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On the Molecular Etiology of Cornelia de Lange Syndrome

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Abstract

Cornelia de Lange syndrome (CdLS) is genetically heterogeneous and is usually sporadic, occurring approximately once per ten thousand births. CdLS individuals display diverse and variable deficits in growth, mental development, limbs and organs. In the past few years it has been shown that CdLS is caused by gene mutations affecting proteins involved in sister chromatid cohesion. Studies in model organisms, and more recently in human cells have revealed, somewhat unexpectedly, that the developmental deficits in CdLS likely arise from changes in gene expression. The mechanisms by which cohesion factors regulate gene expression remain to be elucidated, but current data suggest that they likely regulate transcription in multiple ways.

Keywords

Cornelia de Lange syndrome; cohesin; Nipped-B; NIPBL; Scc2; Scc4; Mau-2; Smc1; Smc3; Rad21; Stromalin; Pds5; CTCF; insulator; enhancer-promoter interactions; DNA-looping; *H19*; *Igf2*; bithorax complex; cut gene; ecdysone receptor; axon pruning; transcription; Drosophila

Mutations affecting sister chromatid cohesion proteins cause Cornelia de Lange syndrome

Cornelia de Lange syndrome (CdLS; OMIM #122470, #300590, and #610759), also known as Brachmann-de Lange syndrome, is a genetically heterogeneous disorder affecting multiple aspects of development. The phenotype is distinctively recognizable but may be highly variable in its expression (Fig. 1). This variability is highlighted by the earliest reports of this entity by Vrolik in 18491 and Brachmann in 19162, who both reported isolated cases of severely affected infants, and in 1933 by de Lange3 who reported two unrelated children with milder manifestations and classified the clinical findings as a diagnostic entity.

Affected individuals typically show slow pre- and postnatal growth, and varying degrees of developmental delays and mental retardation at times associated with autistic features.[4-8] Almost all organ systems can be affected, but typical involvement includes the craniofacial structures, upper extremities, eyes, gastrointestinal system, hearing and to a lesser degree the heart, diaphragm and genitourinary system.[4,5,8,9] The main facial characteristics include arched eyebrows, synophrys, ptosis, long eyelashes, microcephaly, anteverted nares, long

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philtrum and thin upper lip with micrognathia (Fig. 1). In spite of the considerable differences in severity from patient to patient, the consistent facial dysmorphisms have provided the most helpful feature in establishing a diagnosis. The limbs are involved to variable degrees, primarily affecting the ulnar aspects of the upper limbs, and can range from severe reduction defects with almost complete absence of the forearms to small hands with fifth finger clinodactyly and proximally placed thumbs. Hirsutism is commonly seen especially of the face, neck, back and arms. Gastrointestinal issues include reflux, which is almost universally present and malrotation seen in approximately 25%. Cryptorchidsm and hypospadias are commonly seen in affected males.

Developmental delays and mental retardation are close to universal and generally moderate to severe, however with increasing recognition of the milder CdLS phenotype more and more individuals are being identified with mild cognitive delays. Growth is generally retarded with prenatal onset and mean adult heights in males is 156 cm and 131 cm in females.10 Aided by the molecular analysis described below, it has recently been recognized that there are many mild cases of CdLS that display primarily mental retardation without substantial structural differences. This suggests that the brain is the organ most sensitive to the perturbations of sister chromatid cohesion factors described below. This has prompted development of scoring systems to help anticipate the care that will be required in individual cases.8

The recognition of CdLS as a genetically heterogeneous disorder with genotype-phenotype correlation has arisen from breakthroughs in identifying the multiple genes responsible, all of which are involved in sister chromatid cohesion. Most CdLS cases are sporadic and dominant. At least half are caused by loss-of-function mutations in the *Nipped-B-Like (NIPBL)* gene on chromosome 5.6،11¯16 Genotype-phenotype correlations with a large number of patients indicate that more severe *NIPBL* mutations (such as deletions or truncations) usually cause more severe clinical manifestations than missense mutations. In the mildest forms, affected individuals generally have mild to moderate mental retardation and, at least at a young age, typical facial characteristics (Fig. 1).

The fact that *NIPBL* mutations could be identified in only about half the cases of CdLS prompted investigators to look for mutations in other genes with related functions. As described in detail below, NIPBL is required for binding of the cohesin complex that mediates sister chromatid cohesion to chromosomes.17 Thus initial efforts to find other genes responsible for CdLS focused on genes involved in the cohesin complex and its regulation.

