

Published in final edited form as:

Biochim Biophys Acta. 2014 March ; 1839(3): 191–202. doi:10.1016/j.bbagr.2013.11.002.

Mechanisms of cohesin-mediated gene regulation and lessons learned from cohesinopathies

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Abstract

Cohesins are conserved and essential Structural Maintenance of Chromosomes (SMC) protein-containing complexes that physically interact with chromatin and modulate higher-order chromatin organization. Cohesins mediate sister chromatid cohesion and cellular long-distance chromatin interactions affecting genome maintenance and gene expression. Discoveries of mutations in cohesin's subunits and its regulator proteins in human developmental disorders, so-called "cohesinopathies," reveal crucial roles for cohesins in development and cellular growth and differentiation. In this review, we discuss the latest findings concerning cohesin's functions in higher-order chromatin architecture organization and gene regulation and new insight gained from studies of cohesinopathies.

Keywords

cohesin; cohesinopathies; NIPBL; Cornelia de Lange Syndrome; Roberts' Syndrome

1. Introduction

Chromosomes undergo both global and local structural changes during the cell cycle and cellular differentiation. Accumulating evidence indicates that proper structural organization of chromosomes is critical for genome maintenance and functions, including proper chromosome segregation during cell division, DNA replication and repair, and gene expression. Structural Maintenance of Chromosomes (SMC) protein-containing complexes are a unique class of conserved and essential factors that control these processes by altering chromatin structural organization.

The first SMC gene, *Smc1*, was identified in yeast as being essential for mitotic chromosome segregation [1]. SMC proteins have conserved ATPase motifs, and ATP binding and hydrolysis by SMC proteins were shown to be important for the complexes' functions [2–4]. SMC proteins are folded in half at the hinge domain, which brings the conserved head and tail globular domains with divided ATPase motifs together. They form highly stable heterodimers in specific combinations in eukaryotes (SMC1-SMC3, SMC2-SMC4, and SMC5-SMC6) that further interact with specific sets of non-SMC subunits to

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assemble three major complexes: cohesin, condensin and the SMC5-SMC6 complex, respectively.

The common feature of SMC complexes is that they physically associate with chromatin and regulate higher-order chromatin structure. Early studies of condensin and cohesin in a *Xenopus* in vitro system and in yeast established solid biochemical and cell biological grounds to appreciate the intricate cell cycle-specific regulation and essential mitotic function of these two complexes [5, 6]. SMC complexes organize mitotic chromosomes to ensure proper segregation during cell division: cohesin through sister chromatid cohesion and metaphase chromosome congression, and condensin through orderly chromatin compaction and chromosome resolution. Studies in multiple organisms including *S. cerevisiae*, *S. pombe*, *Drosophila*, human and chicken cells, *C. elegans*, and more recently zebrafish and mice, have provided further insight into both conserved and species-specific functions of SMC complexes in genome regulation. Comprehensive reviews of condensin [7–9] and the SMC5-SMC6 complex [10, 11] have been recently published and they will not be discussed in detail here.

We now understand that cohesin has pivotal roles in mitosis, DNA replication, DNA repair, and gene expression, though the underlying molecular mechanisms and implications for development and disease are still under active investigation [12–14]. In somatic vertebrate cells, there are two different cohesin complexes (Fig. 1), and their functional redundancies and distinctions have just begun to be uncovered (see below). In this review, we mainly discuss cohesin's functions and regulation in mammalian cells, but we will not address its role in meiosis.

1.1. Structural features of cohesin

Cohesin consists of the SMC family proteins SMC1 (also known as SMC1A) and SMC3 as a heterodimer with the two non-SMC components Rad21 (also called Mcd1 or Scc1) and Scc3 (also called SA or STAG) [5]. SMC1 and SMC3 interact through their central hinge regions, while their respective paired amino- and carboxyl-terminal globular domains are further bridged by the kleisin family component Rad21 (or Scc1) (Fig. 1) [6, 15]. The primary function of cohesin is to mediate genome-wide sister chromatid cohesion in a cell cycle-regulated manner to ensure proper segregation of chromosomes in mitosis [16–18]. High-resolution microscopy and biochemical studies revealed that cohesin forms a ring structure [19–22]. Further analyses of purified cohesin-circular minichromosome complexes assembled in vivo, in conjunction with various mutational manipulations of cohesin subunits, supports the notion that the cohesin ring traps sister chromatids inside to mediate sister chromatid cohesion with distinct chromatin entry and exit mechanisms [20, 23–26]. However, alternative models of DNA trapping and cohesion by cohesin are still being discussed [27], and the exact mechanism is not yet fully resolved.

1.2. Two cohesin complexes in vertebrates

While a single Scc3 is present in yeast, two SA proteins, SA1 and SA2 (STAG1 and STAG2 in mice), are found in higher eukaryotes to form two distinct cohesin complexes in somatic cells: cohesin-SA1 and cohesin-SA2 (Fig. 1) [28, 29]. Both cohesin-SA1 and cohesin-SA2 contribute to genome-wide sister chromatid cohesion, with SA1 being particularly important for telomeric sister chromatid cohesion in mammalian cells [28, 30–32]. This appears to be determined by the specific interactions of SA1, but not SA2, with the telomere binding proteins TRF1 and TIN2 [30]. Thus, while the exact function of the SA/Scc3 subunit in yeast cohesin function remains elusive, SA proteins appear to dictate the recruitment specificity of cohesins through protein: protein interactions in mammalian cells. More recently, knockout and depletion experiments revealed that SA1 and SA2 have non-

redundant functions in the transcriptional regulation of certain, if not all, genes [32]. Mutations in SA2, with an intact SA1, are associated with aneuploidy in a diverse range of human cancers [33], and SA1 knockout caused aneuploidy and increased cancer risk despite the presence of an intact SA2 [34]. These studies suggest both redundant and distinct functions of the two cohesin complexes that are likely to have emerged to manage the increased complexity of chromosome organization and functions in higher eukaryotes.

2. Cell cycle-specific cohesin regulation in chromatin loading and cohesion

2.1. Cohesin loading onto chromatin by Scc2-Scc4 (NIPBL-MAU2) in telophase

In *S. cerevisiae*, cohesin is loaded onto chromosomes during G1 phase, which requires the heterodimeric cohesin loading factor Scc2-Scc4 [35]. Chromatin loading, but not establishment of cohesion, requires ATP hydrolysis [2, 3, 36]. Despite the early discovery of this cohesin loading factor, the exact loading mechanism remains enigmatic. Human cohesin also requires NIPBL (or delangin, yeast Scc2 homolog) and its partner MAU2 (yeast Scc4 homolog) for chromatin loading (Fig. 2) [37, 38]. Cohesin loading takes place in telophase in higher eukaryotes (see also 4.1.). A recent study suggests that this requires the opening of the SMC dimer at the hinge region, though how Scc2-Scc4 mediates this process is not understood [39].

2.2. Establishment of sister chromatid cohesion in S phase

ESCO1/2 (Eco1 in yeast), sororin and Pds5 are additionally needed to antagonize the cohesin destabilizing factor Wapl and establish sister chromatid cohesion in S phase (Fig. 2) [40, 41]. ESCO1 and ESCO2 (and Eco1p in yeast) are acetyltransferases, and their acetylation of SMC3 is required for antagonizing Wapl and establishment of sister chromatid cohesion [41–46]. Wapl appears to release cohesin from chromatin by opening the gate between SMC3 and Scc1 (Rad21) [26, 47, 48]. A recent structural study suggests that the binding of Wapl to the ATPase head domain of Smc3 may regulate its activity, though the detailed gate opening mechanism is unclear [49]. Interestingly, a sororin homolog has not been found in yeast, and although interfering with Wapl activity is critical for sister chromatid cohesion, how it leads to cohesion of the two sister chromatids is not well understood. Interestingly and somewhat counterintuitively, SMC3 acetylation also facilitates DNA replication fork progression, suggesting that this cohesin modification is also important to switch cohesin to a configuration that does not obstruct fork advancement [50]. ESCO-mediated acetylation of SMC3 is reversed by the deacetylase Hos1 in *S. cerevisiae* and HDAC8 in human cells, which is required for the next cycle of cohesion establishment [51–54].

