted from Ref. [83].

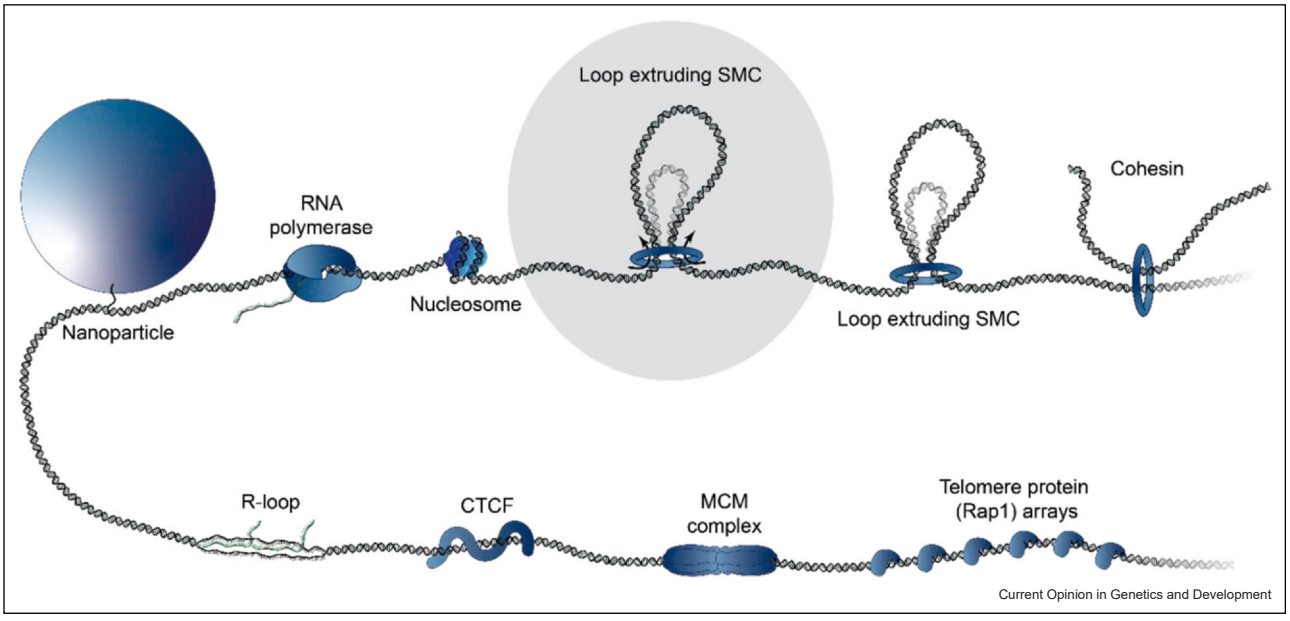
larger than the stalling force of loop extrusion by cohesin (~0.1 pN [29]) and comparable to the forces exerted by other DNA-based motors, for example, RNA polymerase II (~10 pN [48]) and replicating helicases (> 20 pN [49]). In the case of Smc5/6, SMC6-Nse4 (kleisin) serves as an entry gate of DNA for topological loading, which also requires the additional subunit of Nse5/6 [42]. For condensin, Shaltiel et al. reported that none of the SMC/kleisin gate opening is required for DNA entrapment *in vitro*. Instead, condensin can be 'topologically' loaded within the two kleisin chambers created by interactions of Brn1 and Ycs4/Ycg1, respectively [34].

These *in vivo* and *in vitro* evidence of topological interaction between SMC complexes and DNA raise the question of whether such interaction is also needed for loop extrusion. Alternatively, loop extrusion may not need topological loading and instead occur in a manner that SMCs encompass an entire DNA loop within a single SMC-kleisin ring (pseudo-topological loading; Figure 3B), or do not encircle DNA at all and instead bind at the outer interfaces of the ring (nontopological; Figure 3C). Cohesin in which all ring interfaces are covalently linked and therefore cannot open the ring still exhibited loop extrusion activity, thus indicating topological loading is not required for loop extrusion [19]. Similarly, Smc5/6-mediated loop extrusion does not seem to require topological loading. Nse5/6, the Smc5/6-specific subunit required for topological loading of Smc5/6 [42], is not necessary for Smc5/6's loop extrusion activity but rather inhibits it [23]. Furthermore, single molecule experiments showed that covalently linked cohesin can still bypass DNA-bound obstacles that are larger than the ring size, suggesting loop extrusion may occur in a non-topological fashion [27,50]. It is an interesting future question to address how these various topological interaction between SMCs and DNA is regulated and how these interaction modes are linked to different cellular functionalities.