Cohesin, which is conserved from fungi to man, consists of the Smc1, Smc3, and Stromalin (SA, Stag) proteins, and has a ring-like structure (Fig. 2).17¯20 The internal diameter of the cohesin ring is estimated to be some 35 nm, and a leading model is that cohesin encircles both sister chromatids to hold them together topologically (Fig. 2).18،19،21 Alternatives include “handcuff” models, in which a cohesin ring that encircles one sister interacts or interlocks with a cohesin ring encircling the other. In a third idea, cohesin encircling one sister can interact with proteins bound to the other.22 All the leading models involve topological binding, and the demonstration that cohesin can be released from a yeast circular chromosome by linearizing the DNA provides convincing evidence for this idea.23،24

Screening of large cohorts of individuals with a clinical diagnosis of CdLS in whom *NIPBL* mutations were not found revealed that in approximately 5% missense or small in-frame deletion alleles in *SMC1A*, which encodes the SMC1 subunit of mitotic cohesin were causative.25¯27 One individual with a 3 bp deletion in the *SMC3* gene, which encodes another cohesin subunit, was also reported.26 The cases caused by mutations affecting the

cohesin subunits are on the mild end of the CdLS spectrum, showing primarily mild to moderate mental retardation without severe limb or other systemic involvement. Growth also appears to be less impacted by mutations in these two structural genes. The *SMC1A* gene is X-linked, and both hemizygous male patients, and heterozygous female patients have been found. This gene was found to escape inactivation on the inactivated X chromosome in female mice.28 *SMC3* is on chromosome 10, and the mutation is heteroallelic with a wild-type allele.

Combined, mutations in *NIPBL, SMC1A* and *SMC3* have been identified in nearly 60% of CdLS patients with a confident clinical diagnosis. It is possible that mutations affecting the remaining structural components of the cohesin complex [Rad21 or Stag2 (SA)] might occur in some of the patients in which *NIPBL, SMC1A* or *SMC3* mutations have not been found, but these genes have not been extensively screened. There might also be mutations in regulatory sequences for *NIPBL*. As discussed below, there are also other factors besides NIPBL and cohesin that are required for sister chromatid cohesion, and thus could also be potential candidate genes for CdLS. Finally, evidence outlined in a later section strongly suggests that the developmental deficits seen in CdLS likely result from effects on gene expression, and mutations in some of the genes targeted by NIPBL and cohesin could give rise to similar patient phenotypes. Below we discuss in more detail molecular aspects of cohesin and factors that regulate cohesin, and their potential involvement in CdLS.

Components of the sister chromatid cohesion apparatus

Cohesin

As noted briefly above, cohesin forms a large ring-like structure composed of the Smc1, Smc3, Rad21 and SA proteins (Fig. 2).29–31 SMC (structural maintenance of chromosome) proteins are conserved from bacteria to man, and play multiple roles in chromosome structure and function.18,19 Each SMC protein folds back on itself at the hinge region near the middle to form long antiparallel coiled-coil arms. This brings the N and C termini together, which form an *ABC* ATPase head domain in each SMC protein. The hinge domains of Smc1 and Smc3 interact to form a heterodimer. The head domain of Smc1 interacts with the C terminus of the Rad21 kleisin protein, and the head domain of Smc3 interacts with N terminus of Rad21 to form the ring. A kink in the Smc3 arm gives an open structure large enough to encircle chromosomes.29

Missense mutations predicted to inactivate the ATPase activity in the yeast SMC head domains prevent binding of cohesin to chromosomes,32,33 and interaction with Rad21 stimulates the ATPase activity.34 Although not generally seen in electron micrographs, the Smc1 and Smc3 head domains also interact with each other.33,35 Crystal structures of a yeast Smc1 head dimer and a bacterial SMC head dimer show that they “sandwich” ATP between them.36,37 The role of ATP in cohesin binding isn't fully understood, but it could regulate opening of the cohesin ring, SMC head domain interactions, and interactions of Rad21 with the head domains. Studies on bacterial SMC complexes indicate that the SMC hinge domain also controls head domain ATPase activity and binding to chromosomes.38,39 Certain mutations in the yeast Smc1 hinge region form cohesin that binds chromosomes, but fail to establish cohesion,40 while covalent linkage of yeast Smc1 and Smc3 hinge regions prevent binding of cohesin to chromosomes.41

The CdLS-causing *SMC1A* missense and in-frame deletions affect many domains, including the ATPase domain, the junction of the hinge with the coiled-coil arm, and in the coiled-coil arm itself.25–27 None, however, are predicted to inactivate SMC1, consistent with the finding that male CdLS patients, who have only the mutant form, produce normal levels of

SMC1 and survive.25 The potential significance of these mutations for the molecular etiology of CdLS is discussed below in a later section.