There are additional factors that function in sister chromatid cohesion that all relate to DNA replication. These include the Ctf18-RFC complex, the DNA polymerase α -associating Ctf4, Trf4 (DNA polymerase κ PCNA and, more recently, Timeless and Tipin, further suggesting the coupling of DNA replication and cohesion [36, 55–60]. How these factors orchestrate the establishment of sister chromatid cohesion remains obscure. Consistent with the apparent coupling of DNA replication and establishment of sister chromatid cohesion, cohesin newly expressed in G2 phase after the completion of DNA replication fails to establish sister chromatid cohesion despite its loading onto chromatin in *S. cerevisiae* [36, 61]. This observation has not yet been confirmed in higher eukaryotes.

2.3. Cohesin removal and spindle-associated function in mitosis

In higher eukaryotes, cohesin is removed from chromosomes in a two-step process during mitosis that results in chromosome separation in anaphase [62]. The first step is removal of the majority of cohesin from chromatin in prophase, and the second step is destruction of the

residual cohesin remaining primarily at centromeres by separase-mediated Rad21 cleavage at the end of metaphase, which leads to chromosome segregation in anaphase. This mitosis-specific regulation of cohesin was reviewed extensively [63–67] and will not be discussed here in detail. More recent studies indicate that the SMC3-Rad21 gate opening by Wapl is important for cohesin release in prophase [47, 48]. A small population of cohesin associates with centrioles, and a proteolytic cleavage of Rad21 also regulates centriole disengagement [68–70]. In addition, a significant population of cytoplasmic cohesin associates with spindles and spindle poles in a mitosis-specific fashion, contributing to proper spindle assembly and chromosome congression [69, 71]. Thus, cohesin ensures proper congression and segregation of chromosomes during cell division through both chromatin-dependent and -independent actions.

2.4. Non-mitotic functions of cohesin

Cohesin functions in maintaining genome stability through post-replicative DNA double-strand break (DSB) repair, specifically sister chromatid homologous recombination (HR) repair [72, 73]. In mammalian cells, cohesin is also involved in DNA damage checkpoint control [74–77]. An excellent comprehensive review of the regulation and function of cohesin in DSB damage response and repair was recently published [14]. A recent study also indicated that cohesin affects normal DNA replication [78]. In addition, an expanding body of literature is documenting cohesin as a key regulator of gene expression (see below).

3. Mechanism of cohesin-mediated gene regulation

3.1. Long-distance chromatin interactions

3.1.1. CTCF-dependent and -independent long-distance chromatin interactions

interactions—Cohesin was shown to mediate chromatin looping at multiple gene loci important for imprinting and differential gene expression during development [79–85]. These interactions include CCCTC-binding factor (CTCF)-dependent insulator interaction, which blocks enhancer activity and/or inhibits the spreading of heterochromatic domains, as well as distal enhancer-promoter interactions important for gene activation (there are a number of comprehensive reviews, including but not limited to [12, 86, 87]). The roles of CTCF and related insulator binding proteins in *Drosophila* are discussed in a companion article in this issue (Matzat and Lei, this issue). In mammalian cells, the total number of cohesin binding sites vary from ~25,000 to ~120,000 as determined by chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) analyses, depending on the antibody, cell type, experimental conditions, and analytic tools employed [32, 83, 88]. Approximately 50–70% of cohesin sites overlap with CTCF binding sites genome-wide [88–92]. Cohesin mediates chromatin domain organization and insulator functions at many of these CTCF sites, including the *H19/IGF2* locus, the *IFNG* locus, the apolipoprotein gene cluster, the β -globin locus, the *Igh* locus, the *MHC class II* gene cluster, the *HoxA* locus, and the *T-cell receptor α* locus [79–81, 84, 93–97]. However, a significant number of cohesin sites appear to be CTCF-free and often overlap with binding sites for cell type-specific transcription factors [88, 98, 99]. Thus far, however, no significant DNA sequence preference was observed at cohesin binding sites other than the CTCF binding motif. CTCF-free cohesin binding sites coincide significantly with enhancer elements and genes that exhibit tissue/cell type-specific patterns of expression, and cohesin appears to help stabilize transcription factor binding to these sites [88]. It should be noted that a CTCF-associated function of cohesin has not been observed in *Drosophila*, in which cohesin mediates gene regulation in an insulator-independent manner (Matzat and Lei, this issue).

At the β -globin locus, both CTCF-dependent insulator interaction and CTCF-independent enhancer-promoter interactions can be observed [82]. Both types of interaction involve

cohesin in mouse and human erythroid lineage cells as detected by chromatin conformation capture (3C) and 3C combined with ChIP (ChIP-loop) (Fig. 3) [82]. The distal enhancer in the locus control region (LCR) interacts with the developmental stage-specific globin genes, which correlates with their specific expression [100, 101]. The lineage-specific transcription factors EKLf (Klf1), GATA-1, Fog-1, and Ldb1 are required in this process [102–104]. Both Nipbl and cohesin binding rapidly increases at chromatin loop anchoring sites upon cellular differentiation [82]. Depletion of either cohesin or Nipbl decreased both the insulator interaction and the LCR enhancer-promoter interaction, while CTCF depletion only affected the insulator interaction [82]. Consistent with this, cohesin depletion, but not CTCF depletion, decreased β -globin gene expression [82].

3.1.2. Genome-wide analyses of cohesin-mediated long-distance chromatin interactions—Recent studies examined cohesin-mediated chromatin interactions genome-wide using high-resolution high-throughput 3C-based techniques, circular 3C followed by high-throughput sequencing (4C-seq) with and without ChIP [105, 106], 3C carbon copy (5C) [107], and Chromatin Interaction Analysis by Paired-End Tag sequencing (ChIA-PET) [108] (for experimental details, see a recent review [109]).

An SMC1 ChIA-PET study, in which chromatin interactions involving cohesin were selectively analyzed in developing mouse limb, identified over 2,200 interactions at both CTCF-positive and -negative cohesin binding sites [108]. In either the promoter or intergenic/intronic regions, ~65% of chromatin interaction sites coincided with CTCF occupancy. The study revealed that in addition to tissue-specific promoter-enhancer interactions and constitutive chromatin domain demarcations, a subset of promoter-enhancer interactions reflect the poised state in embryonic stem cells (ESCs) and are maintained in multiple tissues even when the genes are not expressed.

Cohesin plays an important role in the maintenance of pluripotency. Cohesin was found to interact with Mediator and colocalize at the anchoring sites of enhancer-promoter interactions at pluripotency genes in mouse ESCs (mESCs), with its depletion causing spontaneous differentiation [83, 110]. High-resolution 5C analysis of the regions surrounding the major developmentally regulated genes during neuroectoderm differentiation was compared to corresponding ChIP-sequencing data for CTCF, cohesin and Mediator [107]. The results revealed that CTCF/cohesin tends to mediate relatively constant long-range chromatin interactions defining megabase-sized topologically associating domains (TADs), while Mediator and cohesin bridge short-range enhancer-promoter interactions, which are often cell typespecific, both within and between TADs [107]. Both 3C and 4C with or without ChIP revealed that cohesin and Mediator are involved in pluripotency-specific chromatin interactions at the *Oct4* and *Nanog* promoters [83, 105, 106, 111]. The interaction patterns are altered during differentiation and restored in induced pluripotency cells (iPSCs). Cohesin recruitment is induced concomitant with the induction of long-distance chromatin interactions during the iPSC reprogramming process. Cohesin depletion disrupts the enhancer-promoter interaction, blocks self-renewal, induces differentiation in pluripotent cells, and interferes with reprogramming of fibroblasts to iPSCs [105, 106, 111].

3.1.3. Chromatin looping: cause or consequence of gene expression?—The aforementioned studies strongly suggest that cohesin-mediated chromatin interactions are critical for gene expression. Furthermore, a recent study also showed that forced induction of distal enhancer-promoter interaction indeed activates β -globin gene expression (albeit to lesser extent than the full activation), demonstrating the pivotal role of long-distance chromatin interactions in gene regulation [112]. Comparison of mESCs and differentiated cells as well as examination of iPSC reprogramming described above also provided

evidence that reorganization of chromatin interactions precedes the actual gene expression changes, supporting the idea that chromatin interactions are causative rather than a consequence of gene expression changes [105, 106, 111]. Whether cohesin is involved in the initiation and/or maintenance of these interactions is unclear.

