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1 **TADs: dynamic structures to create stable regulatory functions**

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3

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7 **Abstract**

8 Mammalian chromosomes are organized at different length scales within the cell nucleus.
9 Topologically Associating Domains (TADs) are structural units of 3D genome organization
10 with functions in gene regulation, DNA replication, recombination and repair. Whereas TADs
11 were initially interpreted as insulated domains, recent studies are revealing that these domains
12 should be interpreted as dynamic collections of actively extruding loops. This process of loop
13 extrusion is subsequently blocked at dedicated TAD boundaries, thereby promoting intra-
14 domain interactions over the surroundings within the Hi-C matrix. In this review, we discuss
15 how mammalian TAD structure can emerge from this dynamic process and we discuss recent
16 evidence that TAD boundaries can have regulatory functions in this process.

17

18 **Keywords:** TADs, Cohesin, CTCF, enhancer-promoter looping, loop extrusion

19 **Introduction**

20 Mammalian interphase chromosomes, together adding up to around 2 meters in length, are
21 subjected to a wide range of biological processes, including transcription, gene regulation,
22 repair and replication. Yet, all this activity takes place in the crowded cell nucleus with a
23 volume of less than $800 \mu\text{m}^3$. How the 3D organization of chromosomes permits, or facilitates,
24 these processes, is a topic of intense interest. Whereas initial discoveries on 3D chromosome
25 organization were made using fluorescent imaging of fixed cells, nowadays a wide range of
26 approaches are used. These approaches incorporate genomics, super-resolution and live-cell
27 imaging, the combination of imaging and genomics for spatial and *in-situ* studies and
28 biophysical polymer models of chromatin organization [1,2].

29 Within the mammalian cell nucleus, 3D organization of chromosomes emerges at
30 different length scales. At the largest scale, as discovered using fluorescence microscopy,
31 individual chromosomes occupy discrete zones called chromosome territories [3]. At a finer
32 scale, the Hi-C assay—a combination of Chromosome Conformation Capture and whole-
33 genome sequencing—has been instrumental to identify additional levels of organization [4].
34 Hi-C first revealed that chromosomes separate into multi-Megabase (Mb) compartments, so-
35 called A and B compartments ([4], Figure 1a). These compartments are characterized by
36 preferred homotypic interactions between compartments (*i.e.* A-A or B-B) over heterotypic
37 interactions (A-B), creating in a checker-board pattern in a Hi-C interaction matrix. At a sub-
38 Mb scale (typically between 500 kb – 1 Mb), a further level of domain organization appears
39 ([5], Figure 1a). The human and mouse genome are divided into several thousands of
40 Topologically Associating Domains (TADs), which exhibit more frequent intra-domain
41 contacts over their surroundings. Comparison of TADs between cell types and mammalian
42 species revealed a considerable degree of conservation, suggesting that TADs are a more
43 constitutive and structural level of chromosome organization [5-7]. However, insulation
44 between TADs is relatively limited, as intra-domain contact enrichment is only in the order of
45 two-fold [5,8]. Within TADs, further structures can be observed, such as sub-TADs, contact
46 domains and DNA loops between enhancers and promoters [9,10]. In the remainder of this
47 manuscript, we will focus on the structure and function of 3D genome organization in
48 mammalian cells at the level of TADs and within.

49 **Main text**

50 TADs as regulatory neighborhoods

51 Soon after the discovery of TADs, it was reported that enhancer-promoter pairs mostly localize
52 within the same domain [11]. The insulated nature of TADs may thus create ‘regulatory
53 neighborhoods’ that restrict inter-TAD loop formation ([8,12], Figure 1a). Indeed, most TAD
54 boundaries are occupied by the CTCF insulator protein, which previously was recognized for
55 its enhancer blocking activity [5]. CTCF binds an asymmetric DNA motif and most TADs are
56 demarcated by convergently oriented motifs, which suggests that binding orientation may play
57 a role in TAD formation ([6,10,13], Figure 1a and below). Since their initial discovery, TADs
58 and other domains restricted by CTCF binding have been identified as units of DNA
59 replication, recombination and repair as well [14-16].