NIPBL/Nipped-B/Scc2/Mis4

NIPBL encodes the homolog of the *S. cerevisiae* Scc2 and *S. pombe* Mis4 proteins, which were discovered for their roles in sister chromatid cohesion.42,43 Using temperature-sensitive alleles, it was shown that the Scc2 and Mis4 proteins are required *in vivo* for binding of cohesin to chromosomes.44,45 Antibody depletion experiments with Xenopus extracts, and siRNA knockdown of human NIPBL in cultured cells, confirm that vertebrate homologs are also required for binding of cohesin to chromatin.46-49 In Drosophila, homozygous lethal mutations in *Nipped-B*, which encodes the NIPBL/Scc2 homolog, strongly reduce sister chromatid cohesion, implying that it has the same function.50

The current evidence indicates that Nipped-B and its orthologs directly regulate cohesin binding to chromosomes. Purification of yeast Scc2, and immune precipitation of Drosophila Nipped-B, shows that they can occur in complexes with cohesin subunits.32,51 Drosophila Nipped-B co-localizes virtually completely with cohesin on chromosomes in both mitotic and meiotic cells, except at meiotic centromeres.51,52 While yeast cohesin does not co-localize with Scc2 on chromosomes, it appears to load at sites that bind Scc2, and then translocate away.53

As mentioned above, all evidence to date indicates that the CdLS-causing mutations in *NIPBL* are loss-of-function alleles, and that the severity of the syndrome generally correlates with the severity of the mutation.13,16 We will discuss in later sections possible mechanisms by which a partial reduction in NIPBL can cause developmental deficits.

Scc4/Mau-2

Scc2 forms a complex with the Scc4 protein, which is also required for chromatid cohesion. 44 Distant relatives of Scc4, including the Ssl3 protein of *S. pombe*, the Mau-2 proteins of *C. elegans* and Drosophila, and human Scc4/Mau-2 homologs, interact with the Scc2 orthologs, and are required for binding of cohesin to chromatin.48,49,54

To date, no mutations in Scc4/Mau-2 have been discovered in CdLS patients.48 If they occur, they may be rare, or it may be that the effects of Scc4/Mau-2 are not as dosage-sensitive as those of NIPBL.50

Eco1/Esco2/ Eso1

The Eco1/Ctf7 acetyltransferase in fungi is required to establish sister chromatid cohesion, but not for binding of cohesin to chromosomes.55-57 The Drosophila Eco1 ortholog affects cohesion specifically at centromeres, and not along the chromosome arms.58 Yeast Eco1 can acetylate cohesin subunits *in vitro*,59 but it remains to be demonstrated that these are authentic targets *in vivo*.

Intriguingly, mutations affecting the human Esco2 homolog of Eco1/Ctf7 cause Roberts/SC phocomelia, a recessive genetic syndrome.60,61 This syndrome is clinically distinct from CdLS, but many of the developmental differences seen in this entity do partially overlap those of CdLS.17 As seen in Drosophila *Eco1* mutants, cell lines derived from Roberts patients show chromosome cohesion defects primarily in the heterochromatic portions of the chromosomes, which has been termed “heterochromatic repulsion”.62

Pds5

S. cerevisiae Eco1 also acetylates the Pds5 protein *in vitro*,59 and the *S. pombe* Eco1 ortholog, Eso1, interacts with Pds5.63 Pds5 was discovered in yeast because it is required to maintain, and possibly establish sister chromatid cohesion.64,65 In *S. cerevisiae* Pds5 is not required for binding of cohesin to chromosomes, and is sumoylated to help dissolve cohesion.64,66 The Drosophila Pds5 ortholog is also essential for cohesion, but not cohesin chromosome binding.67 Vertebrates have two Pds5 genes, and depletion of Pds5 from extracts appears to slightly increase cohesin binding, but reduce cohesion.68

Pds5 also interacts with cohesin, co-localizes with it on chromosomes, and its association with chromosomes depends on cohesin.51,57,64-69 Recently, it was shown that *S. cerevisiae* Pds5 interacts directly with both Rad21 (Mcd1/Scc1) and the Smc1 hinge domain *in vivo*, and that the interaction of Pds5 with Smc1 is Rad21-dependent.35 As described above, genetic evidence shows that the hinge region is important for cohesin chromosome-binding, and establishment of cohesion.40,41 The interaction of Pds5 with the Smc1 hinge provides further evidence that the Smc1-Smc3 hinge interface plays critical roles in cohesin function.

To date, no confirmed mutations in either of the two human *Pds5* genes have been identified in CdLS patients, or in other human syndromes. Knockout of the mouse *Pds5B* gene is homozygous perinatal lethal, but the mutant mice show several developmental deficits reminiscent of CdLS, including growth retardation, facial abnormalities, short limbs, cardiac defects and cleft palate.70 Heterozygous *Pds5B* mutant mice are normal, but it remains possible that rare partial loss-of-function mutations, or mutations in *Pds5A* could cause CdLS, or a similar syndrome.

Wapl

The Wings-apart-like (Wapl) protein was first discovered for its role in heterochromatin organization and chromosome segregation in Drosophila.71,72 The human ortholog interacts with cohesin, and is required for removal of cohesin from chromosome arms during prophase.73,74 Although it appears to have an opposing function to NIPBL regarding the binding of cohesin to chromosomes, it is conceivable that gain-of-function mutations affecting Wapl could have a similar effect as NIPBL loss-of-function mutations, and cause developmental deficits similar to those seen in CdLS patients. The *Wapl* (*WAPAL*) gene has also not yet been screened for mutations in CdLS patients.

