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X-linked mental retardation and epigenetics

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Abstract

The search for the genetic defects in constitutional diseases has so far been restricted to direct methods for the identification of genetic mutations in the patients' genome. Traditional methods such as karyotyping, FISH, mutation screening, positional cloning and CGH, have been complemented with newer methods including array-CGH and PCR-based approaches (MLPA, qPCR). These methods have revealed a high number of genetic or genomic aberrations that result in an altered expression or reduced functional activity of key proteins. For a significant percentage of patients with congenital disease however, the underlying cause has not been resolved strongly suggesting that yet other mechanisms could play important roles in their etiology. Alterations of the 'native' epigenetic imprint might constitute such a novel mechanism. Epigenetics, heritable changes that do not rely on the nucleotide sequence, has already been shown to play a determining role in embryonic development, X-inactivation, and cell differentiation in mammals. Recent progress in the development of techniques to study these processes on full genome scale has stimulated researchers to investigate the role of epigenetic modifications in cancer as well as in constitutional diseases. We will focus on mental impairment because of the growing evidence for the contribution of epigenetics in memory formation and cognition. Disturbance of the epigenetic profile due to direct alterations at genomic regions, or failure of the epigenetic machinery due to genetic mutations in one of its components, has been demonstrated in cognitive derangements in a number of neurological disorders now. It is therefore tempting to speculate that the cognitive deficit in a significant percentage of patients with unexplained mental retardation results from epigenetic modifications.

Keywords: epigenetics • acetylation • methylation • chromatin • mental retardation • X-linked • memory

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Epigenetics: an introduction

A Pubmed search, limited for the last 3 years, for the word "Epigenetic" reveals about 3500 publications of which almost a 1000 are reviews. This high percentage of reviews that often only refer to epigenetics, clearly illustrates that errors in epigenetic regulation are considered as the "golden egg", possibly explaining many of the unsolved cases in current clinical and molecular genetics. Due to the lack of high-throughput, high-resolution, and robust techniques to study these processes in the past, experimental epigenetic research remained relatively scarce.

It is clear that the phenotype of an individual is not solely based on its sequence information but that secondary modifications, independent of the nucleotide sequence, play an important role. The broadest definition of epigenetics is a heritable change in a DNA-dependent process (expression/repression of particular genes, DNA replication, recombination, and repair or chromosome function and stability) that does not rely on the primary DNA sequence. For instance, it is clear that although the sequence of a genome is identical in a liver and a brain cell of an individual, the gene expression profiles in both cells are completely different. This difference mainly relies on epigenetic factors. Epigenetic modifications therefore, can be attributed to changes in the environment or to mutations in genes that affect local chromatin structures. In this review we will focus on those changes that affect gene expression.

In the eukaryotic nucleus, the basic unit of chromatin is the nucleosome that consists of 147 bp of DNA wrapped around an octameric complex of histones H2A, H2B, H3 and H4. Linker histones, like histone H1, bind and stabilize the nucleosome structure and organize the linker DNA located between successive nucleosomes. It is hypothesized that this interaction is required for the formation of the higher-order 30 nm chromatin fibre [1]. The association of linker histones with nucleosomes also hinders the action of ATP-dependent chromatin remodeling enzymes [2]. Proper chromatin function is further dictated by epigenetic marks that constitute the epigenome. These include **1**) DNA methylation of the cytosine residue of CpG dinucleotides, **2**) methylation, acetylation, phosphorylation, or ubiquitylation of the histone tails, **3**) use of histone variants, **4**) nucleosome distribution and interaction, or **5**) higher order chromatin structures (such as loops and scaffolds) [3]. These epigenetic marks are then deciphered by other factors (the ATPase-dependent remodelers; the silencing Polycomb group (PcG) and the activating Tritorax group (TtxG) complexes [4]). Any modification in this epigenetic program can have an impact on the proper function of each of the DNA-dependent processes.