60 The importance of CTCF binding at TAD boundaries to prevent enhancer hijacking—the
61 inappropriate activation of a gene by an enhancer in a neighboring TAD—has been confirmed
62 at several loci (*e.g.* [17,18]). During embryogenesis, correct hand development requires an
63 intra-TAD interaction between the *EPHA4* gene and its distal enhancer. Various instances of
64 structural variation involving the CTCF binding sites on either side of the *EPHA4* TAD
65 (inversions, deletions, duplications) create chromatin configurations where separation between
66 the *EPHA4* enhancer and genes in the neighboring TADs is lost (Figure 1b). For certain genes,
67 this permits their ectopic activation and ultimately the emergence of hand malformations [17].
68 In *IDH*-mutant gliomas, the genome undergoes global hypermethylation, which interferes with
69 site-specific CTCF binding. CTCF is therefore absent from an insulator that separates the
70 *PDGFRA* oncogene from an enhancer in a neighboring TAD, thereby allowing enhancer
71 hijacking and subsequent *PDGFRA* activation ([18], Figure 1b).

72 However, subsequent studies have added further nuance to these observations.
73 Transcriptome analysis in mouse embryonic stem cells where CTCF was rapidly degraded,
74 using the Auxin-inducible degron (AID) system [19], revealed that the nearly complete loss of
75 TAD organization only caused the upregulation of a limited number of genes [20]. Despite their
76 constitutive presence, TADs may thus only prevent a limited number of ectopic promoter-
77 enhancer loops. Explanations for this observation may be that the cell-type specific activity of
78 enhancers limits the potential for ectopic activation in a given cell type or that TADs are
79 required for the establishment of promoter-enhancer loops after mitotic exit but not for their
80 maintenance [21,22]. In parallel, several studies reported that ectopic gene activation and the
81 fusion of neighboring TADs required the removal of multiple CTCF binding sites that were
82 spread out over considerable genomic intervals [23-25]. Conversely, the creation of chromatin
83 configurations where CTCF binding sites were positioned in-between promoters and enhancers
84 revealed that the reduction in gene activity was incomplete [25-28]. Many boundaries between
85 TADs may therefore be composed of sets of CTCF binding sites that are not impermeable and
86 whose impact is restricted to specific cell types.

87 TADs as collections of actively extruding loops

88 The discovery of TADs raised the questions how these insulated domains are formed and how
89 CTCF binding can act as a directional boundary. An important hint came from the previously
90 identified colocalization of the Cohesin complex with CTCF [29,30]. The Cohesin
91 complex—well-known for sister chromatid cohesion after DNA replication—is a member of
92 the Structural Maintenance of Chromosomes (SMC) proteins that were proposed to have
93 motor-protein functions [31]. The role of Cohesin in TAD formation was revealed by polymer
94 simulations that could replicate the TAD structure in Hi-C matrices [32,33]. In these models,
95 Cohesin is loaded on the chromosome followed by the bidirectional extrusion of a DNA loop
96 (Figure 2a). Extrusion creates an increasingly large loop that compacts the chromosome until
97 two possible events. Either a roadblock is encountered that halts extrusion on that side, but
98 permits continued extrusion on the other side, or Cohesin dissociates from the chromosome
99 and the loop dissolves (Figure 2a). Whereas CTCF appears to have evolved as a functional
100 roadblock in this process, the encounter with large DNA-associated complexes (*e.g.* RNA
101 polymerase II) may interfere with loop extrusion as well [6,7,34,35]. Consequently, these

102 polymer simulations revealed that TADs are not insulated domains, but rather a collection of
103 actively extruding loops [32,33].

104 Numerous studies have contributed to the experimental validation of TAD formation by
105 Cohesin-mediated loop extrusion. Live-cell imaging after biochemical reconstitution of the
106 Cohesin complex has confirmed it possess a motor-protein function for bidirectional extrusion
107 of DNA loops [36,37]. The recent observation of ‘jet-like’ structures that sprout perpendicularly
108 from the Hi-C diagonal at narrow sites of Cohesin loading further confirmed the bidirectional
109 nature of loop extrusion [38]. Multiple Hi-C studies confirmed that TAD structure was lost in
110 cells where Cohesin was prevented from loading on the chromosome or where it was degraded
111 (RAD21/SCC1-AID cells) [39-41]. The active and energy-consuming aspect of loop extrusion
112 was confirmed in cells with Cohesin ATPase mutations [42]. Finally, single-cell Hi-C and
113 super-resolution imaging studies revealed a large degree of cell-to-cell variation in TAD
114 structure and intermingling. Instead, to obtain defined TAD structure as observed in a Hi-C
115 matrix, data from large numbers of cells had to be averaged [43-46].