Sister chromatid cohesion defects are unlikely to cause CdLS

Cohesin functions in sister chromatid cohesion in mitosis and meiosis, DNA repair, and gene expression.17-20 The finding that the CdLS-causing mutations all affect proteins required for sister chromatid cohesion naturally raised the idea that the developmental deficits might be caused by cohesion defects, or cell cycle delays. To date, however, the evidence neither supports nor rules out these possibilities. Cell lines derived from CdLS patients do not display overt cohesion defects. In one study, a small fraction of the cells in some 40% of the patient cell lines show cohesion defects, and another study found none (Kaur et al. 2005; Vrouwe et al. 2007).75,76 CdLS patient cell lines with either *NIPBL*76 or *Smc1A* mutations (Antonio Musio, personal communication), however, display mild defects in DNA repair, indicating that at least one of cohesin's functions is measurably reduced.

The Drosophila Nipped-B ortholog of NIPBL regulates gene expression

In contrast to the inconclusive evidence regarding the role of sister chromatid cohesion defects in the etiology of CdLS, there is increasing evidence that many, if not most, of the

developmental deficits in CdLS likely result from changes in gene expression. The first key evidence arose from screens in Drosophila for genes that regulate gene expression in a dosage-sensitive manner. *Nipped-B* loss-of-function alleles were isolated in screens for mutations that dominantly reduce expression of the *cut* and *Ultrabithorax* (*Ubx*) homeobox genes that control multiple aspects of development.77 Homozygous *Nipped-B* mutations are lethal and cause defects in sister chromatid cohesion, while the heterozygous mutations reduce *cut* and *Ubx* expression without detectable cohesion defects.50 It was discovered that heterozygous *Nipped-B* null alleles only reduced *Nipped-B* mRNA by some 25%, and that further reduction to 50% wild-type levels by *in vivo* RNAi was lethal.50 Even with a 50% reduction, however, cohesion defects were not apparent.

Amazingly, the unusual partial dosage compensation for *Nipped-B* seen in Drosophila, whose mechanism is unknown, also occurs in mice and humans. CdLS patient cell lines78 (Jinglan Liu and I.D.K., submitted for publication) and heterozygous *NIPBL* knockout mice (Arthur Lander and Anne Calof, personal communication) also show only a 30% reduction in *NIPBL* mRNA or less. This unusual dosage sensitivity is a critical feature that must be taken into account into studies aimed at determining the molecular mechanisms underlying CdLS. Indeed, if this partial dosage compensation could somehow be improved, it might provide a means to reduce or ameliorate some of the developmental deficits that occur after diagnosis.

Missense *NIPBL* mutations generally cause less severe forms of CdLS than truncating alleles,13 and in Drosophila, missense Nipped-B alleles similar to some CdLS-causing mutations have smaller *in vivo* dominant effects on *cut* and *Ultrabithorax* (*Ubx*) gene expression than truncating or null alleles.51 Thus the milder effects of *NIPBL* missense mutations in humans could reflect milder effects on gene expression.

Cohesin regulates gene expression

The finding that some mild cases of CdLS are caused by missense or small in-frame deletions in the *SMC1A* or *SMC3* cohesin subunit genes clearly links the effects of *NIPBL* mutations to the role of NIPBL in regulating cohesin binding to chromosomes. Because cell lines from patients with cohesin subunit mutations lack cohesion defects, and produce normal levels of SMC125 (Antonio Musio, personal communication), these findings further support the idea that the etiology of CdLS involves effects of cohesin on gene expression.

Evidence that cohesin regulates gene expression is accumulating rapidly. Mutations in the *Rad21* gene of zebrafish reduce expression of *Runx* genes during embryonic development79 and reductions in the dosage of the SA, Rad21 and Smc1 cohesin subunits increase expression of the Drosophila *cut* gene in the developing wing.50›67 The effect of reducing cohesin dosage on *cut* expression is opposite to the effect of reducing Nipped-B, leading to the hypothesis that cohesin might interfere with long-range communication between the wing margin enhancer and the *cut* promoter, and that Nipped-B facilitates long-range gene activation by controlling cohesin dynamics.80

More recently, Drosophila *Smc1* and *SA* mutations were isolated in genetic screens for factors required for axon pruning in the mushroom body.81 During the development of this olfactory organ, certain post-mitotic neurons extend excess axons, which are then pruned back. Using a clever genetic strategy to generate homozygous mutant clones, Schuldiner et al. showed that lack of Smc1 or SA blocked axon pruning.81 It was known that the ecdysone steroid hormone receptor (EcR) is required for pruning82 and these investigators further found that neurons lacking Smc1 had reduced levels of receptor protein.81 Amazingly, the pruning defect could be nearly completely rescued by expression of EcR specifically in post-mitotic neurons, indicating that reduced EcR expression is primarily

responsible for the pruning defect.81 Targeted destruction of Rad21 in the same neurons also causes loss of pruning, indicating that the entire cohesin complex is required.83 Because the effects of cohesin on axon pruning and EcR protein levels occur in post-mitotic cells, they clearly do not arise from effects on chromosome segregation or cell division.

