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# **Chromatin Structure and Intellectual Disability Syndromes**

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## **1. Introduction**

The molecular complex consisting of DNA and its associated proteins is referred to as chromatin. In the central nervous system (CNS), dynamic chromatin remodelling is required for cell division, specification, differentiation, maturation and to respond appropriately to environmental cues (reviewed in 1-4). Modifications to chromatin can act as a form of cellular memory, storing information about a cell's development, differentiation and environment [5].

In humans, the cerebral cortex is required for normal memory, information processing, thought, attention, perception and language. It consists of six horizontal layers of excitatory pyramidal neurons interspersed with inhibitory interneurons that form distinct synaptic circuits. During synaptic transmission, neurotransmitters released by a neuron bind receptors and initiate electrical signals that travel through the axon of the neighbouring neuron, and in this process alter its morphology and behaviour. One of these modifiable neuronal behaviours is the strength of the synaptic response, termed synaptic plasticity. The complex morphological and gene expression changes that are triggered by synapse formation must be maintained so that the maturing neuron can develop its identity and specific role in the nervous system. Dynamic changes in chromatin structure and gene expression underlie many of the above processes. Perhaps not surprisingly, many neurodevelopmental syndromes characterized by intellectual disabilities are caused by mutations in chromatin modifying factors. In this chapter, we provide an overview of the basic concepts behind chromatin structure regulation, followed by the description of three neurodevelopmental syndromes where altered chromatin structure is believed to be a major causative factor: Cornelia de Lange, Rett and ATR-X syndromes. We highlight common features at the phenotypic and molecular level and discuss the implications for the design of therapies.

## 2. Basic concepts in chromatin organization and structure

The packaging of DNA into chromatin occurs at different levels. In the primary structure of chromatin, a stretch of DNA is tightly wrapped around four pairs of positively charged structural proteins called histones. Together, the DNA and histones form the basic unit of chromatin known as the nucleosome [6]. In the secondary structure, the nucleosomal array is tightly coiled into a 30 nm chromatin fiber, although whether this arrangement exists *in vivo* has been questioned [7, 8]. The tertiary structure of chromatin consists of higher order chromatin fibre configurations. The density of chromatin packaging, and its dynamic remodelling, affect the accessibility of the DNA to factors involved in DNA replication, transcription and repair [9]. The molecular determinants that influence the level of compaction of chromatin fibres include DNA methylation, nucleosome composition, histone post-translational modifications (PTMs), ATP-dependent chromatin remodelers, and architectural chromatin-associated proteins.

Methylation of DNA in mammals occurs at cytosine residues, in the context of CpG dinucleotides [10]. DNMT3A and DNMT3B are methyltransferases responsible for *de novo* methylation [11]. Accordingly, they are responsible for the wave of *de novo* DNA methylation that occurs in the early embryo [12]. Another enzyme, DNMT1, maintains DNA methylation patterns by acting on newly synthesized DNA to match the parental strand after DNA replication [13-15]. High levels of DNA methylation seem to be correlated with gene inactivity [16]. DNA methylation is involved in gene and transposon silencing [17, 18] and also constitutes the molecular mark that often distinguishes the two alleles of imprinted genes [19-21]. However, DNA methylome analyses in several species have revealed that methylated cytosine residues are also highly enriched in the exons of transcribed genes [22-25]. While the role of exonic DNA methylation is not yet resolved, evidence suggests that it could aid the spliceosome in the process of defining exons [26, 27]. Several derivatives of 5-methylcytosine have now been identified including 5-hydroxymethylcytosine (5-hmc), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). They are thought to be generated during the 5-methylcytosine demethylation pathway catalyzed by the Ten-eleven translocation (Tet) enzymes [28-30]. Interestingly, 5-hmc is most abundant in the brain, especially in the hypothalamus and cerebral cortex [31], and its genomic distribution in human and mouse brain showed that it is greatest at synaptic genes and intron-exon boundaries, suggesting an important function in gene splicing and synaptic activity in the central nervous system [32].

The canonical nucleosome consists of pairs of the four core histones H2A, H2B, H3 and H4. Histone H1 binds the DNA between nucleosomes, which is known as linker DNA, and stabilizes higher order chromatin folding [33-36]. During developmental processes such as gene imprinting and X chromosome inactivation, the canonical histones in the nucleosome can be replaced by atypical forms to designate chromosomal regions for specific functions (reviewed in [37, 38]). For instance, the largest of the histones, MacroH2A, acts as a strong transcriptional repressor [39]. It is found in heterochromatin and is associated with the inactive X chromosome in females and the inactive allele of imprinted genes [40-44]. H2A.Z is typically found at transcriptional start sites of active and inactive genes, and is thought to be involved

in regulating nucleosome positioning [45-50]. H3.3 incorporation into nucleosomes can remove histone H1 from linker DNA and is thought to facilitate recognition of target sequences by the CCCTC-binding (CTCF) zinc finger protein [51]. Moreover, nucleosomes containing both H2A.Z and H3.3 are particularly labile and often correspond to binding sites for CTCF [52, 53].

Post-translational modifications to core and variant histones are crucial for the dynamic changes in chromatin organization [54, 55]. The amino terminal tails of histones that protrude from the nucleosome core can be marked by methylation, acetylation, ubiquitination and phosphorylation, among a growing list of chemical modifications. These marks are introduced or removed by "writer" and "eraser" proteins, respectively, and are recognized by specific "readers" that alter chromatin properties [54]. For example, histone acetylation is catalyzed by writer enzymes called histone acetyltransferases (HATs) that help open chromatin and correlate with transcriptional activation [56-59]. Conversely, transcription repressor complexes often include histone deacetylases (HDACs) that repress transcription by removing acetyl groups from the histones, promoting a closed chromatin state [60]. Bromodomain-containing proteins are reader proteins that specifically bind to acetylated histones [61]. Methylation marks on histones can be repressive, such as H3K9me3 and H3K27me3 [62-64] or they can denote particular regulatory elements. For example H3K4me3 often flags active gene promoters, which is the start-site of gene transcription [65, 66].