116 Combined with the notion that TADs are collections of extruding loops (see [32,33]), the
117 observed cellular heterogeneity raised the idea that TADs are statistical properties of chromatin
118 ([47,48], Figure 2b). Here, the observation of discrete TADs in population-averaged data is
119 explained by chromosomal configurations where loops can be extruded anywhere in-between
120 two TAD boundaries. In contrast, loops that cross TAD boundaries are rare. Live-cell imaging
121 studies have determined loop extrusion dynamics, by measuring contacts between pairs of
122 nearby CTCF binding sites [49,50]. In population-averaged Hi-C matrices, interactions
123 between such pairs are enriched, creating visible ‘corner dots’ at the summits of TADs (Figure
124 2b). Although the distance between the CTCF binding sites was different between these studies
125 (150 kb versus 505 kb), they both detected prolonged periods of contact (in the order of 10-30
126 minutes). In contrast, the time gap in-between contacts was different and increased with
127 distance [49-51]. Biophysical modeling of looping kinetics indicated that the fully looped state
128 (*i.e.* halting of loop extrusion by both boundaries) was a rare event. Instead, most of the time,
129 the TADs were in a partially extruded state (*i.e.* combinations of loops that are being extruded
130 or halted on one side). These results further confirm that TADs are transient and indicate that
131 intra-TAD loop density is relatively minor [49-51]. The importance of intra-TAD loops was
132 nonetheless confirmed by studies of enhancer-promoter loop formation in cells that lack
133 Cohesin-mediated loop extrusion [52-54]. Whereas local interactions, in the order of 100 kb,
134 were only mildly affected in the absence of loop extrusion, the formation of long-range loops
135 (over 500 kb) was essentially lost. The dynamic nature of TADs is therefore essential to form
136 long-range intra-TAD enhancer-promoter loops, which expands the function of these domains
137 beyond the prevention of inter-TAD enhancer hijacking.

138 TAD boundaries as regulatory units

139 The formation of discrete TADs critically depends on the halting of loop extrusion at dedicated
140 boundaries. This raises the question if TAD boundaries can influence loop extrusion, thereby
141 providing means for regulation [8]. Most TADs carry CTCF binding sites in a convergent
142 orientation at their boundaries, suggesting this has an influence on the blocking process
143 [5,6,10,13]. Upon rapid depletion of CTCF (using CTCF-AID cells), TAD structure and pairing
144 of CTCF binding sites was reduced, without interfering with loop extrusion itself [20,41,49,50].

145 Halting of loop extrusion requires the N-terminus of CTCF, which faces the extruding Cohesin
146 complex when correctly oriented ([55,56], Figure 3). The STAG2 (also known as SA2) and
147 RAD21/SCC1 subunits of the Cohesin complex can subsequently interact with the N-terminus
148 of CTCF. Interestingly, the STAG2 subunit is present only in a variant of Cohesin that is
149 associated with more rapid dissociation and the formation of shorter loops [57,58]. When
150 bound to Cohesin, CTCF competes with the WAPL protein, which is responsible for the
151 dissociation of Cohesin from the chromosome. Indeed, without WAPL, TAD structure was
152 reinforced due to prolonged looping between CTCF binding sites [49,50,59]. For this Cohesin
153 variant we can therefore envision that the CTCF-mediated halting of loop extrusion on one side
154 promotes the formation of longer loops on the other side.