3.2. Role of cohesin in gene repression

Cohesin was found to repress gene expression by enhancer blocking, for example, at the *cut* gene in *Drosophila* [113] and the *IGF2-H19* locus in mammalian cells [89, 91]. Although cohesin is also known to bind to centromeric and non-centromeric heterochromatin repeats [114–116], only a limited number of examples of cohesin's involvement in heterochromatin-mediated gene silencing have been documented. In *Drosophila*, both cohesin and Nipped-B (Nipbl homolog) bind to the *Enhancer of split* and *invected-engrailed* gene complexes coinciding with histone H3 lysine 27 trimethylation (H3K27me3), the repressive histone modification associated with the polycomb silencing pathway [117, 118]. Depletion of cohesin resulted in upregulation of these genes [115]. More recent studies provided additional evidence for the functional interaction between cohesin and polycomb proteins and the effect of cohesin on polycomb silencing in *Drosophila* [119, 120]. In *S. pombe*, cohesin binds to subtelomeric heterochromatin regions harboring H3 lysine 9 methylation (H3K9me) [114]. Cohesin is co-recruited with Swi6, a heterochromatin binding protein 1 (HP1) homolog that recognizes methylated lysine 9 residues, and they function together in gene silencing [114] (see 4.3.3). Similar co-recruitment of cohesin and HP1 γ is observed at subtelomeric heterochromatin repeats in human cells, whose loss is associated with a specific muscular dystrophy (see 7.1).

3.3. RNA polymerase II (RNAPII) occupancy and transition from pausing to elongation

In *Drosophila*, cohesin and Nipped-B bind to a subset of active genes, in particular to genes with a paused RNAPII [121–123]. Cohesin or Nipped-B depletion results in increased RNAPII pausing at cohesin-bound genes, suggesting that cohesin facilitates RNAPII transition to elongation [123]. Whether this is a consequence of cohesin's function in enhancer-promoter bridging or by cohesin's direct effect on RNAPII is currently unclear. Interestingly, cohesin depletion also results in a general decrease of RNAPII pausing and transcription of noncohesin-bound genes [123]. Whether a similar effect of cohesin depletion on non-cohesin-bound genes exists in other organisms is currently unknown, and whether cohesin facilitates RNAPII transition from pausing to elongation in mammalian cells remains to be determined.

3.4. Intragenic cohesin binding and RNA transcription

In contrast to the studies in *Drosophila*, intragenic binding of cohesin together with CTCF appears to cause RNAPII pausing in mammalian cells, resulting in alternative mRNA products. In human cells, cohesin/CTCF binding in intragenic regions functions as a chromatin boundary to block transcriptional read-through of the full-length *PUMA* gene [124]. In addition, RNAPII complexes accumulate at the CTCF-cohesin binding site within the first intron of the latency transcript of Kaposi's sarcoma-associated herpesvirus [125]. This pausing, which also involves the binding of pausing factors SPT5 and NELF-A at the intragenic CTCF-cohesin binding site, appears to be important for proper mRNA processing and production. Although the presence of cohesin was not tested, intragenic binding of CTCF also dictates alternative mRNA splicing of the *CD45* gene [126], and the presence of CTCF at promoter proximal sites was shown to be associated with RNAPII pausing in mammalian cells [127]. Since no significant overlap between cohesin and CTCF binding is seen in *Drosophila* [128] (Matzat and Lei, this issue), how this relates to the observations in *Drosophila* (see 3.3.) is currently unclear.

4. Cohesin recruitment mechanisms

4.1. The NIPBL-Mau2 (SCC2-SCC4) cohesin loading factor

Cohesin is not a canonical sequence-specific DNA binding factor, and how it is recruited to chromatin is critical for both its cell cycle- and differentiation stage-specific functions. In metazoans, genome-wide cohesin loading occurs at the end of mitosis during telophase, which also requires their *Scs2* and *Scs4* homologs (NIPBL (human)/Nipbl (mouse) and MAU2 (human)/Mau2 (mouse), respectively) [38, 129, 130]. In *Xenopus* and human cells, pre-replication complex components, including ORC, Cdc6, Cdt1, and MCM2-7, were shown to be required for loading of *Scs2-Scs4* (NIPBL-MAU2) and subsequent cohesin binding to chromatin [129–131]. This suggests that the initial loading sites for cohesin are at pre-replication complex assembly sites (i.e. replication origins) in higher eukaryotes. In contrast, no obvious relationship between the replication origin (Autonomously replicating sequence (ARS)) and *Scs2-Scs4* binding sites has been demonstrated in *S. cerevisiae*. Interestingly, all three SMC complexes (cohesin, condensin, and the SMC5-SMC6 complex) independently require *Scs2* in *S. cerevisiae* [132, 133]. Condensin additionally requires RNA polymerase (RNAP) III transcription factor TFIIC and is preferentially recruited to RNAPIII genes, such as tRNA genes [133]. In *C. elegans*, loading of condensins and the SMC5-SMC6 complex appears to be *Scs2*-independent despite the partial overlap of *Scs2* and condensin binding sites [134, 135]. The relationship between other SMC complexes and NIPBL-MAU2 is unclear in mammalian cells.

4.2. Cohesin sliding?

In *S. cerevisiae*, cohesin binding appears to be affected by the transcriptional status of nearby genes, and cohesin tends to accumulate at sites of transcriptional convergence [136–138]. Interestingly, ChIP analyses using antibody specific for *Scs2* has revealed that the peaks associated with *Scs2* binding often do not coincide with cohesin peaks, suggesting that cohesin may “slide” from its initial loading sites marked by *Scs2-Scs4* [137, 139]. However, another study using FLAG-tagged *Scs2* revealed the presence of *Scs2* at all cohesin binding peaks, arguing that the loading factor functions at all cohesin binding sites [140]. It should be noted, however, that even in the latter study, the peak signals for *Scs2* binding are not always proportional to cohesin peaks, suggesting that an additional factor(s) impacts cohesin accumulation (e.g., the transcriptional status of the neighboring genes) [140]. If the *Scs2* ChIP efficiency is low, these weak sites may be considered negative and give the impression that cohesin binds to *Scs2*-free regions. Interestingly, the binding of ATP hydrolysis-defective cohesin appears to be more restricted and more closely correlates with the major *Scs2* binding peaks [141]. This suggests that sliding, but not initial loading, of cohesin requires ATP hydrolysis. However, whether cohesin can change its binding sites in the absence of *Scs2-Scs4* has not been explicitly tested.

In higher eukaryotes, there is thus far no clear evidence for cohesin sliding and accumulation at transcriptional convergence sites. In *Drosophila*, Nipped-B and cohesin binding sites virtually overlap and are associated with active genes, often with paused RNAPII [121, 122]. Cohesin was found to be significantly enriched at the promoters and gene regions in mammalian cells [89, 91]. Furthermore, the increase of Nipbl binding closely accompanies the increase of cohesin binding at the adult globin enhancer and promoter regions upon β -globin gene activation [82]. A study in mESCs identified two different populations of cohesin binding sites, one overlapping with CTCF with no apparent Nipbl peaks, and the other coinciding with Nipbl and Mediator [83]. This led to the notion that Nipbl may not load cohesin at CTCF sites. However, specific Nipbl binding peaks can be identified at cohesin-bound CTCF insulator sites by manual ChIP-PCR, and depletion of Nipbl also affects cohesin binding at these regions, suggesting that Nipbl also loads cohesin at CTCF

sites [82]. The difficulty in detecting Nipbl peaks consistently by ChIP-seq at all cohesin binding sites may be due to the fact that Nipbl binds chromatin less stably than cohesin [131]. Recent attempts to reconstitute Scc2-Scc4-dependent cohesin loading in vitro in yeast and human cells are an important first step towards addressing this issue [131, 142].