Extruders can bypass one another and large physical roadblocks

DNA in the cell nucleus is covered by a myriad of DNA-binding proteins, such as histones, polymerases, transcription factors, and other potential roadblocks for SMCs (Figure 4). The question of how SMCs can extrude loops while continually encountering objects in

Figure 4



Roadblocks tested for loop extruding SMC complexes. Loop-extruding SMCs on DNA can encounter other SMCs that are either loop-extruding in cis or holding DNA strands in trans. Multiple roadblocks tested in *in vitro* experiments so far are shown: nucleosomes, RNA polymerases, CTCF, MCM complexes, R-loops, telomere protein arrays, and artificial roadblocks like nanoparticles attached to DNA that are bigger than SMC ring diameter.

their path was addressed by observing loop extrusion by condensin and cohesin on DNA containing various roadblocks. Single nucleosomes and RNA polymerases bound on DNA pose virtually no impact on the loop extrusion activity of condensin [27]. Even obstacles (200 nm nanoparticles) larger than the size of the SMC-kleisin trimeric ring (~35 nm [51]) were incorporated into the loop extruded by condensin and cohesin. Interestingly, however, recent experiments with Wadjet, a complex that cleaves DNA through its loop extrusion activity [25], indicated that a large physical roadblock (~3 μm) can stall loop extrusion activity of Wadjet [52].

Besides other non-SMC interacting protein and physical roadblocks, SMCs can also encounter each other. Kim et al. demonstrated that two condensin complexes extruding loops in close proximity can traverse one another, forming a so-called Z-loop structure [26], a finding that was corroborated by *in vivo* studies for *B. subtilis* condensin [53]. This study also raises the interesting question of whether this bypassing is a general behavior among different SMCs. A recent *in vivo* analysis in combination with polymer simulation indicated that during mitotic chromosome formation, condensins remove loop-extruding cohesins (or push them away from the TAD boundaries), while they bypass cohesive cohesins [54]. Further studies are necessary to dissect intricate interactions between different SMCs in shaping chromosome structure, especially when they encounter one other and more complex DNA structures emerging during chromosome condensation.

Barriers to loop extrusion

While condensin and cohesin can bypass single non-interacting proteins and physical roadblocks with large sizes, recent studies identified several other factors that act as barriers to loop extrusion (Figure 4). The interaction between cohesin and CTCF, a transcription factor that has been long known for its orientation-specific anchoring function at the boundaries of TADs [55,56] has been recently studied at the single-molecule level [29,30]. These studies confirmed the N-terminal-specific loop extrusion blockage of CTCF and that this activity requires interaction between the YDF motif of CTCF and cohesin's STAG1 subunit [30]. Furthermore, Davidson et al. showed that CTCF's blocking ability as well as dynamics after blockage (direction switching, anchoring, and slippage) depends strongly on DNA tension [29]. It remains to be seen how such tension dependence relates to CTCF-cohesin-mediated loops *in vivo*.

Among other proteins that also contain cohesin-binding motif (YxF) like CTCF, the human minichromosome maintenance (MCM) complex, a part of the replication machinery, was shown to impede cohesin-mediated loops *in vivo* and diffusion on DNA *in vitro* [28]. The pausing time upon MCM/cohesin encounter increased

in the presence of the YDF motif [28], likely due to specific cohesin/MCM interaction. Another system that has been indicated as a loop extrusion barrier is RNA polymerase. Computational modeling of Hi-C maps from by *B. subtilis* condensin [57] and human cohesin [58] suggest that active RNA polymerases act as permeable barriers for loop extrusion, while in the presence of single inactive RNA polymerases, loop extrusion seems to be not impacted *in vitro* [27]. The nascent transcript during transcription might be responsible for the active RNA polymerase-specific loop extrusion blockage. In fact, Zhang et al. showed that R-loops, a structure composed of DNA-RNA hybrid and single-stranded DNA, can efficiently block cohesin-mediated loops *in vitro* [30]. Similarly, recent single-molecule experiments showed specific recruitment and stabilization of Smc5/6 on both ssDNA and junctions of ssDNA and dsDNA [59,60], suggesting the possibility of DNA structure-specific stalling of SMCs.

Although loop extruders seem to be able to bypass single roadblocks with varying sizes, it is still an open question how extrusion behavior would be influenced by multiple proteins densely populated along the pathway of loop-extruding SMCs (e.g. nucleosome arrays). Interestingly, a recent study showed that the dense array of Rap1, DNA-binding proteins specific to telomere regions can block loop extrusion by condensin with near 100% efficiency [31]. Reduction in array density by increasing gaps between Rap1 significantly reduced blocking efficiency.

DNA supercoiling (over-/under-winding of DNA) and loop extrusion

Cellular DNA undergoes torsional stresses generated by processes like transcription and replication [61,62]. As a result, DNA adopts overwound or underwound structures, known as positive or negative supercoiling. Supercoiling also leads to the formation of plectonemes in which the DNA helix is coiled onto itself. Although earlier studies reported different DNA supercoiling-specific interactions of SMC complexes [63–67], direct visualization of loop-extruding SMCs on supercoiled DNA has been only recently demonstrated [32,33]. Yeast condensin and Smc5/6 have been shown to preferentially bind the tips of positive supercoils and extrude loops on them [32,33]. During extrusion, multiple plectonemes are absorbed into a single plectonemic loop stabilized by SMCs. *In vivo* analysis shows Smc5/6 accumulates and links transcription-induced positive supercoil-rich genomic regions [33]. Interestingly, recent *in vitro* studies [68–70] show that eukaryotic SMCs induce a negative twist on DNA during loop extrusion. This negative supercoiling occurs upon ATP binding [68–70], specifically during DNA clamping for cohesin [69], and does not involve the Ycg1 subunit in the case of yeast condensin [68]. It remains to be understood the relation between negative supercoiling generated by SMCs and their preferential loading and interactions with positively supercoiled DNA.