155 In parallel, the STAG1 (or SA1) containing variant of the Cohesin complex, which is
156 more stably associated with the chromatin, is subject to a different regulation at CTCF binding
157 sites ([41,57,58,60], Figure 3). The STAG1 variant is acetylated by the ESCO1 protein,
158 promoting the association of Cohesin with the PDS5A protein. PDS5A interferes with the
159 ATPase activity of Cohesin, thereby acting as a brake for loop extrusion. Upon halting at a
160 CTCF binding site, this more stable Cohesin variant therefore reduces loop extrusion in the
161 other direction as well. Combined with the regulation of Cohesin variant loading or
162 processivity [61], the balance between loop extrusion promotion (STAG2 Cohesin variant) and
163 reduction (STAG1 variant) may help to fine-tune the diversity of extruding loops, thereby
164 regulating TAD structure and function.

165 Besides protein-protein interactions, CTCF binding at TAD boundaries itself may also
166 influence the halting of the Cohesin complex. CTCF has an average DNA residence time in the
167 order of 1-2 minutes, which is about ten-fold shorter than the Cohesin complex [62]. Upon
168 CTCF dissociation, Cohesin may thus restart extrusion, resulting in the formation of longer
169 loops. This possibility was confirmed when Cohesin dissociation was perturbed by removing
170 WAPL, which dramatically increased the contacts between neighboring TADs [59]. The
171 permeability of CTCF binding sites is further supported by the biophysical modeling of pairing
172 between nearby CTCF binding sites, which requires the incorporation of a low chance
173 (~12.5%) of loop extrusion blocking [49]. To improve the chance of successful halting of loop
174 extrusion at a boundary, thereby improving the separation between neighboring TADs, multiple
175 CTCF binding sites can be grouped together. Indeed, strong TAD boundaries are characterized
176 by multiple closely-spaced CTCF binding sites [63,64]. Moreover, the complete intermingling
177 between certain neighboring TADs is only achieved upon the removal of multiple CTCF
178 binding sites [23-25]. In summary, the combination of protein-protein interactions, which
179 particularly reduces loop extrusion capacity of the STAG1 variant, with sequential and
180 prolonged halting at multiple DNA-encoded CTCF binding sites may sufficiently tip the
181 balance toward Cohesin dissociation, thereby improving separation between TADs.

182 **Conclusions and future perspectives**

183 The discovery of TADs and their involvement in gene regulation has raised a tremendous
184 interest in their structure-function relationships. Combinations of genomics, imaging and
185 biophysical modeling have identified an active and dynamic mechanism for TAD formation
186 that incorporates the creation of DNA loops through active extrusion by the Cohesin complex,

187 with halting at dedicated CTCF binding sites [32,33]. Whereas loop extrusion is essential for
188 long-range intra-TAD enhancer-promoter loops [53,54], the sequential halting and regulation
189 of loop extrusion at CTCF binding sites and ultimate the dissociation of Cohesin from the
190 chromosome is sufficient to create separation between neighboring domains [17,18].
191 Combined, this intricately regulated process explains how dynamic and active loop extrusion
192 can create TADs with stable regulatory functions.

193 An open question is if loop extrusion can maintain stable regulation over prolonged
194 periods of time, for instance in post-mitotic human neurons where transcriptional programs are
195 maintained for decades. Studies in post-mitotic mouse cells, several days after withdrawal from
196 the cell cycle, confirmed that TAD structure is maintained [20,38,53]. Similarly, a recent Hi-C
197 comparison of young (2 months) versus geriatric (over 28 months) mouse skeletal muscle stem
198 cells confirmed that most TAD boundaries were preserved [65]. Yet, a considerable reduction
199 in insulation at these boundaries was detected as well, suggesting that the efficiency of loop
200 extrusion halting was compromised. It remains to be determined what is the underlying cause
201 for this reduction and if this occurs in other post-mitotic cell types as well.

202 **CRedit author statement**

203 **J. A. d. C-N. & D. N.** Conceptualization.

204 **J. A. d. C-N. & D. N.** Writing- Original draft preparation.

205 **J. A. d. C-N. & D. N.** Writing- Reviewing and Editing.