4.3. Cohesin recruitment through protein and RNA interactions

4.3.1. Modulation of cohesin recruitment to CTCF sites—The majority of cohesin binding sites contain the CTCF motif in mammalian cells [89–92], which appears to be sufficient to recruit cohesin [91]. The SA proteins (both SA1 and SA2) interact with CTCF [143]. CTCF depletion decreases cohesin binding to some of these sites, suggesting that cohesin is recruited to these sites by CTCF though this relationship is not observed in *Drosophila* (Matzat and Lei, this issue). However, not all the CTCF sites in mammalian cells are co-occupied with cohesin [96, 144], suggesting that an additional factor(s) dictates cohesin binding at CTCF sites. Indeed, the cohesin and CTCF interaction is modulated by the DEAD-box RNA binding protein p68, together with its associated non-coding RNA (ncRNA) called steroid receptor RNA activator (SRA), and promotes insulator function, for example, at the *Igf2/H19* locus [145]. ATR-X, mutated in the Alpha-Thalassemia mental Retardation, X-linked (ATR-X) syndrome, together with methyl-CpG binding protein 2 (MeCP2), also interact with cohesin and CTCF in the brain, affecting their binding and postnatal imprinting function at the *Igf2/H19* and *Gtl2/Dlk1* loci [146].

4.3.2. Other factors that dictate cohesin recruitment—Cohesin was shown to interact with Mediator, Nanog and Klf4, suggesting that these interactions may mediate the specific recruitment of cohesin [83, 105, 110]. Cohesin was also found to interact with NF-E2, which is specifically recruited to the LCR enhancer and the promoter regions of the adult β -*globin* locus coinciding with cohesin [82, 147]. In addition, cohesin was found to be part of the human ISWI (SNF2h)-containing chromatin remodeling complex together with the Mi2/NuRD complex, and bind chromatin together in an SNF2h ATPase activity-dependent manner in human cells [148]. Rad21 directly interacts with SNF2h [148]. Recently, *Drosophila* Mi-2 was also found to recruit cohesin to polytene chromosomes in salivary glands [149]. In addition, cohesin was reported to bind to the non-coding RNAs (ncRNAs) transcribed on enhancer regions, termed enhancer RNAs (eRNAs) [150]. Ligand-activated estrogen receptor (ER) upregulates transcription of eRNAs, which act *in cis* to promote upregulation of nearby ER target genes. The eRNAs bind to cohesin and increase cohesin recruitment to the enhancer regions in response to the ER ligand estradiol, and stimulate the enhancer-promoter interactions in MCF7 breast cancer cells [150]. Though the exact mechanism is unclear, this raises the intriguing possibility that other ncRNAs may also affect long-distance chromatin interactions through recruitment of cohesin.

4.3.3. Cohesin recruitment to heterochromatin repeats—In *S. pombe*, cohesin is recruited to both pericentromeric and subtelomeric heterochromatin via the H3K9me-Swi6 (HP1) pathway, except that the recruitment of cohesin and Swi6 is mutually dependent at subtelomeric heterochromatin [114, 151, 152]. While cohesin is recruited by Swi6 to mediate centromeric sister chromatid cohesion with no role in gene silencing at pericentromeric heterochromatin [151, 152], cohesin co-recruited with Swi6 to the subtelomeric heterochromatin participates in gene regulation [114]. Interestingly, similar co-recruitment of cohesin and one of the HP1 variants, HP1 γ , was observed at subtelomeric heterochromatic D4Z4 macrosatellite repeat regions marked by H3K9me3 in human cells, whose loss is closely associated with a muscular dystrophy (see 7.1) (Fig. 4A). Though it was controversial whether the H3K9me-HP1-cohesin pathway is conserved at mammalian centromeres [153, 154], a recent study demonstrated that cohesin recruitment to pericentromeric heterochromatin indeed involves HP1 in human cells [155]. While mainly

HP1 α and also HP1 γ are involved in pericentromeric heterochromatin recruitment of cohesin, HP1 γ is specifically involved in cohesin co-recruitment at subtelomeric D4Z4 heterochromatin. NIPBL, but not cohesin, was shown to directly bind to all three HP1 variants [116, 154, 156]. More recently, the Suv4-20h histone methyltransferase that specifically mediates H4K20 trimethylation (H4K20me₃) was shown to interact with cohesin and functions in cohesin recruitment to pericentromeric heterochromatin in mouse cells in a catalytic activity-independent manner, which is important for centromeric sister chromatid cohesion and proper segregation of chromosomes in mitosis [157]. Suv4-20h recruitment to pericentromeric heterochromatin itself is dependent on H3K9me₃ and HP1. Thus, cohesin recruitment to heterochromatin appears to be more complex than previously thought (Fig. 4B).

4.3.4. Cohesin, NIPBL and MAU2 can each specify cohesin recruitment sites—

Artificial centromeric tethering of an Scc4 fusion protein is sufficient for the recruitment of Scc2 as well as cohesin in budding yeast, indicating that Scc4 can also be a determinant for binding site specificity [158]. Although the pre-replication complex-dependent loading of NIPBL in telophase is cohesin-independent in human cells [131], cohesin is reciprocally required for Scc2/Scc4 recruitment to centromeres in yeast, supporting the notion that cohesin can dictate its loading site [158].

Collectively, these results suggest that increased binding of either cohesin or NIPBL or MAU2 can trigger cohesin's accumulation at specific genomic regions. With many potential interaction surfaces available on subunits of cohesin, NIPBL, and MAU2, differential targeting of cohesin may be achieved by interactions with sequence-specific transcription factors, chromatin remodelers, specific histone mark readers, and even with RNA. Many of these interactions may occur at a specific subcellular and/or genomic location and often in a cell cycle- or differentiation stage-specific manner. These differential interactions may be regulated by post-translational modifications or availability of the interacting proteins. This allows cohesin to be recruited to multiple sites in different cell types and contexts, providing further versatility to its actions.

5. Cohesinopathies

Human syndromes caused by cohesin and cohesin-associated factor mutations, resulting in cohesin dysfunction, are called “cohesinopathies” (Fig. 2) [159, 160]. The two classic examples are Roberts' Syndrome (RBS) and Cornelia de Lange Syndrome (CdLS).

5.1. Roberts' Syndrome

RBS (OMIM 268300) (more recently, Roberts' Syndrome/SC phocomelia) is caused by mutations of both alleles of *ESCO2* (Fig. 2) [161]. RBS patients have a wide range of clinical phenotypes that include upper and lower limb defects, growth retardation, craniofacial anomalies, and mental retardation with limited similarity to the CdLS phenotype [161, 162]. Importantly, RBS chromosomes exhibit premature centromere separation and heterochromatin puffing, indicative of a sister chromatid cohesion defect [163]. Centromeric cohesion defects and cell cycle aberrations are observed in *ESCO2* knockout mice and zebrafish [164, 165].

5.2. Cornelia de Lange Syndrome (CdLS)

CdLS (OMIM 122470, 300590, 610759) is a dominant multisystem developmental disorder characterized by facial dysmorphism, hirsutism, upper limb abnormalities, cognitive retardation, and growth abnormalities [166, 167]. Mutations in the *NIPBL* gene on chromosome 5p13 are linked to more than 55% of CdLS cases (Fig. 2) [168, 169].

Frameshift or nonsense mutations of *NIPBL* that result in *NIPBL* haploinsufficiency often exhibit more severe phenotypes compared to missense mutations [170]. Mutations in the cohesin subunits *SMC1* (human *SMC1* (*hSMC1*), *SMC1A*) and *hSMC3* were also found in a minor subset of clinically milder CdLS cases (~5% and <1%, respectively) [171, 172]. *SMC1* or *SMC3* mutations are always missense mutations and patients often show mental retardation as the primary symptom, with other abnormalities being fewer and/or milder [172]. More recently, mutations in *HDAC8*, which regulates cohesin dissociation from chromatin in mitosis, were also found in a subset of CdLS patients (OMIM 300882) [53]. *HDAC8* functions to deacetylate *SMC3* and therefore facilitates cohesin displacement from chromatin during mitotic progression (also see 2.2) [53]. Nonsense or missense mutations that cause loss of *HDAC8* activity resulted in *SMC3* hyperacetylation and chromatin retention of the cohesin complex during mitosis [53]. CdLS patients with *HDAC8* mutations display similar phenotypes as the patients with *NIPBL* mutations [53]. Furthermore, cohesin component *Rad21* mutations were found in patients with a CdLS-like phenotype (OMIM 614701) [173]. In contrast to *SMC1* and *SMC3* mutations, patients with *RAD21* mutations exhibit classical CdLS physical phenotypic characteristics (growth retardation, minor skeletal anomalies, and facial features) but have mild or no cognitive impairment [173]. Taken together, mutations of cohesin subunits and the regulators of cohesin loading to chromatin cause phenotypically related developmental disorders [167, 174].