The positioning and density of nucleosomes along the DNA influences many cellular processes, including gene transcription [67]. A shift in nucleosome location can expose regulatory sequences of DNA that contain recognition sites for transcription factors or other regulatory proteins. Nucleosome positioning *in vivo* is dictated by the DNA sequence, the structure of neighbouring chromatin, transcription factors, transcriptional elongation machinery and ATP-dependent chromatin remodeling proteins [67-69]. The displacement of nucleosomes is controlled by protein complexes containing histone chaperones and ATP-dependent nucleosome remodellers. These complexes bind specific histones via the chaperone, and harvest energy from ATP using the ATPase to introduce or displace histones (reviewed in [70]). One example is the Swi/Snf-like ATPase called ATRX that forms a complex with the Daxx histone chaperone to incorporate the histone variant H3.3 into telomeric chromatin [71, 72]. ATRX is known to be involved in human cognition, as mutations in the gene cause intellectual disabilities [73, 74].

Architectural proteins are involved in organizing DNA in the three dimensional space of the cell nucleus [75]. Regulatory sequences like enhancers bind transcription activating protein complexes that interact with distant transcription machinery at the gene promoter through chromatin looping [76, 77]. These chromatin conformations are specific to cell-type and developmental context, as they depend on which transcription and chromatin-associated factors are available in the cell. The activation effect of an enhancer can be blocked by what is known as an insulator sequence. In eukaryotes, CTCF is the only protein known to bind insulator sequences to elicit this blocking effect [78]. CTCF-bound insulators function via the formation of a physically different looping structure, in which the regulatory element can no longer encounter the gene promoter [79, 80]. This type of long-range chromatin fibre interac-

tion also involves the cohesin ring complex, which is believed to encircle DNA strands, as well as ATP-dependent chromatin remodelling proteins [81-83].

Cohesin is a ring complex composed of four proteins: SMC1, SMC3, RAD21 and SA1/SA2 and is genetically conserved from fungi to humans [84]. The ring structure of the complex is formed by interactions between SMC1, SMC3 and RAD21 [85]. The fourth component (either SA1 or SA2) attaches to the ring through interaction with RAD21 and targets cohesin to specific genomic sites [86]. Cohesin and CTCF binding sites largely overlap across the genome, especially near active genes [83, 87]. The current model proposes that CTCF is targeted to its consensus sequence, and cohesin is recruited to the same sites via the SA1/2 subunit [88]. There are now multiple studies demonstrating that cohesin cooperates with CTCF in the formation and stabilization of chromatin looping structures to alter gene expression, including studies at the *H19/Igf2* locus [89], the *IFNG* locus [90], the Beta-globin locus [91], and the MHC class II locus [92]. Depletion of either CTCF or cohesin at these sites results in altered loop formation. In addition, CTCF and cohesin have been demonstrated to mediate interactions of genes and elements on different chromatin fibres [93]. However, cohesin may have a CTCF-independent role in tissue-specific enhancer interactions (reviewed in [94]). A study of murine embryonic stem cells revealed that cohesin localized to a subset of active promoters with a transcriptional coactivator called Mediator, in the absence of CTCF. These genes were expressed in embryonic stem cells through enhancer-promoter interactions that were formed through cohesin-mediator complexes [95]. Cohesin is also required for homologous-recombinational repair of DNA damage following DNA replication [96]. Subunits of cohesin become SUMOylated upon exposure to DNA damaging agents or presence of DNA double-strand breaks by the SUMO E3 ligase Nse2, a subunit of the related Smc5-Smc6 complex [97]. Cohesin was also shown to antagonize binding of the histone variant  $\gamma$ H2AX at double-stranded breaks, which may allow for the chromatin remodelling necessary for DNA repair [98].