Methylation of DNA is a hallmark for gene silencing. Indeed, methyl binding proteins (MBP) that bind to methylated CpGs (reviewed in [5]) will induce the repression by recruitment of co-repressors such as a histone deacetylase (HDAC) in the case of MECP2 [6]. Methylation of cytosine at position C5 in CpG dinucleotides [5] is conferred by DNA methyltransferases (DNMTs) of which *DNMT1* is the maintenance DNMT while *DNMT3a* and *DNMT3b* are *de novo* DNMTs [7]. The contribution of DNMT2 in methylation is still questionable [8]. Recently, a link between histone H1 and DNA methylation was discovered in mouse [9, 10]. Reduction of the H1 levels by 50% in mouse embryonic stem cells not only affects chromatin structure but the expression of specific genes as well. A large part of these deregulated genes represent either imprinted genes or genes located on the X chromosome that are known to be regulated by DNA methylation. Intriguingly, the authors found that DNA methylation at specific CpG dinucleotides is reduced in the mutant mouse ES-cells while no alterations in global DNA methylation are noted. This strongly suggests that a certain level of H1 protein is required for the maintenance or establishment of specific DNA methylation patterns leading to gene repression. Evidence is thus accumulating that linker histones participate in the epigenetic regulation of gene expression.

Also in humans, disturbances in methylated DNA-induced gene silencing might lead to disease (reviewed in [11]). Aberrant DNA methylation patterns have now been demonstrated not only in cancer [12, 13] but also in constitutional diseases [11, 14]. The best known epigenetic factor is imprinting (reviewed in [11]) in which one of both parental alleles is methylated and therefore, inactivated. Imprinting defects has been demonstrated in tumors (*e.g.* Wilms tumor) [15] as well as in constitutional disorders *e.g.* Prader-Willi and Angelman syndromes [16]. Moreover, it became apparent that imprinted regions are also characterized by differences in chromatin conformation, histone modification, repli-

cation timing and recombination rate clearly pointing to an integrated epigenetic modification network that influences multiple DNA-dependent processes [17]. Many key players in these processes have been identified including DNMTs, histone acetyltransferase (HAT), HDACs, histone methylases, chromatin-modifying enzymes and members of the SWI-SNF family that are part of the ATP-dependent chromatin remodeling complexes [18–20].

Besides DNA, also the other constituents of the basic chromatin unit, the histones, can be modified to functionally and structurally alter chromatin organization. The histone tails are subject to covalent post-translational modifications such as lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, lysine ubiquitylation and sumoylation, and ADP-ribosylation. These modified histone tails present a code that is 'read' by regulatory factors, thereby generating altered chromatin structures and functions. This mechanism, which is superimposed on the genetic code, was named "the histone code" [21, 22]. Evidence validating this hypothesized theory is accumulating. In accordance with this theory, protein domains that specifically recognize modified histone tails have been identified. The complexity of the histone code is increased by the co-existence of different histone tail modifications in cis and by mechanisms as mono-, di- and tri-methylation of lysine residues [23]. The net electrical charge of histone proteins can be altered by covalent modifications like acetylation and ADP-ribosylation. Modification of the electrostatic interactions between histones and the negatively charged DNA phosphate backbone alter the conformational and functional state of the genome [24]. In this regard, polyADP-ribosylation of histone H1 by polyADP-ribose-polymerase-1 (PARP-1) has been suggested to enable the transcription needed for long-term memory formation in Aplysia [25] and to promote neurotrophic effects in rats [26]. PolyADP-ribosylation of H1 causes fast and transient chromatin relaxation rendering the DNA accessible to repair and transcription factors [27].

In general, methylated DNA, deacetylated histone tails and methylated H3K9 allow the binding of factors that cause a closed chromatin environment and hence, a repressed transcriptional state [17]. On the other hand, unmethylated DNA, acetylated histone tails and methylation of H3K4 dictate an open chromatin structure, which allows for gene transcription at this region. Therefore, alteration of these epigenetic marks will lead to altered gene expression and eventually to disease [11, 14, 18, 28–30]. The interplay of histone acetylases (HATs) and histone deacetylases (HDACs) will generate an acetylation imprint on Lysine residues. Similarly, methylation marks are placed by histone methyltransferases (HMTs) and Arg methyltransferases (PRMTs) [31, 32], and are removed by H3K4 demethylase LSD1 [33], and a new family of JmjC domain-containing histone demethylation proteins (JHDM1) that has recently been identified [34, 35].