5.3. Mutations of additional genes in the cohesin pathway?

While mutations in these proteins (*NIPBL*, *HDAC8*, *SMC1A*, *SMC3*, and possibly *RAD21*) may explain approximately 65% of CdLS patients, the cause of the remaining 35% remains unclear. For example, mutations in *Pds5A* and *Pds5B*, additional factors important for proper cohesin function in sister chromatid cohesion, also result in phenotypes in mouse models reminiscent of those observed in CdLS patients. However, no significant association of *Pds5A* or *Pds5B* mutations with CdLS has been observed [175, 176]. Nevertheless, mutations in additional genes involved in the cohesin pathway are expected to contribute to CdLS' pathogenesis.

6. Mechanism of cohesinopathies

6.1. *NIPBL* haploinsufficiency causes CdLS

NIPBL haploinsufficiency is the major cause of CdLS (see above) [167, 177, 178]. *Nipbl* heterozygous mutant (*Nipbl*^{+/-}) mice exhibit wide-ranging defects characteristic of CdLS, including small size, craniofacial anomalies, microbrachycephaly, heart defects, hearing abnormalities, low body fat, and delayed bone maturation, confirming that partial reduction of *Nipbl* is sufficient to cause a CdLS-like phenotype [179]. The mutant mice demonstrated only a 25–30% decrease in *Nipbl* transcripts, suggesting compensatory upregulation of the intact allele, which apparently is not sufficient to block development of the phenotype. Consistent with this, as little as a 15% decrease in *NIPBL* expression was shown to cause CdLS, though mild, in patients [180, 181]. These observations indicate the extreme sensitivity of mammalian development to *NIPBL/Nipbl* gene dosage.

6.2. *NIPBL* haploinsufficiency exhibits no significant sister chromatid cohesion defect

There appears to be a functional hierarchy for cohesin in which the most essential function, which is resistant to partial reduction of cohesin, is its role in sister chromatid cohesion and proper segregation of chromosomes (reviewed in [12]). The differential sensitivities of cohesin functions to cohesin depletion were most systematically demonstrated in yeast with different degrees of cohesin protein reduction [182]. Namely, mitotic sister chromatid cohesion is most resistant to partial reduction of cohesin. Similar observations were made in *Drosophila* and in human cells, in which partial depletion of cohesin by siRNA does not lead

to any significant sister chromatid cohesion defect [69, 115]. Consistent with these findings, CdLS patient cells do not exhibit any obvious sister chromatid cohesion abnormalities [183–186]. This is in contrast to RBS, in which premature sister chromatid separation serves as a prototypical cellular phenotype for the disorder [163]. Though it is currently unclear how sister chromatid cohesion defects specifically contribute to the pathogenesis of RBS, distinct mechanisms are likely involved in the development of this cohesinopathy as opposed to CdLS.

6.3. DNA repair

Increased DNA damage sensitivity appears to be a general feature of cohesinopathies, as it has been reported in RBS, CdLS, and CdLS-like disorder patient cells [173, 184, 186–188]. A study in yeast suggested that an RBS-associated *ESCO2* catalytic mutation impairs HR repair [189]. While no obvious HR repair defect was detected in NIPBL-mutated CdLS patient cells, increased chromosome aberrations indicative of a DNA repair defect were observed in SMC1- and SMC3-mutant CdLS patient cells [184, 186]. Cells with the Rad21 mutation found in the CdLS-like disorder also exhibited a repair defect, although impairment of the HR repair pathway was not specifically confirmed [173]. Nevertheless, the defect does not appear to result in prominent genome instability and/or increased cancer incidence [190]. Thus, how the increased DNA damage sensitivity contributes to the disorder's pathogenesis is currently unclear.

6.4. Nipbl reduction results in decreased cohesin binding and gene expression changes

As discussed above, NIPBL mutations in both CdLS patient cells and in mouse models cause little or no chromatid cohesion defect, suggesting that the developmental abnormalities are a result of defective cohesin-mediated gene regulation [179, 181]. In both patient lymphoblasts and *Nipbl*-mutant mouse tissues and cells, the partial decrease of *Nipbl* expression is associated with pervasive, though small, alterations in gene expression. It was proposed, therefore, that diffuse, relatively mild expression perturbations collectively contribute to the developmental defect phenotype. Supporting this model, combined depletion of *Nipbl* target genes indeed recapitulates the *Nipbl* depletion phenotype in zebrafish [191]. As noted above, it was shown that *Nipbl* haploinsufficiency causes both decreased cohesin binding at the β -globin locus in embryonic liver as well as decreased long-distance chromatin interactions (involving both CTCF sites and non-CTCF sites). In particular, reduced chromatin interactions between the enhancer and adult globin genes appear to contribute to decreased globin gene expression [82]. One can envision that diminished cohesin-mediated long-distance chromatin interactions could affect gene regulation genome-wide, resulting in widespread disruption of normal gene expression in a cell type- and differentiation stage-specific manner.

6.5. Cohesinopathy may be a ribosomopathy?

Mutations of genes that impair ribosomal RNA (rRNA) transcription or ribosome biogenesis were found to be associated with various human genetic disorders, many of which are accompanied by growth and mental retardation. These include Treacher Collins Syndrome, Bloom's and Werner Syndromes, Cockayne Syndrome, and Shwachman–Diamond Syndrome, which can all be considered to be “ribosomopathies” [192].

Recently, the effects of cohesinopathy disorder mutations of *ESCO2* (RBS), *NIPBL* (severe CdLS) and *SMC1* (mild CdLS) genes were evaluated by introducing analogous mutations in the corresponding homolog genes *Eco1*, *Scs2*, and *Smc1* in *S. cerevisiae* [193]. It was found that *Eco1* and, to lesser extent, *Smc1* mutations (but not *Scs2* mutation), caused decreased rRNA production and ribosomal biogenesis resulting in translational defects [193]. Similar defects were observed in RBS patient cells in which *ESCO2* (the *Eco1* homolog) is mutated,

raising the intriguing possibility that RBS is in fact a ribosomopathy [193]. Although cohesin is known to bind to ribosomal DNA [116, 194], the underlying mechanism of the defect caused by *ESCO2* mutation is currently unknown. Whether similar defects contribute to CdLS with *NIPBL* haploinsufficiency has not been addressed. However, since growth and mental retardation appear to be common phenotypes shared between various ribosomopathies and CdLS, it is possible that defective nucleolar/ribosomal function significantly contributes to CdLS and CdLSlike disorders as well.

6.6. NIPBL function beyond cohesin loading?

The wide range of defects observed in CdLS shows that abnormalities of cohesin-related functions have significant impact throughout development and on multiple cellular differentiation processes. Mutation of the genes involved in cohesin function and regulation can result in overlapping but not identical phenotypes. Zebrafish mutant analyses of *ESCO2*, *Nipbl*, *SMC1*, and *Rad21* revealed only a modest overlap of affected genes [164, 195]. Despite the evidence that cohesin function is affected by *NIPBL* haploinsufficiency, CdLS cases with *NIPBL* mutations/haploinsufficiency tend to have a more severe phenotype compared to those with cohesin mutations (increased severity of mental retardation, growth impairment, or structural abnormalities of the limbs and other organ systems) [171–173]. This raises the possibility that NIPBL may in fact govern other pathways in addition to cohesin loading. For example, NIPBL may dictate the chromatin loading of other SMC complexes as seen in yeast [132, 133], though, unlike in yeast [133, 196], no obvious chromosome condensation defect (indicative of condensin dysfunction) was reported to be associated with *Nipbl* mutation in mammalian cells.