Pruning defects in mushroom body axons are not the only effect of cohesin deprivation on nervous system development in Drosophila. Schuldiner et al. also found excessive dendrite outgrowth and poor dendrite targeting,81 and Pauli et al. found that destruction of the Rad21 cohesin subunit in cholinergic neurons caused abnormal larval locomotion, without obvious effects on mitosis.83 While a nearly complete loss of a cohesin subunit in nerve cells is unlikely to occur in CdLS patients, these experiments nevertheless emphasize the importance of cohesin in nervous system development and function, and it is tempting to predict that many of the mental and other nervous system deficits (*e.g.* high pain threshold) in CdLS patients arise from related mechanisms.

Nipped-B and cohesin bind to active genes

Recent evidence argues that the effects of cohesin on EcR protein levels in Drosophila post-mitotic mushroom body neurons, and on *cut* expression in the developing wing margin likely involve direct effects on transcription. Misulovin et al. mapped Nipped-B and cohesin binding genome-wide in multiple cell lines, and found that Nipped-B and cohesin bind to the full length of the *EcR* transcription unit in MLDmBG3 (BG3) cells line derived from central nervous system.52 If Nipped-B and cohesin bind the *EcR* gene in the same way in mushroom body neurons, this would argue that cohesin facilitates *EcR* transcription. This is opposite to the negative effects of cohesin on *cut* gene expression in the developing wing margin. Both *EcR* and *cut* are transcribed in BG3 cells (C. Vandebunte, Z. Misulovin, D.D., unpublished), and Nipped-B and cohesin also bind to a 150 kb region that extends from the remote wing margin enhancer through the entire transcription unit of *cut*.52 Thus it is likely that Nipped-B and cohesin also directly regulate *cut* transcription. Combined, these finding suggest that cohesin has both positive and negative effects on transcription that are context-dependent.

Genome-wide mapping of Nipped-B and cohesin in Drosophila cell lines provided other critical new information. Nipped-B and cohesin were found to co-localize throughout the genome, consistent with the idea that Nipped-B can dynamically regulate cohesin binding.52 Strikingly, Nipped-B and cohesin were also found to bind preferentially to active genes. They extensively overlap RNA polymerase II (PolII) binding and are almost completely excluded from silenced genes.52 Nearly 500 genes were found to bind Nipped-B and cohesin between the three cell lines assayed, and in most cases, these genes also bind PolII. In more than a hundred cases, a gene bound Nipped-B and cohesin in one cell line and not another. In most of these cases, PolII only bound the gene in the cell line in which cohesin binds.52 Based on these findings, it was hypothesized that transcription unwinds the chromosome, allowing it to be encircled by cohesin, which then can affect multiple aspects of transcription, including gene activation and transcriptional elongation.

A key example that illustrates most of the major findings is the *Abd-B* gene in the bithorax *HOX* gene complex (BX-C) that controls abdominal segment identity and limb development (Fig. 3). The BX-C is of particular interest because expression of the *Ultrabithorax* (*Ubx*) gene in this complex is highly sensitive to Nipped-B dosage *in vivo*.51،77 The entire BX-C is silenced in BG3 cells, and Nipped-B, cohesin or PolII binding is not detected (Fig. 3). In Sg4 cells, however, *Abd-B* is highly transcribed.52،84 Silencing by Polycomb group proteins results in coating of the silenced regions by the histone H3 lysine 27 trimethylation (H3K27Me3) modification.84 In Sg4 cells, the *Ubx* and *abd-A* genes of the BX-C are

covered by the H3K27Me3 silencing mark, but there is low H3K27Me3 over a 75 kb region that includes the active *Abd-B* transcription unit and its downstream regulatory domain.84 PolII, Nipped-B and cohesin bind precisely to this region with low H3K27Me3 (Fig. 3).52

Intriguingly, the cohesin-binding domain associated with the active *Abd-B* gene begins near a site that binds the CCTC-binding factor (CTCF) zinc finger protein near the distal *Abd-B* promoter,85 and extends down to the Fab-7 boundary, or insulator sequence. The CTCF protein and the Fab-7 boundary are both known for their ability to insulate, or attenuate long-range enhancer-promoter communication, and thereby interfere with gene activation.86›87 The curiously precise containment of the *Abd-B* Nipped-B and cohesin-binding domain between a known insulator and a CTCF binding site raises the intriguing possibilities that insulators might control the localization of cohesin, and/or that cohesin might contribute to insulator function. As discussed next, a functional connection between cohesin and the CTCF insulator protein has recently been documented in mouse and human cells.