It is thought that several more proteins involved in chromatin remodeling will be discovered in the near future. The recruitment of these histone tail modifying enzymes to particular genes or DNA regions is in part driven by the presence of enhancer or repressor regulatory sequences in the DNA, but is in part also driven by non-coding RNAs. The best known link between non-coding RNA and chromatin modification is the inactivation of one of both X chromosomes in female mammals to allow for dosage compensation between both sexes (reviewed by [36]). This process is well-studied in mouse embryos and embryonic stem cells. Before blastocyst stage of the female embryo, the paternal X chromosome is inactivated by an imprinted mechanism [37]. During blastocyst stage, this inactivated paternal X chromosome becomes reactivated in the cells of the inner cell mass that give rise to the embryo proper and one of both X chromosomes will subsequently be silenced by a random X inactivation mechanism. A required region in the X inactivation process is the X inactivation center (Xic). Xic generates multiple non-coding RNAs, such as Xist and its antisense RNA Tsix, which regulates the expression of Xist. Once it is decided which X chromosome will be inactivated, a process, that is dependent on a transient colocalization of both Xics [38, 39], will downregulate Tsix leading to the in cis spreading of Xist RNA on the X chromosome that will be inactivated. Subsequently, different chromatin-remodeling factors are recruited that will place epigenetic marks on nucleosomes of the X chromosome to be inactivated resulting in a transcriptionally repressive state.

Other potential links between non-coding RNAs and chromatin remodeling in mammals are represented by RNA-interference-mediated heterochro-

matin assembly, *e.g.* at pericentromeric regions, and gene silencing (reviewed by [40]).

To date, still little is known about the genome-wide distribution of DNA methylation, histone modification or other epigenetic factors in health or disease. Recently however, novel methods that combine existing and new technologies will speed up the search for epigenetic mechanisms underlying different disease states. Ongoing clinical trials for epigenetic therapy are currently focused on inhibitors of DNA methylation and histone deacetylation but future knowledge might direct therapeutic concepts into new directions (reviewed by [14]). In this review, we will focus on mutations that lead, directly or indirectly, to an altered epigenetic status leading to constitutional neurodegenerative diseases.

Epigenetics in neurodegenerative disease

Evidence for epigenetics in neurons

Recent studies on primary modifications of histone tails in neuronal cells provided ample evidence for a role of epigenetic factors that control proper neuronal development and functioning. The RE1 silencing transcription factor (REST) binds to the repressor element 1 (RE1) conserved motif to repress the transcription of neuron-specific genes in differentiated nonneuronal cells [41, 42]. It acts in conjunction with the co-repressors SIN3A and CoREST. The REST/SIN3A complex associates with HDAC1, HMT G9a and LSD1 [43, 44], while the REST/CoREST repressor interacts with HDAC2 [45], MeCP2, HMT SUV39H1, HP1 and possibly SWI-SNF [46]. In addition, CoREST was recently shown to play a role in H3K4 demethylation [47]. All these findings strongly indicate that the REST-dependent silencing of neural-specific genes in non-neuronal cells is epigenetically regulated with subsequent chromatin reorganization. Moreover, DNA methylation was also found in differentiated non-neuronal cells [48] although the binding of REST to RE1 is methylation-independent. Neuronal-specific gene expression in differentiated neurons is thought to rely on the absence of binding of REST. The exact mechanism however, is not known yet. The two current models are; **1**) degradation of the REST complex and dissociation from the RE1 sites, and **2**) displacement of REST from RE1 by a small double-stranded non-coding RNA (reviewed by [49]). In addition to the REST complex a novel silencing mechanism was recently reported in nonneuronal cells. The transcription factor AP4 was shown to form a functional complex with the co-repressor geminin that recruits the co-repressor SMRT as well as HDAC3 to repress the expression of target genes including *PAHX-AP1* and *DYRK1A*, in nonneuronal cells [50]. Finally, several learning models in mice provide evidence that acetylation of histones H3 and H4 have regulatory roles in memory formation (reviewed by [51]).