7. Facioscapulohumeral dystrophy (FSHD) as a new cohesinopathy disorder?

FSHD is the third most common heritable muscular dystrophy in the U.S. It is characterized by progressive wasting of facial, shoulder, and upper arm musculature, which can spread to the abdominal and foot-extensor muscles [197–199]. The genetics underlying FSHD are highly unusual; the majority of FSHD cases (>95%) are associated with monoallelic deletion of D4Z4 macrosatellite repeat sequences clustered at the subtelomeric region of chromosome 4q (4qter D4Z4) (FSHD1 (MIM 158900)) [197, 200]. There are between one and ten repeats in the contracted 4qter allele in FSHD1 patient cells, in contrast to 11–150 copies in normal cells. In the more rare form of FSHD (<5% of cases) (FSHD2) there is no D4Z4 repeat contraction, though phenotypically FSHD1 and FSHD2 are largely identical [201].

D4Z4 is a 3.3 kb repeat that contains an open reading frame (ORF) for the double-homeobox transcription factor *DUX4* retrogene [202–204]. Artificial overexpression of the fulllength *DUX4* (*DUX4*fl) protein caused a myoblast differentiation defect in human myoblasts and mouse C2C12 cells [205, 206]. Only those individuals with a 4qA haplotype with specific single-nucleotide polymorphisms in the region distal to the last D4Z4 repeat (creating a canonical polyadenylation signal for the *DUX4* transcript) develop FSHD, strongly suggesting that *DUX4*fl mRNA expression is critical for FSHD pathogenesis [207].

7.1. FSHD is associated with disruption of transcriptionally repressive chromatin organization at 4qD4Z4

D4Z4 chromatin normally harbors the transcriptionally repressive histone modification marks histone H3 lysine 9 trimethylation (H3K9me3) and H3K27me3 (Fig. 4A) [116]. Interestingly, H3K9me3 is significantly diminished at D4Z4 repeat regions in both FSHD1 and FSHD2 patient cells, but not in other muscular dystrophies [116, 208]. This change is

also found in FSHD patient lymphoblasts, indicating that the loss is not an epiphenomenon of the dystrophic phenotype and suggesting that it occurs early in development before lineage separation [116].

D4Z4 DNA was also shown to be hypermethylated in normal cells (Fig. 4A). Intriguingly, D4Z4 DNA is hypomethylated in both FSHD1 and FSHD2 [209]. D4Z4 DNA, however, is also severely hypomethylated in the Immunodeficiency–Centromeric instability–Facial anomalies (ICF) syndrome, which is phenotypically distinct from FSHD [209, 210]. Thus, the loss of DNA methylation alone is insufficient to cause FSHD. It should also be noted that H3K9me3 is intact in ICF cells or 5-AzaC-treated (DNA methylation-inhibited) cells, indicating that the H3K9me3 loss is not a downstream consequence of DNA hypomethylation [116]. Whether DNA hypomethylation is triggered by H3K9me3 loss is not known. Nevertheless, concomitant loss of H3K9me3 and DNA methylation at D4Z4 indicates that FSHD is an epigenetic abnormality disease.

Although the molecular basis for heterochromatin loss is unclear, the concomitant loss of DNA methylation and H3K9me3 may be directly related to the etiology of FSHD. Cohesin and HP1 γ are recruited to D4Z4 in an H3K9me3-dependent manner and are therefore lost in FSHD cells [116]. Interestingly, the two factors require each other for D4Z4 binding, demonstrating the active role of cohesin in heterochromatin organization in human cells (Fig. 4A) [116]. This is analogous to the subtelomeric heterochromatin repeats in *S. pombe* in which cohesin and Swi6 are recruited in a mutually dependent manner and function in gene silencing [114]. Thus, FSHD can also be considered to be a cohesinopathy, in which D4Z4 heterochromatin-associated cohesin function is specifically disrupted. It is speculated that the loss of heterochromatin contributes to the expression of DUX4fl in FSHD. However, this has not been explicitly demonstrated.

A single copy of D4Z4 repeat sequence was shown to recruit CTCF and A-type lamins and to function as an insulator [211]. However, this binding was lost as the D4Z4 repeats were multimerized, simulating normal non-contracted D4Z4 alleles. Furthermore, CTCF binding is known to be DNA methylation-sensitive [212]. Thus, CTCF binding may be induced in FSHD cells in which D4Z4 heterochromatin, including DNA methylation as well as cohesin binding, is largely lost. Cohesin binding to D4Z4 heterochromatin, therefore, is through an H3K9me3-HP1 γ pathway and is independent of CTCF.

7.2. An SMC homolog, SMCHD1, is mutated in FSHD2 and in severe cases of FSHD1

A recent study found that SMCHD1, an epigenetic gene silencer involved in the maintenance of DNA methylation and X inactivation [213, 214], binds to D4Z4 and plays a role in *DUX4* gene repression (Fig. 4A) [215]. Importantly, this gene is mutated in many FSHD2 patients (OMIM158901) as well as in severe cases of FSHD1 in conjunction with D4Z4 contraction [215]. How SMCHD1 is recruited to D4Z4 remains unclear, but a recent study indicated that SMCHD1 is recruited to H3K9me3 domains through interaction with HBiX1, an HP1 binding protein, and contributes to the compaction of the inactive X chromosome [198]. This raises the intriguing possibility that in normal cells SMCHD1 is recruited to D4Z4 by the H3K9me3/HP1 γ /cohesin heterochromatin, the loss of which in FSHD results in decreased binding of SMCHD1 and subsequent derepression of the *DUX4* gene.

8. Concluding remarks

The recent explosion of genome-wide analyses as well as mechanistic studies have established cohesin as a key chromatin organizer in both mitosis and in interphase, influencing virtually all aspects of genomic function. Studies of cohesinopathies highlight

the exquisite sensitivity of developmental processes in multiple cell types to subtle dysfunction of cohesin and its associated factors. Further interrogation of mechanistic details (e.g., in vitro reconstitution of cohesin-mediated processes) and how cohesin-mediated genomic organization is integrated into and controlled by other biological processes and signaling pathways, are two exciting areas for further investigation. This will provide significant insight into both basic questions concerning chromosome dynamics and the mechanisms underlying cohesinopathies.

Acknowledgments

The work in the Yokomori laboratory described was supported in part by NIH AR058548, HD062951, MDA4026, and the David and Helen Younger Research Fellowship Research Grant from the FSH Society (FSHS-DHY-001). We apologize to the researchers whose work we could not cite due to space limitations.

List of abbreviations

ChIP-loop	3C combined with ChIP
ATR-X	Alpha-Thalassemia mental Retardation, X-linked
ARS	Autonomously replicating sequence
CTCF	CCCTC-binding factor
ChIP	Chromatin immunoprecipitation
ChIA-PET	Chromatin Interaction Analysis by Paired-End Tag sequencing
3C	Chromosome conformation capture
5C	Chromosome conformation capture carbon copy
4C-seq	Circular chromosome conformation capture followed by high-throughput sequencing
CdLS	Cornelia de Lange Syndrome
DSB	DNA double-strand break repair
eRNAs	Enhancer RNAs
ER	Estrogen receptor
H3K9me	H3 lysine 9 methylation
H3K9me3	H3 lysine 9 trimethylation
H3K27me3	H3 lysine 27 trimethylation
H4K20me3	H4 lysine 20 trimethylation
HR	Homologous recombination repair
iPSCs	Induced pluripotency cells
LCR	Locus control region
mESCs	Mouse embryonic stem cells
ncRNA	Non-coding RNA
rRNA	Ribosomal RNA
RNAPII	RNA polymerase II
RNAPIII	RNA polymerase III

RBS	Roberts' Syndrome
SRA	Steroid receptor RNA activator
SMC	<u>Structural Maintenance of Chromosomes</u>
TADs	Topologically associating domains

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Highlights

- Cohesin is a key chromatin organizer in both mitosis and in interphase.
- Cohesin regulates gene expression through different mechanisms and chromatin contexts.
- Differential protein and RNA interactions dictate cohesin targeting and function.
- Dysfunction of cohesin and its regulators causes related developmental disorders.
- Facioscapulohumeral dystrophy (FSHD) may be a new cohesinopathy disorder.