206 **D.N.** Supervision

207 **Declaration of competing interest**

208 The authors declare that they have no known competing financial interests or personal
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216 **Data availability**

217 No data was generated for the research described in the article.

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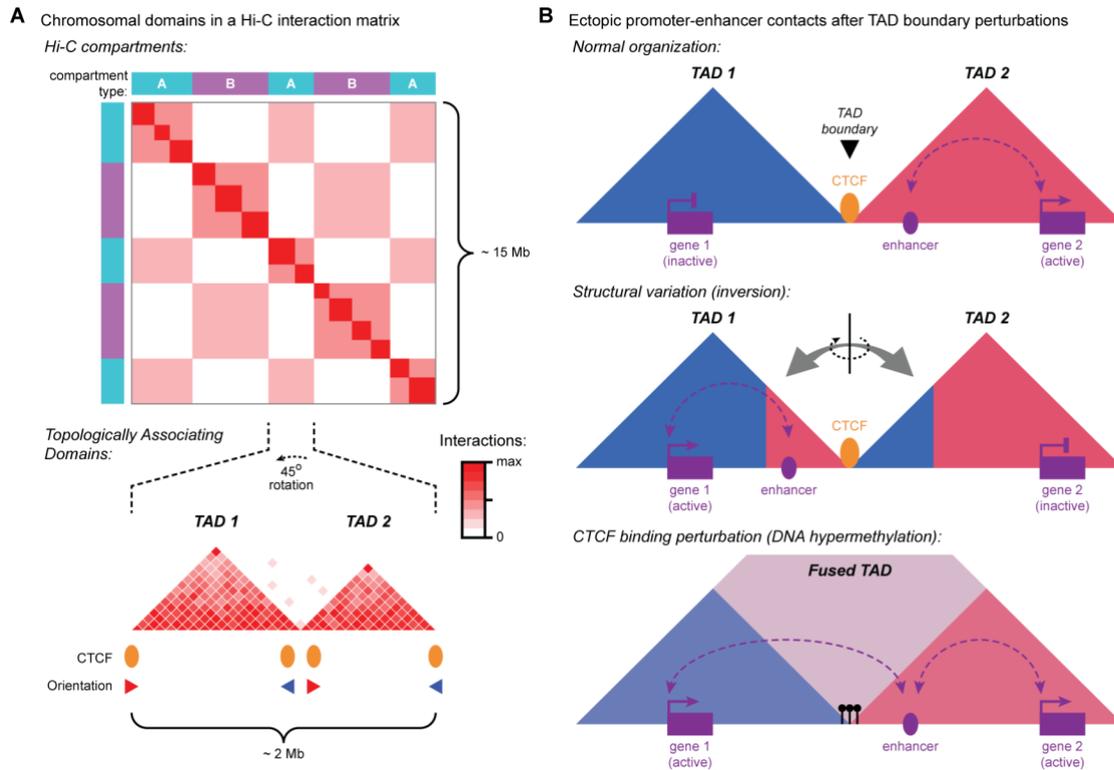
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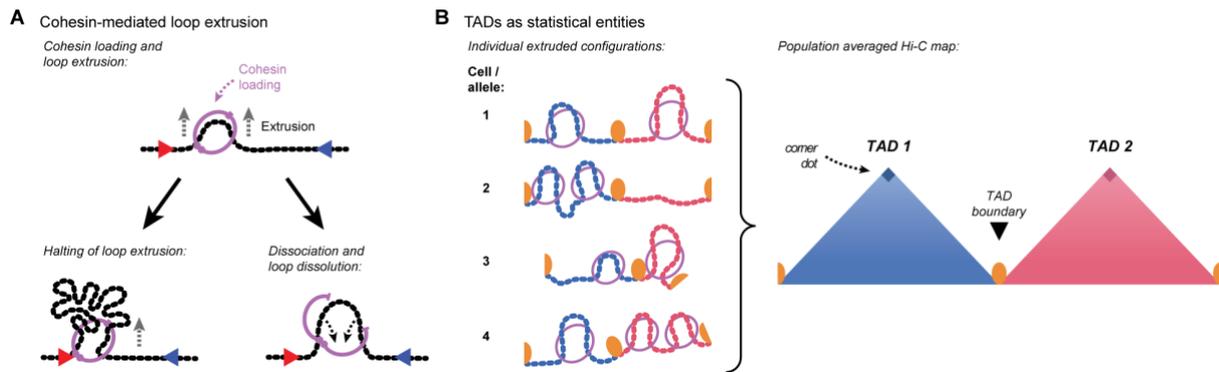
623 **Figures and legends**