Imprinting and disease

Imprinting is the process in which expression of a gene is restricted to only one of the parental alleles. Paternal or maternal imprinting means that an allele inherited from the father or the mother, respectively, is not expressed in the offspring. A few dozen of imprinted genes are currently known. These genes are mostly organized in clusters and often coordinately regulated by imprinting centers (ICs) [52]. The most consistent feature of imprinted regions is DNA methylation although other epigenetic marks also occur, including histone modifications, antisense transcripts, boundary elements, silencers and condensed chromatin structures [53, 54]. The two best known examples for which deregulation of imprinting leads to congenital disorders associated with mental impairment are Prader-Willi/Angelman syndrome at 15q11–q13 [55], and Beckwith-Wiedemann syndrome at 11p15.5 [56]. In both regions, ICs regulate expression bidirectionally over distances up to 1 Mb. Deletions at 15q11–q13 cause different phenotypes depending on the parental origin of the affected chromosome. Deletions at the paternal chromosome result in Prader-Willi syndrome while those on the maternal counterpart lead to Angelman syndrome. The imprinted expression of several genes present in the deleted region are therefore candidate genes for the different phenotypes of these syndromes. Loss of the genes *SNRPN*, *NDN*, *MAGEL2* and *MKRN3* that are expressed solely from the paternal allele results in Prader-Willi syndrome while the genes coding for UBE3A and ATP10A are expressed exclusively from the maternal allele and their loss results in Angelman syn-

drome. In Beckwith-Wiedemann syndrome the imprinted region at 11p15.5 is about 1 Mb and involves the *KIP2/LIT1* and *IGF2/H19* regulated domains. The genomic aberrations and epigenetic changes that mark these syndromes are recently reviewed in detail [57]. However, the precise mechanisms by which ICs mediate their imprinting marks in these regions still remain largely unknown. Recently, a cell lineage-specific imprinting has been demonstrated in the brain [58].

Autosomal genes and neurodegeneration

RELN and schizophrenia

Several studies have demonstrated that the mRNA levels of the reeling gene (*RELN*), located at 7q22.1, are significantly reduced in neurons from patients with schizophrenia and bipolar disorders compared to nonpsychiatric subjects [59, 60]. RELN is an extracellular matrix protein that plays a role in neuronal migration, axonal branching and synaptogenesis during brain development. It is expressed in cerebellar granule neurons and acts as a regulator of *Arc* mRNA translation in synaptoneurosomes [61]. In these cells, an increase of Dnmt1 was also noticed [62]. Evidence for a role of changes in methylation at the *Reln* locus came from experiments in mice where administration of methionine induced a downregulation of *RELN* due to an increase of methylation at its promoter [63, 64]. Interestingly, treatment with antisense *Dnmt1* or valproic acid, an HDAC inhibitor, counteracted the decrease of *RELN in vitro*. Administration of valproic acid to mice even corrected the schizophrenia-like behaviours induced by methionine [65]. The corresponding hypermethylation of the *RELN* promoter has now been demonstrated in schizophrenic and bipolar patients by two independent groups [66, 67] suggesting that this enhanced methylation compromises promoter activity with a subsequent reduction in expression. Recent data also demonstrated hypomethylation of the promoter of the membrane-bound catechol-O-methyltransferase (*MB-COMT*) (22q11.21) in these patients, resulting in enhanced expression of *COMT* and a potential increase of dopamine degradation in their frontal lobe [68]. The availability of heterozygote *Reln* knockout mice, which demonstrate cognitive dysfunction and an altered hippocampus synaptic complex [69], can now be used to further investigate the underlying mechanisms. These initial findings in this field stress the importance of epigenetic changes in complex diseases.

CBP and neurodegenerative disorders

CREB-binding protein (CBP) is a transcriptional co-activator that binds phosphorylated cyclic AMP response element binding protein (CREB), which then induces gene transcription. CBP is a large nuclear protein that comprises several different domains including a bromodomain and a histone acetyltransferase (HAT) domain [70]. Therefore, it may form the link between transcription and DNA- or chromatin-binding factors. This protein plays an important role in many biological processes including neural plasticity [71]. It is believed that activated CREB connects the neuronal activity-induced protein kinase pathway with the transcription of genes important in learning and memory formation. Rubinstein-Taybi syndrome (RTS) is a rare disorder caused by dominant mutations in *CBP* at 16p13.3 [72, 73]. It is characterized by mental retardation and many other physical abnormalities [74]. Impaired function of the co-activator CBP, might affect the proper establishment of long-term memory in RTS patients [75–77]. The HAT activity of CBP may guide the transcriptional processes by relaxing repressive chromatin structures [78, 79]. Therefore, it is likely that defective CBP or parts of CBP act as a dominant negative protein in RTS patients. Indeed, mice that are heterozygous for a C-terminal truncation of CBP have impaired long-term memory [80, 81].