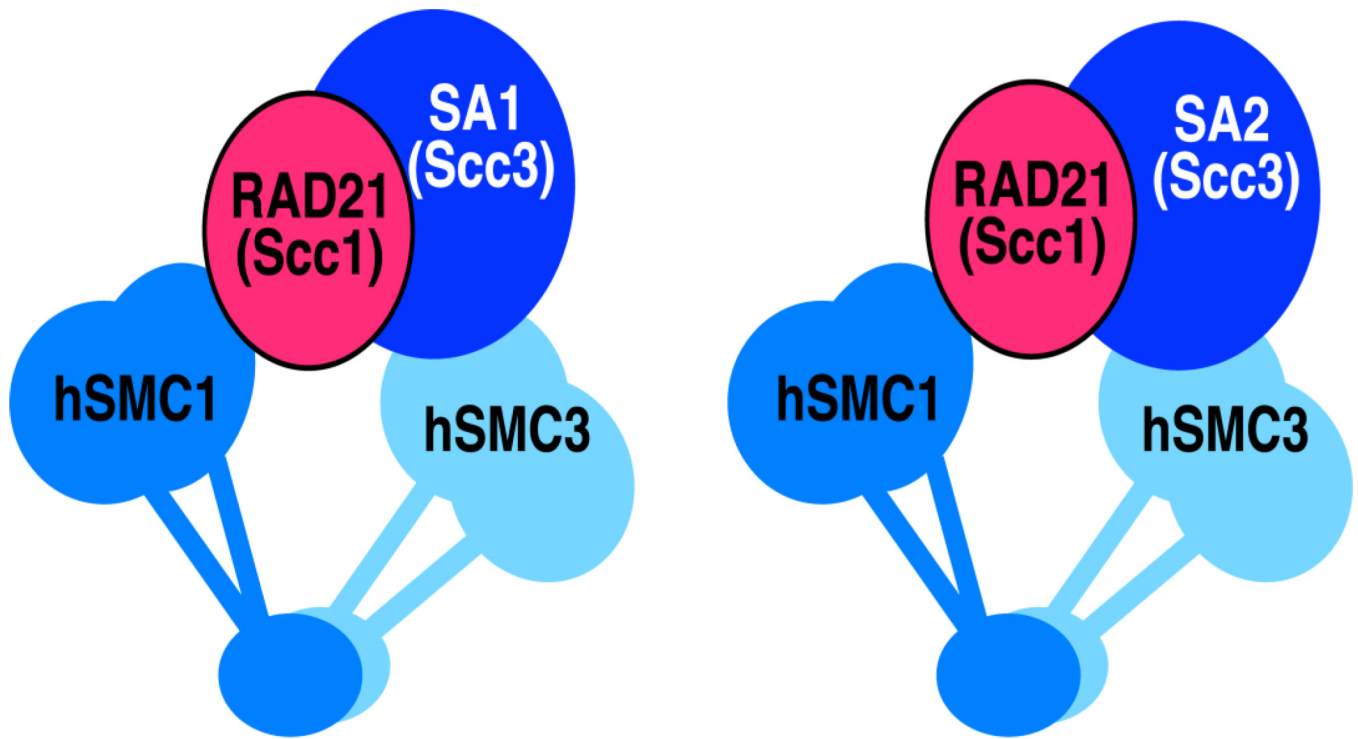


Figure 1.
Schematic diagrams of cohesin-SA1 and cohesin-SA2.

Genome-wide cohesin loading

Establishment of sister chromatid cohesion

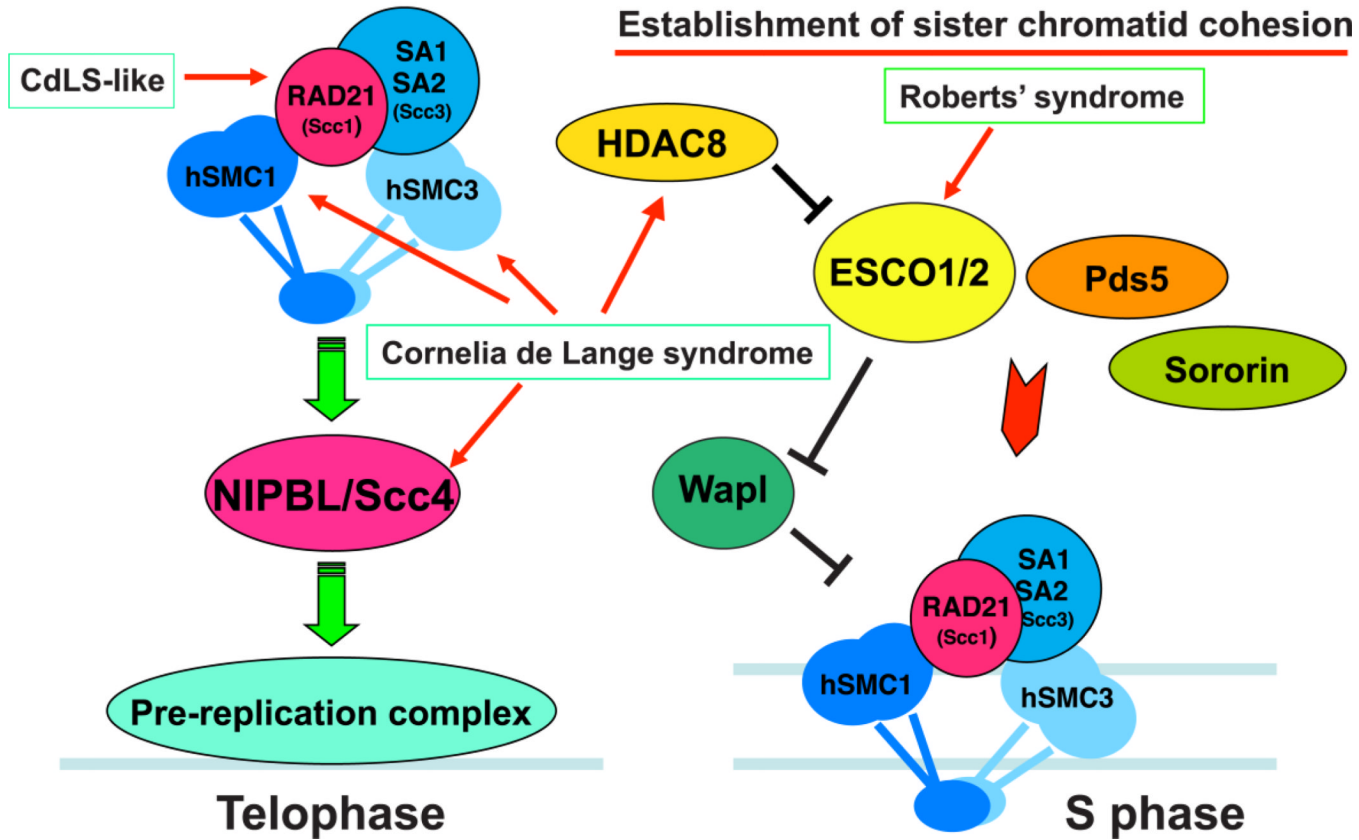


Figure 2. Regulators of cohesin loading and establishment of sister chromatid cohesion. The cohesin loading factor, NIPBL-MAU2 (Scs2-Scs4), is required for cohesin loading onto chromatin in telophase in mammalian cells. The initial loading of NIPBL-MAU2 is dependent on the pre-replication machinery. In S phase, the establishment of sister chromatid cohesion requires sororin and Pds5A/B as well as the ESCO1/2 (Eco) acetyltransferases that coordinately antagonize the activity of the cohesin destabilizing factor Wapl. ESCO-mediated acetylation of the cohesin subunit SMC3 must be reversed by histone deacetylase HDAC8 in order to refresh and recycle cohesin for the subsequent cell cycle. Mutations associated with the cohesinopathies RBS and CdLS are indicated.