Functional association between cohesin and CTCF in mammalian cells

Wendt et al. mapped the binding of the Rad21 cohesin subunit and CTCF genome-wide in human HeLa cells,88 and Parelho et al. mapped cohesin and CTCF binding in some 3% of the genome in mouse lymphocytes.89 Both studies revealed a substantial overlap in peaks of cohesin and CTCF binding. Wendt et al. found some 14,000 CTCF sites in the human genome, consistent with previous studies,90›91 and nearly 60% of these correlate with cohesin peaks.88 They also found that some 90% of cohesin peaks are also CTCF binding sites.88 Parelho et al. found that 65% of cohesin peaks in mouse pre-B cells and thymocytes bind CTCF, and that almost 80% of CTCF bindings sites are also cohesin peaks.89 In another study, Stedman et al. found that well-known CTCF binding sites near the *c-myc* gene promoter, between the *H19-Igf2* loci, and a CTCF binding site in Kaposi's sarcoma-associated herpes virus genome, all bind cohesin.92

Despite the extensive co-localization, these studies revealed that CTCF and cohesin are not required for each other to bind. Knockdown of CTCF does not reduce overall cohesin binding to chromatin, but reduces its localization to CTCF binding sites.88›89 Knockdown of the Rad21 cohesin subunit reduced CTCF binding to some sites, but not others, suggesting that the effect may be through an effect on chromatin structure, or perhaps by cooperative interactions.88›89 In favor of the latter, Stedman et al. found that CTCF and cohesin could be co-immunoprecipitated from nuclear extracts under some conditions.92

Importantly, Wendt et al. and Parelho et al. further showed that cohesin contributes to the enhancer-blocking activity of CTCF using reporter gene constructs.88›89 The most compelling evidence for cohesin's role in insulator activity was the effect of reducing Rad21 levels on the endogenous *H19* and *Igf2* genes in HeLa cells (Fig. 4).88 A CTCF binding region between *H19* and *Igf2*, located just upstream of the *H19* promoter, is regulated by imprinting (Fig. 4).93¯95 On the maternally-derived chromosome, CTCF binds to this region, and prevents an enhancer located downstream of *H19* from activating *Igf2* (Fig. 4). On the paternal chromosome, however, the DNA in this region is methylated, which prevents CTCF binding. Thus the insulator no longer functions, and the enhancer now activates *Igf2* instead of *H19*. Wendt et al. found that knockdown of either CTCF or Rad21 had the effect expected from reduced insulator function, increasing *Igf2* transcription and decreasing *H19* expression.88 Consistent with these findings, an increase in *H19* expression was detected in expression microarray analysis of *NIPBL* heterozygous mutant mice, suggesting that CTCF or insulator function might also be affected by partial loss of NIPBL activity (Arthur Lander and Anne Calof, personal communication).

Thus while CdLS patients are unlikely to suffer significant reductions in cohesin levels, the functional interaction of cohesin and CTCF predicts that at least some of the developmental deficits seen in CdLS might arise from changes in insulator activity. Currently, evidence is accumulating in support of the idea that CTCF facilitates the formation of long-range chromosomal loops and interchromosomal interactions.86،87،96،97 These include demonstrations that the *H19-Igf2* insulator forms loops with transcriptional enhancers and even interacts with genes on other chromosomes.98¯101 This raises the exciting possibility that cohesin might help form or stabilize such long-range interactions using mechanisms similar to those proposed for how it mediates sister chromatid cohesion. If so, then even subtle changes in the efficiency of looping could potentially have significant consequences for gene expression.

Is cohesin chromosome-binding dynamics important for gene expression?

Perhaps the most puzzling aspect of CdLS is how such small reductions in *NIPBL* expression (<30%), or subtle changes in cohesin function caused by single amino acid changes in SMC1 can have such substantial effects on human development. The finding that CdLS cell lines do not display significant defects in sister chromatid cohesion, and only slight effects on DNA repair indicates that the sister chromatid cohesion apparatus is structurally functional in CdLS patients.25،75،76 These considerations have led to the hypothesis that changes in the dynamics of the binding of cohesin to chromosomes is responsible for the effects on gene expression that likely underlie the developmental deficits in CdLS.17،27

In addition to the small reductions in NIPBL needed to cause CdLS, scrutiny of the CdLS-causing *SMC1A* and *SMC3* mutations supports the idea that a change in cohesin function or dynamics, as opposed to reduced cohesin activity, are central to CdLS etiology.27 As mentioned above, CdLS-causing mutations occur in the ATPase head domain, the coiled-coil arm and the hinge domain.25¯27 An SMC1 residue at the arm-hinge junction is a mutation hotspot, with three independent patients identified that have missense mutations in this residue. Strikingly, none of the CdLS-causing mutations in *SMC1A* are predicted to inactivate the ATPase, interfere with interactions with other cohesin subunits, or disrupt the coiled-coil arm. This is consistent with the overall picture that the patients produce cohesin that supports sister chromatid cohesion, and that is only slightly impaired for DNA repair.