Huntington disease (HD) is caused by a CAG expansion in exon 1 of the huntingtin (*htt*) gene at 4p16.3. This polyglutamine (polyQ) expansion results in a mislocalization of the protein in the form of aggregates in the nucleus [82]. The expanded polyQ tract subsequently binds to the HAT domain of CBP resulting in a significant reduction of transcription and ultimately leading to neuronal death [82, 83]. Moreover, upon reversal of hypoacetylation, rescue of cell death and prolonged survival of Huntington's disease mice was demonstrated, pointing towards a crucial role of reduced HAT activity in Huntington patients [84, 85].

Alzheimer's disease (AD) is a late-onset neurological disease that progresses to severe memory deficits and ultimately neuronal death. Mutations in the amy-

loid precursor protein (*APP*) and the presenilin genes 1 and 2 (*PS1* and *PS2*) have been related to the increase of the β-amyloid (βA) peptide although the precise mechanisms are not known yet [86, 87]. The neuronal loss in Alzheimer's' disease has been connected to epigenetics in two ways. First, several studies demonstrated that mutations in *APP* and *PS1* affect the CREB/CBP pathway (reviewed in [88]). βA deposits impair phosphorylation of CREB [89] and this can be restored *in vivo* [90, 91]. The conditional knockouts of *PS1* and *PS2* in excitatory neurons of the forebrain of mice lead to decreased levels of *CBP* and subsequently, a reduced transcription of *CREB*-dependent genes in the brain [92]. Second, the intracellular domain of APP (AICD) that is obtained after cleavage with β- and γ-secretases [93] regulates transcription through recruitment of Fe65 and the histone acetyltransferase TIP60, which was recently shown to be implicated in chromatin remodeling and DNA repair [94].

From these studies it is clear that disturbance of the CBP/CREB pathway might result in neurodegeneration through an epigenetic effect on transcriptional regulation of brain-specific genes. Similarly, the CBP/CREB proteins have been implicated in depressions too [95].

Two other examples of how epigenetics can regulate neuronal function are circadian rhythmicity and seizures. Circadian rhythmicity is a continuing transcription-translation feedback loop regulated by the circadian clock [96] and has been shown to depend on histone acetylation [97]. Second, seizures induce many transcriptional changes in the brain, which might result from histone modifications, including at the *CREB* promoter, and subsequent chromatin remodeling [98, 99]. A schematic representation of the CBP/CREB transcriptional activation pathway is shown in Fig. 1.

Other autosomal MR-related genes

Mutations in the *CHD7* gene at 8q12.1, a member of the chromodomain helicase DNA binding family, causes CHARGE syndrome of which mental and somatic developmental delay is one of the hallmarks [100, 101]. It contains two N-terminal chromodomains, a SNF2-like ATPase/helicase domain and a DNA-binding domain [102]. This family of proteins is thought to have important roles in chromatin remodeling, and thus gene expression, in early embryogenesis [103]. As a second example, the euchromatin histone methyl transferase 1 (*EHMT1*) gene was found to be closely related to the enzyme G9a. These enzymes appear to be H3K9 HMTases, present in euchromatic regions that form complexes with heterochromatin protein 1, E2F-6, and Polycomb group (PcG) proteins [104, 105]. These proteins have also been shown to interact with the XLMR protein, ATRX as will be discussed in the section 'ATRX syndrome'. Deletions at 9q34 that include EHMT1 have been related to the mental impairment in patients [106].