Regulation of SMCs and loop extrusion

Although loop extrusion is a general mechanism shared among different SMC complexes, its regulation mechanisms are specific to individual complexes. So far, several regulatory processes have been identified mainly for cohesin and condensin, which leads to modulation of the proteins' residence time on chromatin, activation or repression of the complexes, or their subcellular localizations. At the molecular level, these regulations are achieved through interactions between the SMCs' subunits and regulatory factors, or interactions within the subunits of SMCs.

One of the well-studied regulatory factors for cohesin is WAPL, a helical repeat protein that removes cohesin from DNA through interaction with cohesin's HAWK subunits STAG and PDS5 [71,72]. This WAPL-induced release of cohesin is counteracted in multiple ways. Acetylation of SMC3 subunit by acetyltransferase ESCO1/ESCO2 and recruitment of sororin protect cohesive cohesin from WAPL during DNA replication [73,74]. CTCF also competes with WAPL by binding cohesin's STAG2 subunits via its YxF motif, thus stabilizing interphase chromatin loops formed by cohesin at CTCF sites [75]. Protection of cohesin at centromeres is achieved by the interaction between STAG2 and SGO1 protein through its YxF motif, which allows for maintaining sister chromatid cohesion in mitosis [76].

While the activity of condensin is clearly cell cycle regulated, the mechanisms underlying this cell cycle–specific activation have remained elusive. Recent *in vitro* studies revealed the condensin's HAWK subunit, NCAPG/CAP-G2 (the human analog of the Ycg1), plays a major function in the regulation of condensin. The binding of NCAPG/CAP-G2 to the N-terminal of kleisin NCAPH/CAP-H2 or to the C-terminal of NCAPD/CAP-D3 (the human analog of the Ycs4) leads to the self-suppression of condensin [35,77–79]. Inhibiting these interactions *in vitro* by entire or partial deletion of these subunits or via phosphorylation activates condensin's chromatin loading and compaction [78,79]. Furthermore, two recently identified condensin activators, KIF4A (for condensin I [36,80]) and M18BP1 (for condensin II [37]), as well as condensin II inhibitor MCPH1 [81] seem to also achieve their function through competitive binding for NCAPG/CAP-G2 subunit.

Limitation of *in vitro* studies and future outlook

Thanks to the recent surge of *in vitro* and single-molecule experiments overviewed here, we are rapidly

advancing our understanding of SMC complexes and their working principles. While these experiments are optimal for dissecting molecular mechanisms of SMCs, they intrinsically lack the cellular complexity. Thus, their *in vivo* relevance is often a primary question. For example, most of the studies mentioned here use bare DNA as a substrate, whereas *in vivo* loop extrusion must be performed on chromatin or nucleoid DNA and in the presence of myriads of chromatin (nucleoid) associated proteins. Therefore, it is possible that some of the major conclusions from *in vitro* studies are subject to change once the complexity of the system increases. Recently demonstrated ~10-fold lower loop extrusion rate for cohesin *in vivo* (~0.1 kb/s [82]), compared to the rate determined from *in vitro* experiments (~ 1 kb/s [19]), may arise from such *in vivo versus in vitro* differences.

In the future, it will be therefore important to precisely characterize the dynamics of loop extrusion on native chromatin *in vitro* and revisit the major findings from the previous *in vitro* experiments. This includes for instance determining stoichiometry of loop-extruding complexes, extrusion rate, symmetry and directionality, as well as SMC loading and loop initiation preferences. This will not only allow us to understand the possible regulatory role of chromatin on loop extrusion but also provide a basis for addressing more complex questions, such as influence of chromatin modifications, chromatin remodeling, and transcription on loop extrusion.

Having stated that, increasing complexity of *in vitro* experiments similar to *in vivo*–like conditions would be a nearly impossible quest. Therefore, cross-validating the findings from *in vitro* experiments using *in vivo* assays, such as live-cell imaging or genomic analysis, would be another ideal direction to increase the *in vivo* relevance. In this regard, recent combined studies of Hi-C and single-molecule experiments [28,33] would be excellent examples.

We believe that these *in vitro* experiments will continue to provide us with ever more detailed insights of SMC-mediated chromosome dynamics. Looking further, bringing these mechanistic understandings at the protein structural level will allow us to build more accurate model, which will in turn provide a better prediction of the role of SMCs on chromatin organization and genome function. These multiscale and integrative approaches will enable us to further challenge, develop, and refine the current still largely incomplete understanding of these fascinating molecular machines.

Data Availability

No data was used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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- • of special interest
- •• of outstanding interest

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This study demonstrated that CTCF is a polar barrier for DNA loop extrusion by cohesin and cohesin's diffusion on DNA. It also showed that the outcomes of CTCF and cohesin interaction, such as bypassing CTCF, direction switching, loop extrusion blockage and loop shrinkage, are dependent on DNA tension.

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