624

625 **Figure 1: Chromosomal domains and TAD reorganization in disease.**

- 626 a. Appearance of mammalian chromosomal domains in Hi-C matrices. Top: Hi-C
 627 compartments within an ~ 15 Mb chromosomal region. Hi-C compartments engage in local
 628 and long-range homotypic interactions, resulting in a checker-board pattern of interactions.
 629 Compartment A and B identity is indicated on the top and left. Bottom: Zoom in on TADs
 630 in an ~ 2 Mb chromosomal region. TADs are domains with enriched intra-domain
 631 interactions and frequent convergent CTCF binding at the boundaries.
- 632 b. Examples of enhancer hijacking due to TAD boundary perturbations. Top: normal TAD
 633 organization. TAD 1 contains inactive gene 1 and TAD 2 contains active gene 2 that forms
 634 a DNA loop with its enhancer. Middle: Example of a chromosomal inversion that brings
 635 the enhancer and gene 1 together in the same TAD, thereby allowing the formation of a
 636 promoter-enhancer loop and gene activation. Example based on [17]. Bottom: Example of
 637 CTCF binding perturbation, caused by DNA hypermethylation (lollipops), that causes the
 638 fusion of neighboring TADs. Gene 1 and the enhancer are in the same TAD, thereby
 639 allowing the formation of a promoter-enhancer loop and gene activation. Example based
 640 on [18].



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Figure 2: Loop extrusion and TADs as statistical properties of chromatin.

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a. Steps of Cohesin-mediated loop extrusion. Top: The Cohesin ring (purple) loads on the DNA, followed by bidirectional loop extrusion (arrows). Bottom left: Loop extrusion is halted on one side when a roadblock is encountered (red arrow: occupied CTCF binding site in the correct orientation). Loop extrusion will continue unidirectionally on the other side, until a roadblock is encountered as well. Bottom right: The Cohesin ring dissociates from the DNA and the extruded loop will dissolve.

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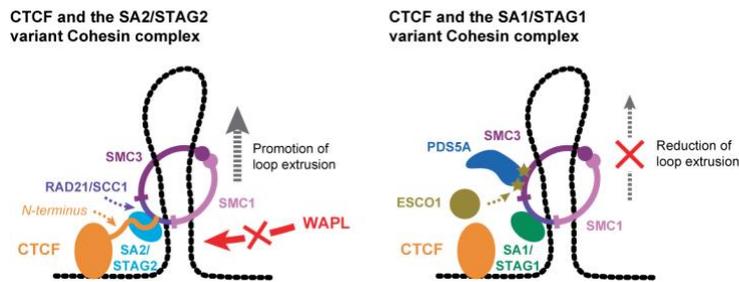
b. TADs as statistical properties of chromatin. Left: At a given time, different configurations of loops are extruded in individual cells. The density of loops is relatively minor and loops that cross the boundary are rare. Blue and red lines indicate the different regions covered by two TADs. Orange ovals indicate the boundary. Right: the averaged extruded configurations from large numbers of cells appear as an insulated TAD in a Hi-C matrix.

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655 Figure 3: Different regulation of variant Cohesin complexes at CTCF binding sites.

656 Left: Promotion of loop extrusion of the SA2/STAG2 variant Cohesin complex at CTCF
 657 binding sites. Unidirectional loop extrusion is promoted through the direct interaction of the
 658 CTCF N-terminal tail with the SA2/STAG2 and RAD21/SCC1 Cohesin complex subunits. This
 659 interaction prevents the interaction of the Cohesin complex with WAPL, thereby preventing its
 660 dissociation from the chromatin. Figure compiled after [57,58]. Right: Reduction of loop
 661 extrusion of the SA1/STAG1 variant Cohesin complex at CTCF binding sites. The SMC3
 662 Cohesin complex subunit is acetylated (stars) by ESCO1, followed by the recruitment of
 663 PDS5A and inhibition of Cohesin ATPase activity. Figure compiled after [58,60].