X-linked mental retardation genes

Mental retardation (MR) is one of the most common disorders affecting 2–3% of the human population. It is a non-progressive cognitive impairment mostly affecting normal brain development marked by learning (IQ < 70) and behavioural disabilities. Since more males than females are suffering from MR (1.3/1 ratio), the search for disease-associated genes has, up to now, predominantly been focused on the X chromosome (XLMR) [107–110]. For this, the clinical genetics group at our Centre in Leuven has collected, in collaboration with the EuroMRX Consortium (http://www.euromrx.com/), many hundreds of DNA samples from presumed XLMR families. Although combined efforts in molecular genetics, positional cloning and mutation screening have led to the identification of tens of MR genes on the X chromosome, linkage analysis data and mutation screening of the known XLMR genes strongly indicate that the contribution of each yet identified gene is very small (< 1%). Hence, many more genes or mechanisms should be involved in MR [108]. Therefore, the X chromosome can be used as a model to study novel approaches. One such approach was array-CGH for which new genes and mechanisms have been identified [111–114], but it is also tempting to believe that changes in the epigenetic codes in the brain will result in cognitive deficits since several XLMR genes identified to date seem to play determining roles in chromatin remodeling. Each of those will be briefly discussed. A schematic overview of the different methods to identify genetic mutations that result in disease is given in Fig. 2.

Fragile X syndrome

Fragile X mental retardation is characterized by moderate to severe mental retardation,