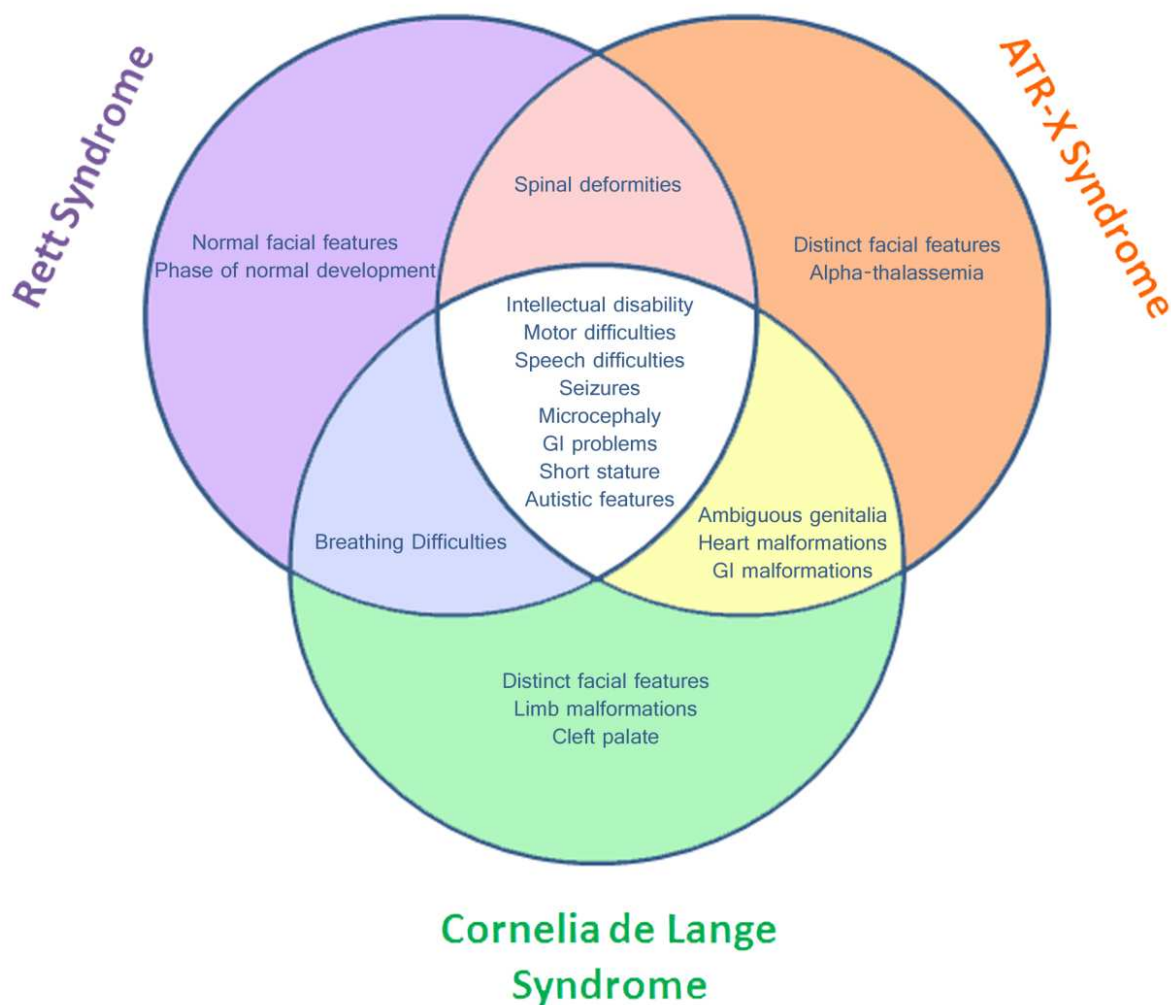
Normal development and maturation of the human brain relies heavily on the dynamic nature of chromatin and therefore on many of the factors mentioned above. In the next few sections, we discuss particular examples of human disorders with overlapping phenotypes, where mutation of chromatin structure regulators leads to birth defects and intellectual disability (Figure 1).

### 3. Cornelia de Lange syndrome

Cornelia de Lange Syndrome (CdLS) is a multi-organ developmental disorder characterized by intellectual disability, distinct facial features, growth impairment, short stature and upper limb defects (reviewed in [99]). CdLS causes birth defects in both males and females, and occurs in 1/10,000 to 1/100,000 live births [100, 101]. Clinical manifestations of CdLS range substantially (reviewed in [102, 103]). Facial features and intellectual disability tend to occur in all patients, but limb malformations of the upper extremities present in approximately one third of CdLS patients and range from oligodactyly to absent forearm [104]. About one quarter of patients are affected by a congenital heart defect [105-107] or cleft



palate [104]. Gastrointestinal abnormalities, diaphragmatic hernia and ambiguous genitalia have also been reported [105, 108, 109].



**Figure 1.** Common clinical features of CdLS, RTT and ATR-X syndromes. Each syndrome is represented by a circle. Features common to two or all three syndromes are listed in the areas of overlap. Multiple clinical features, including Intellectual Disabilities, seizures and microcephaly are shared by all three syndromes.

Central nervous system abnormalities in CdLS include cognitive delay, seizures, self-injurious behaviour, obsessive-compulsive behaviours, attention deficit disorder with or without hyperactivity, and depression. The incidence of structural brain anomalies is unknown, but cerebellar abnormalities have been reported in rare cases [108]. Mild to moderate cases of CdLS are commonly reported to have features of autism [99].

CdLS is caused by mutations in the components of the cohesin complex or its regulatory proteins (reviewed in [84]). Cohesin is responsible for keeping sister chromatids linked during mitosis and meiosis, a process termed sister chromatid cohesion, until they are pulled apart into separating daughter cells (reviewed in [110]). However, abnormal cell division does not satisfyingly explain the molecular cause of CdLS, as only a small fraction of cells from less than

half of CdLS patient-derived cell lines show defects in sister chromatid cohesion [111]. Rather, accumulating evidence suggests that a deregulation of gene expression is likely to be the biggest contributor to the symptoms in CdLS [112, 113].

Haploinsufficiency for *NIPPED-B-LIKE* (*NIPBL*) is the most frequent cause of CdLS, with *NIPBL* gene mutations occurring in more than half of all cases [114-116]. *NIPBL* is a highly conserved protein that facilitates cohesin loading onto DNA [117]. The causative mutations tend to occur *de novo*, and a single mutant allele is sufficient to result in the most common autosomal dominant form of CdLS [118]. Mutations in *SMC1A* and *SMC3*, which belong to the family of structural maintenance of chromosomes proteins, account for an additional 5-10% of CdLS cases [119]. Recently, histone deacetylase 8 (*HDAC8*) was identified as the vertebrate protein responsible for the deacetylation of *SMC3* and the dissolution of the cohesin complex at anaphase [120]. Loss-of-function mutations in *HDAC8*, an X-linked gene, were identified in six of 154 individuals affected by CdLS, including two females [120]. *HDAC8* mutations were also identified in 7 males of a Dutch family affected by a novel syndrome characterized by intellectual disability, hypogonadism, obesity, short stature and distinct facial features reminiscent of Wilson-Turner Syndrome (WTS) [121]. Together the findings from this and the Deardorf et al study suggest that WTS may be an X-linked variant of CdLS, or that CdLS and WTS share a causative molecular pathway.