The genetics also support the idea that there is something special about the CdLS-causing *SMC1A* mutations. *SMC1A* is X-linked, and there are equally affected male and female patients with heterozygous missense alleles. The male patients demonstrate that the cohesin with the mutant SMC1 is largely functional, because a significant reduction in activity would be lethal. More importantly, no female patients have been identified that have a heterozygous truncation or null alleles, yet these types of mutations should occur more frequently than missense or small in-frame deletions. While one might suspect that random X-inactivation would cause loss-of-function alleles to be dominant lethal in females, *SMC1A* is one of the genes that escapes X-inactivation in mice.28 If the X-inactivation seen in mice also holds true for humans, then it is presumed that loss-of-function mutations in females would result in no phenotype (the normal allele would counter-balance the effect) and would be early embryonic lethal in males, and therefore these types of mutations would not be identified in screening CdLS probands. Thus it can be deduced that simply reducing SMC1 levels does not cause CdLS, but that the mutant SMC1 proteins have properties that allow them to interfere with gene expression and development, possibly by altering cohesin dynamics.

Another point in favor of the idea that cohesin dynamics could be more critical for gene expression than for sister chromatid cohesion is that cohesin shows unusually strong chromosome binding. Most DNA binding proteins bind to chromosomes with residence half-lives of less than 15 seconds.102 In contrast, cohesin binds to chromosomes in multiple modes, with residence half-lives ranging from several minutes up to a few hours.74،103 It is postulated that the most stable binding, which occurs in G2, is the mode that mediates sister chromatid cohesion.103 Gene transcription occurs throughout interphase, and the rates at which genes are turned on and off are in a range similar to those seen for cohesin dynamics. Thus, both the distribution of cohesin into different binding modes, and the rates at which cohesin can be removed from chromosomes, rebind chromosomes, or converted to a different binding mode could all influence the ability to activate and transcribe a gene. If so, it is feasible that a small reduction in NIPBL, or a point mutation in SMC1 that slightly alters its ability to hydrolyze ATP or its ability to interact with NIPBL or other factors, could affect the distribution of cohesin into different binding modes or chromosomal on-off rates enough to cause a change in gene expression large enough to alter development. It is also possible that small changes in the expression of multiple genes, which might be predicted by the binding of Drosophila Nipped-B and cohesin to hundreds of active genes, or the co-localization of cohesin with thousands of CTCF binding sites in human cells, could significantly alter cell fate.

Prospective

In the end, it is the “consequence of multiple small effects” idea that holds out hope in the future for individuals with CdLS and their families. If the above ideas regarding the effects of cohesin on gene expression and the role of cohesin dynamics in these effects are confirmed, it may be possible to make a slight correction to NIPBL levels or counteract the effects of a slight NIPBL reduction and thereby simultaneously steer multiple aspects of development back on course. Although many of the developmental defects occur pre-natally, an accurate diagnosis at birth may still allow amelioration of some the physical and mental growth deficits that continue post-natally.

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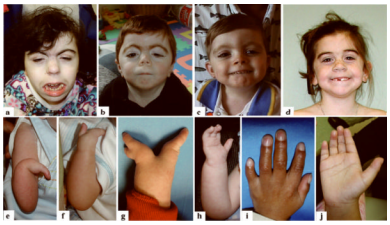

Fig. 1. Phenotypic spectrum in CdLS

Characteristic facial features in two individuals with CdLS and *NIPBL* mutations (**a** and **b**) and in two children with *SMC1A* mutations (**c** and **d**). Note the arched eyebrows with synophrys, long eyelashes, ptosis (more noticeable in `**a**' and `**b**'), the short upturned nose and long philtrum with thin upper lip. These features are still present in the *SMC1A* mutated individuals, but to a more subtle degree. The variability of the phenotype in CdLS is highlighted by the range of involvement of the upper limbs as demonstrated in **e-j**. In `**e**' the more severe end of the spectrum of upper limb involvement is depicted with severe ulnar hypoplasia of the forearm with only a single digit and underdevelopment of almost all boney structures. **f-h** depicts variable forms of oligodactyly and **i-j** demonstrates the milder end of the spectrum with small hands, 5^{th} finger clinodactyly and proximally placed thumbs.