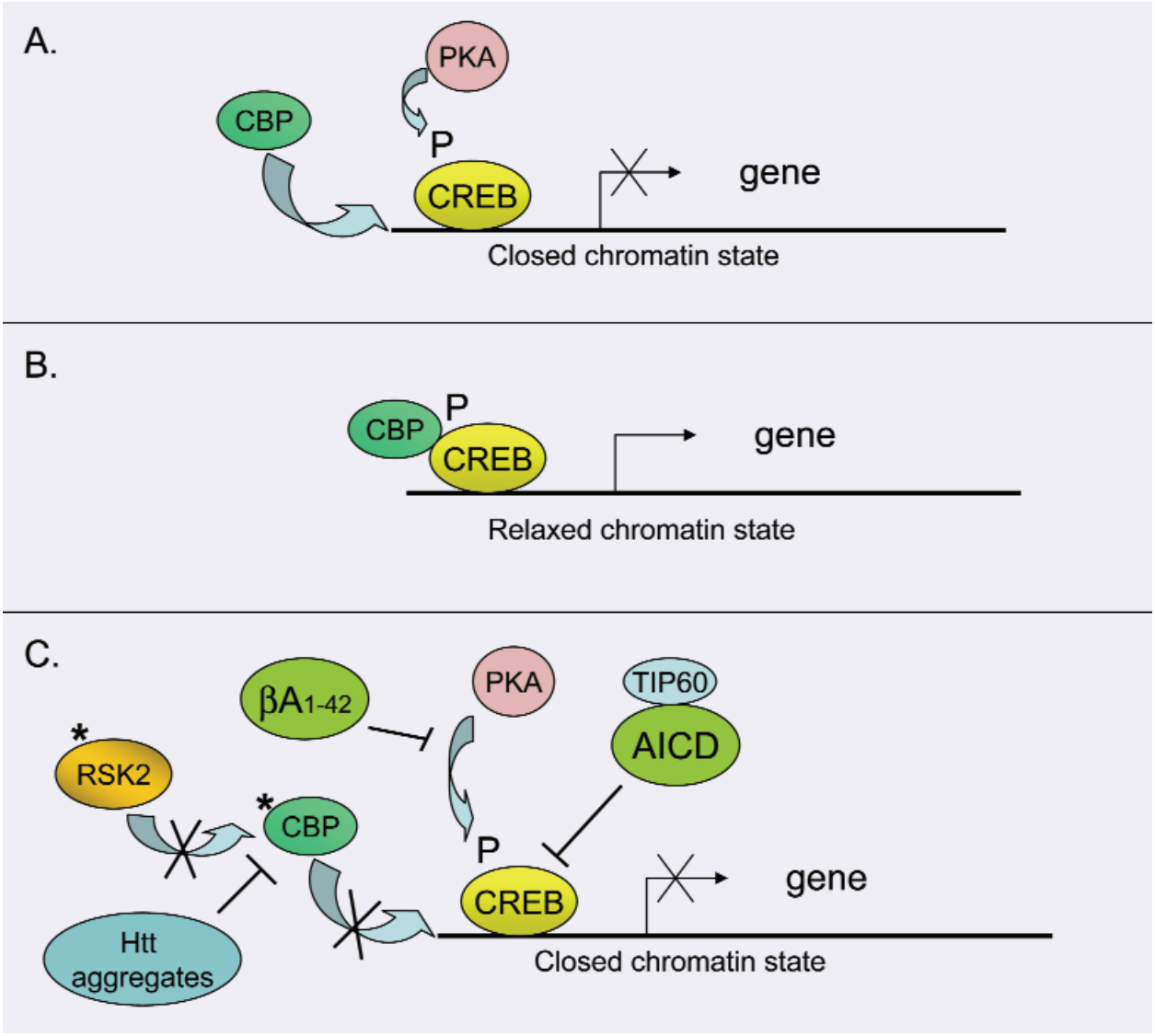

Fig. 1 CBP/CREB-dependent transcriptional regulation. **A**. In unstimulated conditions, chromatin regions containing CBP/CREB-regulated genes are in a closed configuration. The histone tails are not acetylated and gene expression is silenced. **B**. Upon activation, the CREB protein binds to its consensus sequence in promoters of regulated genes. It is phosphorylated by protein kinase A (PKA) and subsequently interacts with CBP. The HAT domain of CBP acetylates the histone tails and opens up the surrounding chromatin with gene expression as a consequence. **C**. Different mutations (indicated by an asterisk on CBP and RSK2), or side products due to mutations (Htt aggregates, $\beta A_{1\text{-}42}$ and AICD) in neurological diseases prohibit the normal trancriptional activation of the CBP/CREB complex at different levels as indicated in the schematic representation.

macroorchidism, large ears, prominent jaw, and high-pitched jocular speech, and is caused by mutation of the *FMR1* gene at Xq27.3. More than 95% of the fragile X cases are due to an expansion of the CGG repeat at the 5' end of *FMR1*, resulting in transcriptional silencing of *FMR1*, and thus, the absence of the FMR1 protein (FMRP) [115, 116]. FMRP is involved in the metabolism of neuronal mRNAs, and therefore, absence or mutation of FMRP will lead to spine dysmorphogenesis and impairment of synaptic plasticity (reviewed in [117]). Extensive analysis of differences in epigenetic modifications in lymphoblastoid cell lines of normal and fragile X individuals has led to some well-defined observations. Next to methylation of the CpGs of the repeat tract and *FMR1* promoter