Mice carrying one mutant copy of *Nipbl* have characteristic features of CdLS including facial anomalies, small size, behavioural disturbances and heart defects [113]. Modest changes in the expression of hundreds of genes were reported in both the mutant mice and in CdLS cell lines [113]. This suggests that perhaps the combination of many small changes in expression culminates into the observed pathology. Experimental manipulation of *NIPBL* target genes in a zebrafish model indeed revealed additive and synergistic interactions on phenotypic outcomes [112]. Similarly, a lymphoblastic cell line generated from one of the CdLS patients with an *HDAC8* mutation showed that the gene expression profile was strongly correlated with that seen in *NIPBL* mutant cell lines, and not cell lines from control individuals [120]. Gene expression profiling of *Nipbl* mutant embryonic brain tissue revealed a marked down-regulation of the *Protocadherin-beta* (*Pcdh-β*) genes [113]. In mice, the *Pcdh-α*, *-β*, and *-γ* genes are arranged in tandem arrays on chromosome 18 [122]. The clustered *Pcdh* genes comprise >50 putative synaptic recognition molecules that are related to classical cadherins and highly expressed in the nervous system. They are located at both pre- and post-synaptic terminals, making them ideal participants in synapse formation. Only a small subset of protocadherins are expressed in each neuron from the time they are born and a combinatorial effect of protocadherin expression is generated by alternative splicing and promoter usage and is postulated to instruct future synaptic connections and shape the brain's neuronal circuitry [123, 124]. A similar effect on *Pcdh-β* was reported in the SA1-null embryonic brain, and also in CTCF-null pyramidal neurons [113, 125]. SA1 is largely responsible for cohesin accumulation at promoters and at sites bound by CTCF, emphasizing the linkage between these proteins and their importance for normal protocadherin gene expression in the brain [126].

*NIPBL* might regulate gene expression by controlling loading and unloading of cohesin onto chromatin, thus counteracting its insulating functions [127]. However, it can also recruit

HDAC1 and HDAC3, suggesting that it may promote chromatin remodelling in this way, leading to gene silencing [128]. Genome-wide analysis of DNA methylation in cell lines derived from CdLS patients show specific methylation patterns that differ from controls, specifically on the X chromosome [129]. It is not clear why DNA methylation is affected in CdLS and whether this impacts gene expression changes seen in the disorder.

#### 4. Rett syndrome (RTT)

Rett syndrome (RTT) is a neurodevelopmental disorder characterized by intellectual disability, autistic features, increased risk of epilepsy, and a loss of previously achieved motor and language milestones. RTT affects about one girl in 10,000-15,000, making it the second leading cause of intellectual disability in females, after Down syndrome [130]. In 1999, Amir et al found that RTT was caused by mutations of the Methyl-CpG-binding protein 2 gene (*MeCP2*) [131]. RTT is mainly sporadic and the majority of mutations appear to be of paternal origin. Mutations alter protein sequence or result in truncated versions of MeCP2 with residual function [131]. Males carry only a single copy of the *MeCP2* gene due to its location on the X chromosome. Since at least one functional copy of the gene is required, males with mutations in *MeCP2* are rarely affected with RTT but rather exhibit severe encephalopathy [132-134]. In a typical course of the disease, RTT patients experience normal development up to 6-18 months of age, followed by a period of arrested developmental progress and eventual regression with poor social contact and finger skills (reviewed in [135]). In early childhood, the majority of patients have gastrointestinal problems including difficulty swallowing, which likely contributes to malnutrition and pervasive growth problems [136]. In addition, about half of patients have head circumferences below the 3<sup>rd</sup> percentile (microcephaly), curvature of the spine (scoliosis), and are unable to walk [137].

Much debate still surrounds the question of MeCP2 function at the molecular level, perhaps due to the confounding effects of various post-translational modifications of the protein. MeCP2 is an intrinsically disordered protein that binds methylated DNA via the methyl-binding domain (MBD). One of the roles ascribed to MeCP2 is that of a transcription repressor that binds methylated gene promoters and recruits repressive factors including HDAC1, HDAC2 and Sin3A [138]. For example, recruitment of this complex by MeCP2 regulates the expression of brain-derived neurotrophic factor (BDNF), a protein with important roles in neuronal survival and synaptic plasticity (reviewed in [139]). Neuronal activity leads to demethylation of the *Bdnf* gene, dissociation of the MeCP2-HDAC complex, and increased gene transcription [140, 141].

Despite this somewhat satisfying and simple model of MeCP2 function as a transcriptional repressor, identification of over-expressed target genes in MeCP2-deficient tissue has been difficult. Even more unsettling was the discovery that half of the genes with MeCP2 bound within the promoter in wild type brain actually showed decreased expression in MeCP2-null tissues [142]. The explanation for these discrepancies may come from emerging data indicating that MeCP2 may regulate the organization and compaction of chromatin at a more global level.