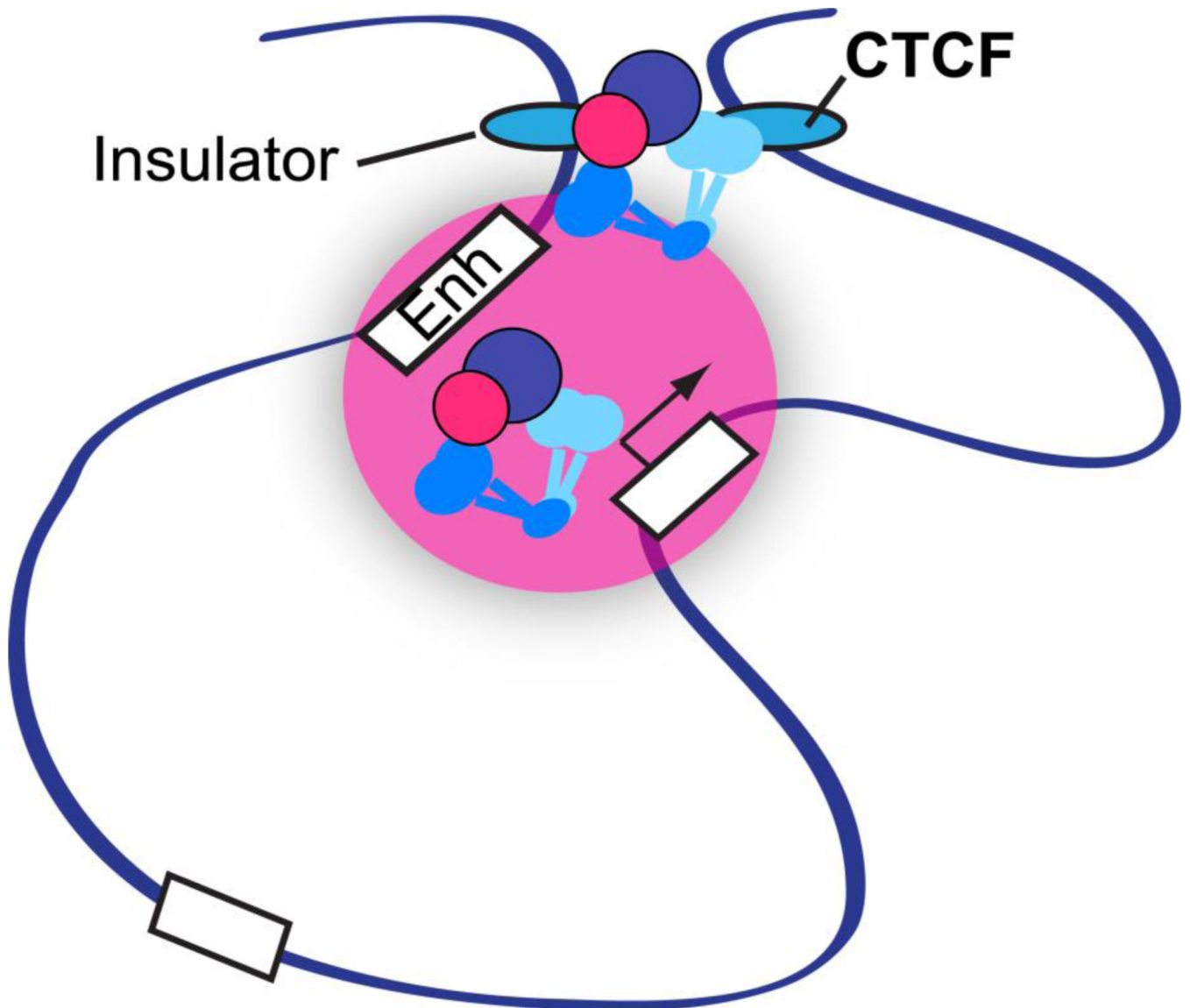


Figure 3. CTCF-dependent and -independent chromatin loop formation at the β -globin locus. Cohesin binds to and mediates the long-distance interactions of CTCF-bound insulator elements flanking the locus as well as between the distal enhancer (Enh) in the locus control region and the adult globin genes (white box with an arrow) [82]. The pink circle represents the presence of various transcription factors involved in globin gene expression, such as EKLF (Klf1), GATA-1, Fog-1, Ldb1, and NF-E2 [82, 102–104, 147]. A white box without an arrow represents the inactive gene, which is not interacting with the enhancer.

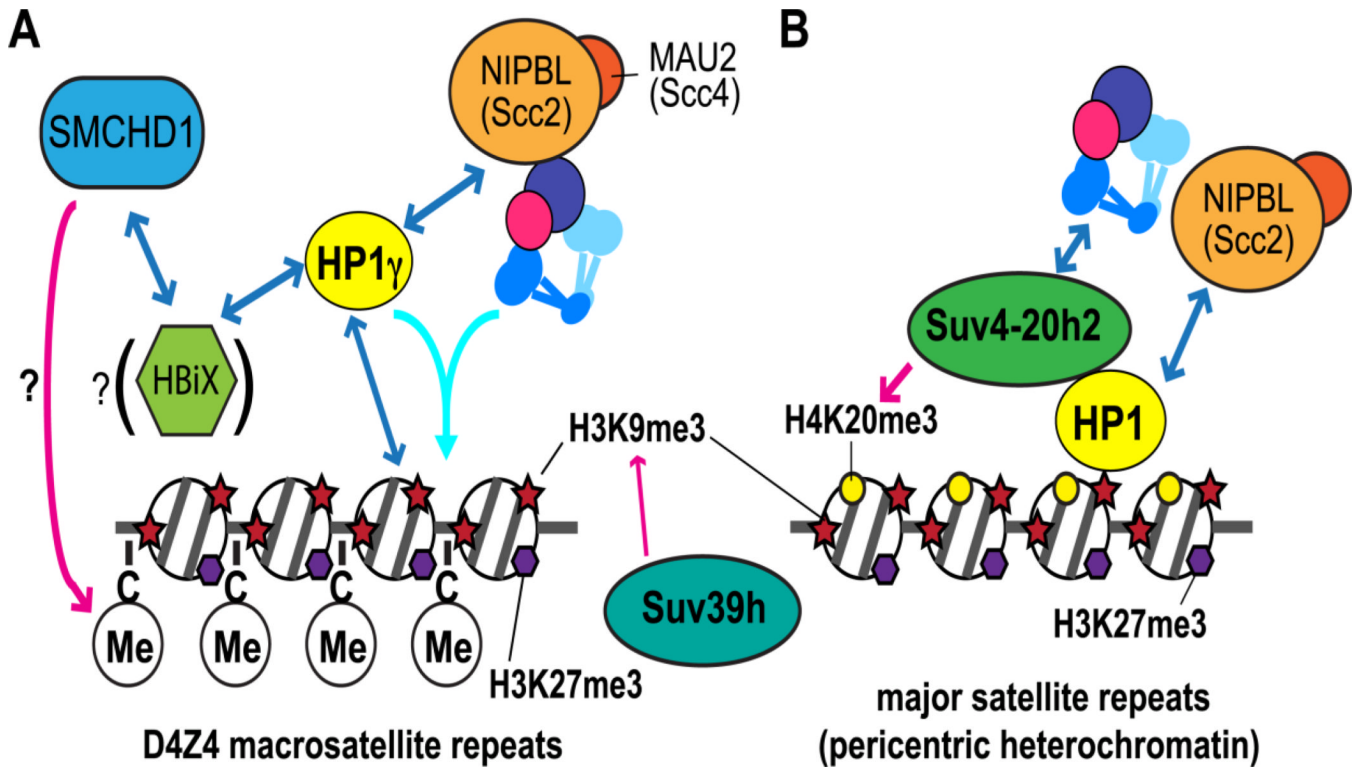


Figure 4.

Cohesin recruitment to heterochromatin. Blue double-sided arrows indicate the interactions reported. Pink arrows indicate downstream effects. **A.** Cohesin and HP1 γ require each other to bind to the D4Z4 subtelomeric heterochromatin in a SUV39H-mediated H3K9me3-dependent manner [116]. Direct interaction of NIPBL with HP1 may contribute [116, 156]. The light blue arrow indicates co-recruitment of cohesin and HP1 γ to D4Z4 [116]. In addition, an SMC homolog, SMCHD1, binds to D4Z4 [215]. Whether this binding is mediated by HBiX1, an HP1-interacting protein, as observed at the inactive X chromosome [198] is currently unclear. SMCHD1 was shown to be important for the maintenance of DNA methylation [214]. Whether it contributes to DNA hypermethylation at D4Z4 has not been determined. **B.** Cohesin is recruited to pericentromeric heterochromatin via interaction with histone methyltransferase Suv4-20h2, which mediates H4K20me3. Suv4-20h2 localization is dependent on HP1 bound to methylated H3K9 mediated by SUV39h [157]. The relevance of the NIPBL-HP1 interaction [156] to pericentromeric cohesin recruitment is unclear.