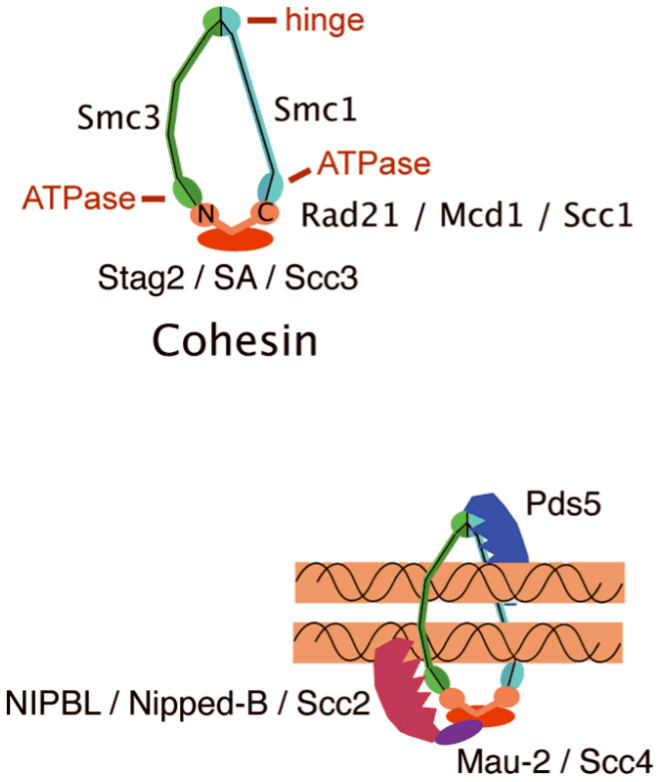

Fig. 2. The cohesin complex and regulatory factors

(Upper diagram) Cohesin consists of two SMC (structural maintenance of chromosome) proteins, Smc1 and Smc3, the kleisin Rad21 (Mcd1, Scc1) and Stromalin (Stag2, SA, Scc3).[17-20] Each SMC protein folds back on themselves at the hinge region to form long antiparallel coiled-coil arms. The N and C termini of each SMC protein come together to form an *ABC*-type ATPase head domain. The N and C termini of Rad21 interact with the head domains of Smc3 and Smc1, respectively. A kink in the Smc3 arm creates an open ring-like structure with an internal diameter of ~35 nm.

(Lower diagram) A leading hypothesis is that cohesin mediates sister chromatid cohesion by encircling the two sister chromatids. NIPBL (Nipped-B, Scc2, Mis4) interacts with the Mau-2 (Scc4) protein and is required for binding of cohesin to chromosomes. The interactions of the NIPBL/Mau-2 complex with cohesin shown in the diagram are speculative. The Pds5 protein interacts with the hinge domain of Smc1 and Rad21 (not shown), and is required for sister chromatid cohesion, but not binding of cohesin to chromosomes.

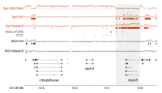

Fig. 3. Nipped-B and cohesin associate with the transcribed *Abdominal-B (Abd-B)* gene in the Drosophila bithorax complex (BX-C)

The BX-C contains the *Ultrabithorax (Ubx), abdominal-A (abd-A)* and *Abd-B* homeobox genes (transcribed from right to left) that control segmental identity and limb development. *Abd-B* is highly transcribed in Sg4 cells of embryonic origin, but not in MLDmBG3 (BG3) cells derived larval central nervous system.52,84 The upper three tracks show the histone H3 lysine 27 trimethylation (H3K27Me), a mark of Polycomb group (PcG) silencing, across the BX-C in Sg4 cells,84 and the binding of RNA polymerase II (PolII) and Nipped-B, the Drosophila NIPBL ortholog.52 The pattern of cohesin binding (not shown) is identical to that of Nipped-B.52 The middle tracks show the locations of know insulator/boundary elements in the BX-C,86 and binding sites for the CCTC-binding factor (CTCF).85 The binding of PolII and Nipped-B is restricted to the region between a known insulator (Fab-7, not labeled), and a CTCF insulator protein binding site near the *Abd-B* distal promoter, which also demarcate the boundaries between the active *Abd-B* region and the flanking silenced domains. The two bottom tracks show the lack of significant PolII or cohesin binding to the inactive BX-C in BG3 cells.

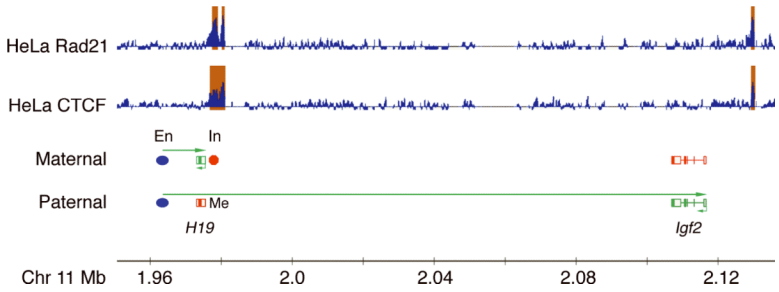

Fig. 4. Cohesin contributes to insulation by CTCF at the imprinted insulator in the human *H19-Igf2* locus.

The *H19-Igf2* locus contains a CTCF-dependent insulator that is regulated by imprinting.87 In the maternally-inherited chromosome (Maternal), CTCF binds the insulator, preventing an enhancer located downstream of *H19* from activating *Igf2*, and forcing it to active *H19* (transcribed from right to left). In the paternally-inherited chromosome (Paternal), the insulator DNA is methylated (Me), which prevents binding of CTCF. The insulator no longer functions, allowing the enhancer to activate *Igf2* (transcribed from right to left) instead of *H19*. Cohesin co-localizes with CTCF at many sites in mammalian cells, including the *H19-Igf2* insulator.88,89,92 The two tracks above the gene map show the binding of the Rad21 cohesin subunit and CTCF to the *H19-Igf2* locus in HeLa cells.88 Knockdown of either CTCF or Rad21 in HeLa cells simultaneously increases *Igf2* transcripts and decreases *H19* transcripts, consistent with a reduction in insulator activity.88