A study by Skene et al. proposed that MeCP2 does not act only in a locus-specific manner, but displays a histone-like distribution across the genome in neurons [143]. Moreover, they found large-scale chromatin changes in neurons of MeCP2-null mice, including elevated histone H3 acetylation and doubling of histone H1 in chromatin. Supporting data comes from *in vitro* studies of MeCP2, showing that it can induce compaction-related changes in nucleosome architecture that resemble the classical zigzag motif induced by histone H1 and considered important for 30-nm-fiber formation. The doubling of histone H1 in MeCP2-null neurons may be explained by the finding of Ghosh et al, which suggests that MeCP2 competes with H1 for common binding sites [144]. Consistent with a broader role in chromatin structure organization, MeCP2 is homologous to the attachment region binding protein (*Arbp*) gene in chicken, which has roles in chromatin looping [145]. ARBP has high affinity for specific DNA sequences known as MAR/SARs which it organizes onto a nuclear matrix scaffold [146]. This suggests that ARBP, and by extension perhaps also MeCP2, is involved in chromatin loop organization. MeCP2 loss-of-function was indeed shown to rearrange chromatin fibre interactions at the *Dlx5* locus in mouse brain cells using the chromatin conformation capture technique [147]. These results may be highly relevant to RTT pathology given that the DLX5 protein is an important regulator of GABAergic interneuron development [148]. GABAergic signalling plays a vital role in modulating the activity of the cerebral cortex, and alterations in interneuron position and/or migration have been linked to mental retardation, autism, schizophrenia, epilepsy and Down syndrome [149]. Two GABAergic interneuron-specific MeCP2 knockout mouse lines were generated that exhibited reduced GABA levels in their cortices and displayed repetitive behaviours reminiscent of RTT, including hindlimb clasping, forelimb stereotypies and over-grooming leading to fur loss. In addition, these GABA-specific MeCP2 knockout mice showed progressive motor dysfunction [150].

The methyl binding domain (MBD) of MeCP2 targets the protein to methylated DNA and allows for clustering of pericentric heterochromatin *in vivo* [151]. Analysis of 21 RTT patient mutations showed that two thirds of these decreased the ability of MeCP2 to cluster heterochromatin in mouse cells [152]. This led to the question whether heterochromatin aggregation is impaired in these mutants because of the inability to bind methylated DNA or because of a different function of MeCP2. MeCP2 has multiple chromatin-interacting domains as well as a methylation-independent DNA binding domain *in vitro* [153]. There was some evidence suggesting that the ability of MeCP2 to control chromatin condensation did not require methylated DNA [147, 154, 155], but it was a study by Casas-Delucchi et al that demonstrated that the role of MeCP2 in heterochromatin condensation was independent of DNA binding [156]. They designed an assay in which different mutant MeCP2 proteins from RTT patients were artificially targeted to heterochromatic regions in living cells by fusion to a heterochromatin-binding protein. This allowed for the effects of MeCP2 mutants on chromatin dynamics and organization to be observed *in vivo*. Some RTT mutations led to exclusive decreases in methylated DNA binding, without influencing the ability of MeCP2 to cluster heterochromatin, while other mutations affected both functions. In those mutants that were able to cluster heterochromatin, fusion of large heterochromatic structures (over several micrometers in size) were visualized *in vivo*, providing evidence for the ability of MeCP2 to mediate large-scale chromatin rearrangements [156].

Several mutant mouse models have been generated to study the effects of MeCP2 deficiency (reviewed in [157]). Each RTT model strain has a slightly different time of adverse phenotype onset, but the males usually begin displaying abnormal behaviours between 4-6 weeks after birth. The defects observed in these strains of mice generally recapitulate the symptoms of RTT female patients: laboured breathing, reduced exploratory activity, seizures, cognitive deficits and decreased synaptic plasticity [157-160]. RTT mice clasp their hind paws when suspended by the tail, which is a common sign of neurological deficits [161]. They also have decreased brain weight, smaller cortical neurons with increased neuronal cell density, and reduced dendritic arborisation compared with controls [162, 163]. Importantly, transgenic mice that overexpress MeCP2 also exhibit behaviours of anxiety and impairments in learning and memory, demonstrating that neurons are highly susceptible to either decreased or increased levels of MeCP2.

Several studies provide clues as to the cause of intellectual disability seen in RTT patients. Some of the findings show deficits in long-term potentiation and long-term depression and reduction in spontaneous neurotransmission in cortical and hippocampal neurons of *Mecp2*-null mice [164-167]. Furthermore, post-mortem tissue displays immature neuronal dendrite morphology predicted to result from altered synaptic activity [162, 168, 169].

## 5. Alpha-Thalassemia, mental Retardation X-linked syndrome (ATR-X)

ATR-X syndrome is a rare genetic disorder characterized by moderate to severe intellectual and motor disability, mild alpha-thalassemia in a subset of cases, as well as specific developmental abnormalities including facial, skeletal and urogenital defects [170, 171]. ATR-X syndrome affects very few individuals as it frequently results from familial mutations in the *ATR-X* gene on the X chromosome that are passed on to sons by carrier females. In 2009, there were over 200 known male patients [172]. Females are rarely affected due to skewed X chromosome inactivation, in which the X chromosome carrying the mutant *ATR-X* gene is preferentially selected for condensation [173, 174].

Manifestation of ATR-X syndrome can be quite variable [175, 176]. Typically, affected males have severe global delay from birth, developing very little language and motor abilities. Approximately one third of patients have seizures, and microcephaly is not uncommon (reviewed in [177]). These patients also often have gastrointestinal abnormalities, including difficulty swallowing and gastro-esophageal reflux, which has been known to cause death by asphyxiation in multiple ATR-X cases [178]. Some patients have anatomical abnormalities that can cause stomach torsion or cause severe constipation [178]. Genital abnormalities exist in about 80% of cases, and can present as undescended testes, hypospadias, ambiguous genitalia or normal female genitalia [177].

ATR-X syndrome is caused by mutations in the X-linked ATP-dependent chromatin remodelling protein called ATRX [73]. Mutations in the *ATR-X* gene have also been identified in previously characterized mental retardation disorders: Juberg-Marsidi syndrome [179], Carpenter-Waziri syndrome [180], Smith-Fineman-Myers [176] and X-linked mental retarda-

tion with spastic paraplegia [181]. These disorders were mistakenly thought to be distinct from ATR-X syndrome, as there were mild differences in patient presentations. Identification of *ATR-X* mutations in these cases exemplifies the clinical variation that can occur in ATR-X syndrome.

The *ATR-X* mutations identified to date alter protein sequence or code for truncated forms of ATRX, resulting in reduced protein function or protein level [73, 182]. Nearly all of these mutations are found within the two functional domains of ATRX, located at the end termini of the protein [73, 182, 183]. The domain located at the N-terminus is known as the ADD (ATRX-DNMT3-DNMT3L) domain [184]. It consists of DNA-binding zinc fingers, a protein-binding plant homeodomain finger, and a globular region [185]. The ADD domain is a histone H3-binding module that is selective for the combinatorial readout of H3K9 trimethylation and the lack of H3K4 trimethylation [186, 187]. The domain located at the C-terminus displays ATPase and helicase activity, and is homologous to protein regions found in Swi2 / Snf2 family members. This region allows for Swi2/Snf2 proteins to modulate histone-DNA interactions using energy from ATP hydrolysis [188]. ATRX protein-interactions are consistent with a role in chromatin regulation as ATRX has been shown to interact with HP1 $\alpha$  [189, 190], EZH2 [191], Mecp2 [155, 192], Daxx [193, 194] and cohesin [155]. Together, the domain analyses and protein interactions of ATRX suggest a role in ATP-dependent alteration of chromatin. ATRX interacts with EZH2 at repetitive sites in centromeres, telomeres and at ribosomal DNA to control heterochromatin formation [195-197]. Heterochromatin formation is further induced by the interaction of ATRX with HP1, a protein that functions in binding and maintaining heterochromatic marks like trimethylated lysine 9 of histone 3 (H3K9Me3) and trimethylated lysine 20 of histone 4 (H4K20Me3) [198].

Forebrain-specific deletion of ATRX causes increased p53-dependent neuronal apoptosis, resulting in reduced forebrain size and hypocellularity of the cortex and hippocampus [199, 200]. Many of the mutant mice die in the neonatal period of unknown causes. In contrast, mice expressing a truncated form of ATRX (ATRX( $\Delta$ E2) mice) survive and reproduce normally [201]. Behavioural analyses of these mice showed that they have defects in contextual fear memory with dysfunction of calcium/calmodulin-dependent protein kinase II (CaMKII) and GluR1 [201]. Further studies showed abnormally increased CamKII activity in the prefrontal cortex of the ATRX( $\Delta$ E2) mice [202]. In addition, their prefrontal cortex contained neurons with longer and thinner dendritic spines than those found in controls, which is consistent with other mouse models of intellectual disabilities [202].

Previous work has also shown that genes are deregulated in cells of ATR-X patients and ATRX mutant mice [203, 204]. Two possible mechanisms by which ATRX can act as a transcriptional regulator have been demonstrated [193, 205]. The presence of Daxx relieves the repressive effect of ATRX, but not through alteration of its ATPase activity [193]. This is now understood to occur through the role of Daxx as a chaperone for histone variant H3.3, a marker of active chromatin. Daxx assists in H3.3-H4 tetramer deposition at nucleosomes at PML nuclear bodies, ribosomal DNA, pericentric DNA and telomeres [71, 206]. One theory is that ATRX directs Daxx to deposit H3.3 at specific chromatin regions that have been made accessible by ATRX through ATP-dependent remodelling. ATRX also acts as an inhibitor of macroH2A deposition

into chromatin [205]. In ATRX-null cells, macroH2A accumulates at the HBA gene cluster and leads to reduced  $\alpha$ -globin expression [205]. This is thought to contribute to the symptom of  $\alpha$ -thalassemia seen in ATR-X syndrome patients.

The mechanism by which ATRX may be able to direct Daxx to specific sites is unknown. One possibility is that ATRX localizes to specific loci through the ADD domain [186, 187, 207]. The ADD domain of ATRX was shown to contain two binding pockets for histone 3 modifications: one for unmodified lysine 4 and the other for trimethylated lysine 9 [207]. The combination of these two histone 3 marks is associated with heterochromatin / silent gene promoters and methylated DNA (reviewed in [208]). This combinatorial binding is required for ATRX localization *in vivo* [207]. Further, mutations in ATRX that disrupt the interaction of the ADD domain with H3K9me3 cause a loss of ATRX targeting to heterochromatin [187].

The localization of ATRX to H3K9me3 is strengthened by interaction with HP1 $\alpha$ , which also binds H3K9me3 [207]. In addition, ATRX has been shown to be recruited by MeCP2 [192], which binds the methylated DNA associated with these histone modifications. In fact, loss of MeCP2 in mice results in a loss of ATRX localization at heterochromatic sites in neurons [192]. In addition, a subset of RTT patient MeCP2 mutations interfere with ATRX-MeCP2 interaction [192], which suggests that RTT can be caused in some cases by the inability of MeCP2 to recruit ATRX to specific chromatin sites.

Recently, a family was identified with two men affected by concomitant duplication of both *MeCP2* and *ATRX* [209]. These men did not exhibit signs of ATRX duplication syndrome (short stature, and hypoplastic genitalia), but instead presented with severe mental retardation, muscular hypotonia, and other characteristic features of MeCP2 duplication syndrome. This finding supports the idea that MeCP2 acts upstream of ATRX. However, there was an added feature (cerebellar atrophy) in these patients that was inconsistent with MeCP2 duplication syndrome, which suggests that ATRX may have some additive effect, and not always function in a pathway with MeCP2.

We previously reported that ATRX, MeCP2 and cohesin might cooperate in transcriptional regulation in the brain [155]. In this study, we utilized mice that lack the ATRX protein specifically in the forebrain, by Cre-loxP recombination [199]. These mice have reduced cortical and hippocampal size, reduced number of GABAergic interneurons and exhibit gene expression changes [199, 200, 203]. In control mice, we could show that ATRX and MeCP2 localize to the maternal allele of the *H19* imprinted gene at the upstream imprinting control region (*H19* ICR). In the absence of ATRX, *H19* gene repression in the postnatal period was lessened and correlated with reduced occupancy of cohesin and CTCF at the *H19* ICR. These findings suggest that ATRX is required for optimal gene repression through the recruitment of CTCF and cohesin or by promoting their stable binding to chromatin. A link between ATRX and chromatin cohesion was not only found in the context of gene regulation, but also during mitosis and meiosis. Depletion of ATRX protein in human somatic cells resulted in several mitotic defects, such as mis-congression, reduced cohesion and condensation, mis-segregation of chromosomes and the formation of micronuclei [210]. Abnormal chromosome congression and segregation may in part explain the reduced brain size of forebrain-specific ATRX knockout mice [210].



Genome wide assessment of ATRX protein binding was performed in mouse embryonic stem cells and human erythroblast cells [195]. ATRX binding was often seen at high GC-rich regions of the genome, including the telomeres. These DNA sequences have a high probability of forming unusual DNA structures called G-quadruplexes, or G4-DNA, and recombinant ATRX protein was able to bind these structures *in vitro*. G-quadruplex structures are believed to influence many cellular processes such as transcription elongation and DNA replication and could prove to be an important feature in understanding CNS defects caused by the loss of ATRX protein activity.

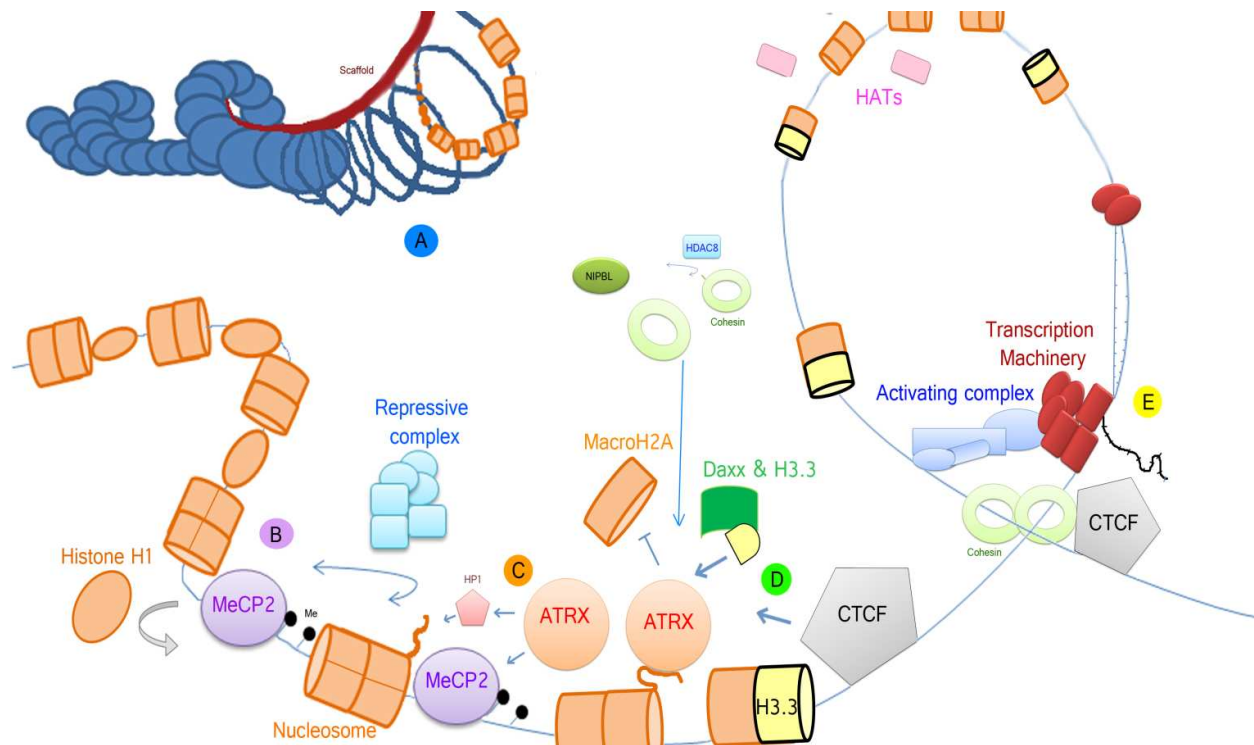
## 6. Therapeutic implications

The shared phenotypic features of CdLS, RTT and ATR-X syndrome (Figure 1) in combination with the molecular findings that place cohesin, MeCP2 and ATRX together in the same physical and functional context (Figure 2) suggest that these three syndromes are in part due to aberration of the same molecular pathways. In particular, the shared feature of intellectual disability and the joint role of MeCP2, ATRX and cohesin in chromatin organization demonstrate that the regulation of chromatin structure is essential for the development of the brain and its complex functions. The study of chromatin structure regulation in the brain, and the identification of defects in gene expression that are caused as a result of abnormal chromatin organization, have been valuable not only to our understanding of human syndromes, but also to the development of therapeutics. This has been especially true of MeCP2 and RTT, the most studied of the three syndromes.

One interesting feature of RTT is that MeCP2-null neurons in the brain do not undergo programmed cell death, or apoptosis [211]. In fact, mounting evidence suggest that RTT is not a neurodegenerative disease, but rather a disorder of neuronal activity (reviewed in [212]). The changes in synaptic maturation and neuronal activity are in part a result of impaired chromatin regulation. Chromatin modifications are dynamic and reversible, which led to the hypothesis that RTT defects may be reversible as well. In 2007, Guy et al. demonstrated that activation of MeCP2 expression in adult MeCP2-deficient mice, even at an advanced stage of illness, reversed neurological symptoms [213, 214]. Replication studies have also shown reversal of RTT morphological features, including neuronal size and dendritic complexity, as well as improvement in functional RTT symptoms such as respiratory function, grip strength and rotarod performance, with the reactivation of MeCP2 in mice [215]. These results have since revolutionized the way in which intellectual disability syndromes are understood [212, 216].

However, there are many obstacles for which gene therapy cannot currently be considered in RTT patients (reviewed in [217]). Gene dosage is one important consideration; since MeCP2 is located on the X chromosome, the number of neurons affected in each patient is dependent on X-inactivation. Providing excess MeCP2 to neurons that already express the non-mutant allele has negative consequences on brain function. This has been observed in mice [214], as well as in humans where severe intellectual disability caused by MeCP2 duplication has been documented (MeCP2 Duplication Syndrome [134, 218, 219]). Therefore a specific dose of the





**Figure 2. Regulation of chromatin organization by cohesin, ATRX and MeCP2.** A: DNA is wrapped around histones in a complex known as the nucleosome. The nucleosome-covered DNA is coiled to form a 30 nm fibre which then further coils and loops to form higher order structures. These chromatin structures are attached to scaffolds in the nucleus. B: MeCP2 competes for linker DNA with histone H1 at methylated cytosine residues. MeCP2 recruits repressive complexes which contain HDACs that deacetylate histone tails. Unacetylated histone 3 Lysine 9 becomes trimethylated and attracts HP1alpha. C: ATRX is recruited by MeCP2. MeCP2 and HP1alpha both directly interact with ATRX. D: ATRX has binding sites for H3K9me3 and unmodified H3K4. ATRX inhibits macroH2A incorporation into nucleosomes and recruits Daxx, which is a chaperone for Histone H3.3. Histone H3.3 is incorporated into nucleosomes and marks active chromatin. ATRX recruits CTCF and cohesin. Cohesin is loaded onto DNA by NIPBL. E: Cohesin and CTCF interact to stabilize looping structures. These loops allow transcription machinery to interact with distant activating complexes bound to enhancers.

gene is required. This issue is not unique to MeCP2; over-expression of ATRX in mice led to disorganization of the cells in the brain at the ventricular zone, seizures and death soon after birth [220]. Case studies in humans report that ATRX duplication is associated with severe intellectual disability, genital anomalies and short stature [221, 222].

Due to these and other issues, therapeutic approaches in RTT have had to focus on pathways downstream of MeCP2. The understanding of how MeCP2 perturbs gene expression through its effects on chromatin has been indispensable to these advances. For example, a link between MeCP2 and the regulation of Brain-derived neurotrophic factor (BDNF) expression led Tsai et al. to test the administration of BDNF on the phenotypic outcomes of MeCP2 mutant mice [223, 224]. BDNF is a secreted factor of the neurotrophin family that promotes survival of neurons but also growth and differentiation of new neurons and synapses. BDNF injection led to a slower progression of disease in the RTT mouse model. Potentially, intravenous injection of BDNF in RTT patients could increase BDNF levels in the brain and slow the progression of symptoms. In particular, breathing dysfunction leads to increased mortality and morbidity in

RTT. Reduced levels BDNF in the brain of mice is associated with increased tachypneas and apneas [225-228]. Pharmacological activation of the BDNF receptor TrkB in RTT mice restored wild-type breathing, which demonstrates another potential avenue for therapy in RTT [225]. However, the treatment with the most promise in current literature is the administration of Insulin-like Growth Factor 1 (IGF-1). IGF-1 is an important regulator of synaptic plasticity and maturation that is widely expressed in the brain (reviewed in [229]). Multiple studies have supported the hypothesis that dendritic spines are altered in RTT, implicating synaptic maturation as a major deficit. Treatment of RTT model mice with IGF-1 N-terminal tripeptide, known as GPE, partially restores dendrite spine number, and improves the cortical plasticity levels to that of wild type mice [230]. In addition, it improves gait and breathing patterns of the MeCP2 mutant mice.

## 7. Conclusions

Animal models have provided an extensive knowledge about the three syndromes discussed above. However these animal studies cannot recapitulate all of the complexities of human brain disorders. Neurodevelopmental disorders have been difficult to study in humans because of the limited supply of post-mortem brain samples and studying peripheral cells from patients such as lymphocytes is problematic because they do not accurately portray defects of the target tissue [231]. Human induced pluripotent stem cells (iPSCs) are a novel technology that may provide a potential solution to this issue. iPSCs are a type of stem cell that are produced by genetic reprogramming of a differentiated somatic cell [232, 233]. These iPSCs can be derived from healthy individuals or from those afflicted by a genetic condition, and then differentiated into the cell type desired for research. Studies of neuronal cells derived from iPSCs of RTT patients have provided valuable complimentary information to the findings from *in vivo* animal studies. Specifically, modeling RTT with iPSCs has allowed for medications like IGF1 to be tested for efficacy in human RTT patient neurons [234]. Administration of IGF1 was shown to rescue synaptic defects in this model and is currently in clinical trials for treatment of RTT for which primary outcome measures will be available in 2013 (<http://clinicaltrials.gov/ct2/show/record/NCT01253317?term=rett+syndrome>). It has not been shown whether IGF1 is affected by ATRX or NIPBL knockdown, or whether similar therapies would be beneficial in ATR-X or Cornelia de Lange syndromes. However, since there is evidence that ATRX, cohesin and MeCP2 function together in regulating gene expression and brain development, it is possible that downstream targets, like IGF1, are similarly affected in all three syndromes.

Although more work remains, the study of chromatin modifiers in brain development have provided insight into inherited forms of intellectual disabilities, as well as target pathways for future clinical interventions. Continued investigation of chromatin regulation in neurological and psychiatric disease will help to identify more commonalities between disorders and further our knowledge of potential treatment avenues.

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