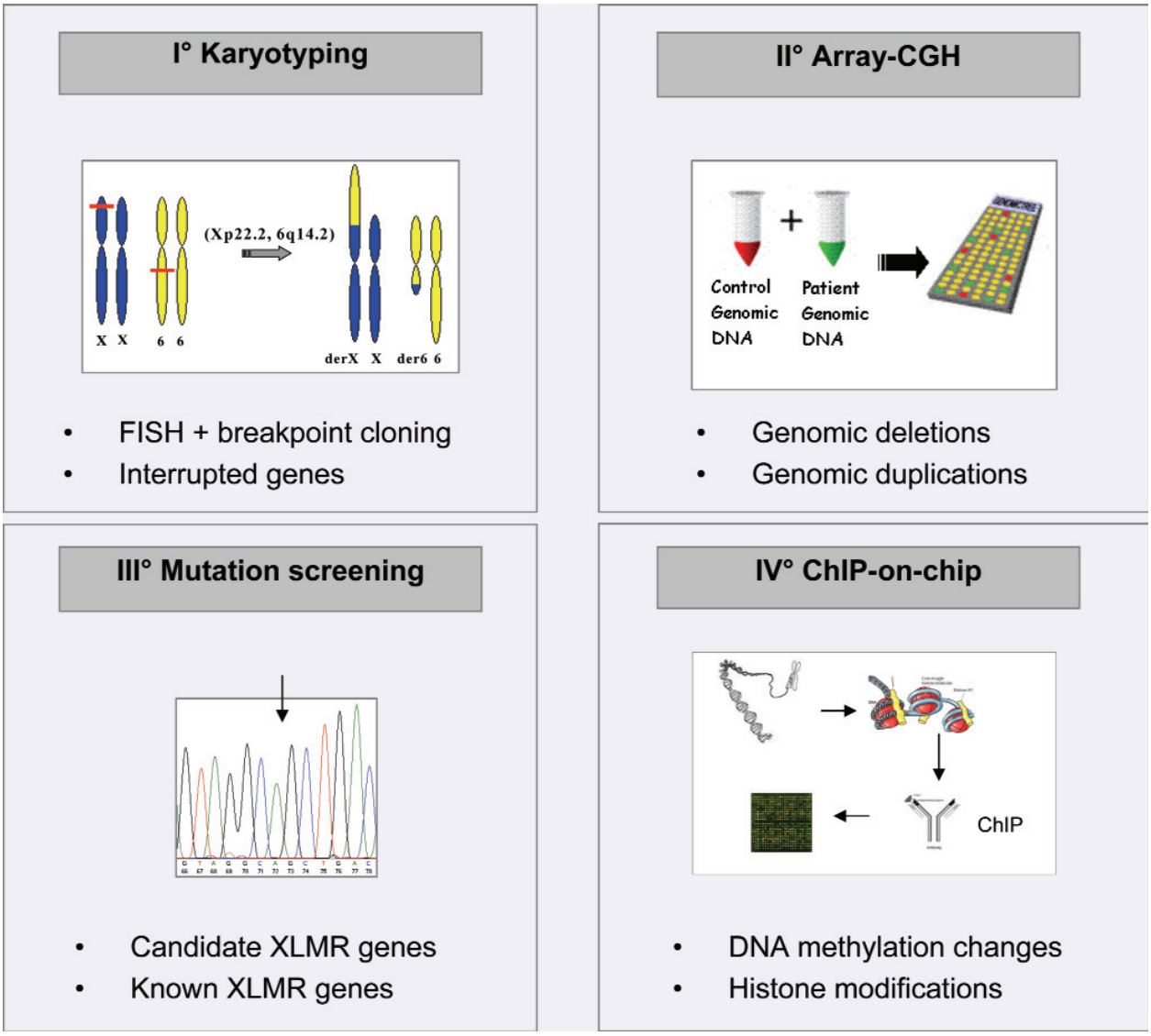

Fig. 2 Four different methods to detect causal mutations. Current methods to identify genetic mutations are standard karyotyping (I), array-CGH analysis (II) and mutation screening (III). With the development of novel techniques such as ChIP-on-chip, it becomes now feasible to investigate the contribution of epigenetic modifications (IV).

sequences, reduced levels of acetylation of H3 and H4 at *FMR1* were observed in cells from fragile X patients when compared to controls [118]. The same group also demonstrated hypomethylation at H3K4 as well as hypermethylation of H3K9 at *FMR1* [119]. Acetylation of histones and methylation of H3K4 are typical markers of an active chromatin structure, while methylation of H3K9 is associated with a repressive state of the chromatin. These data all point to a tightly repressed state of the chromatin at the *FMR1* locus in fragile X patients, also reflected in the observed lack of *FMR1* transcription. A model was suggested in which DNA methylation of the expanded CGG-repeat triggers binding of MBPs, thereby recruiting HDACs and other components of the transcriptional repression machinery to *FMR1*, resulting in chromatin condensation and repression of gene transcription. Recently, this model was extended *via* chromosome conformation capture (3C) assays [120]. In normal cells where *FMR1* is active, chromatin interactions are reduced in a 50 kb region around the *FMR1* promoter. Histone modifications on the other hand, were shown to occur over much

shorter distances. It was suggested that these local histone modifications contribute to the reduced chromatin interactions and as such establish a more open chromatin structure allowing active genes to loop out and interact with other genes [120]. A slightly different order of epigenetic events occurring at the *FMR1* promoter, is proposed by the analysis of a unique cell line derived from a full mutation *FMR1* carrier with normal intelligence [121]. Similar to fragile X cells, histone deacetylation and H3K9 methylation were present. However, DNA methylation was missing as well as demethylation of H3K4. Normal levels of *FMR1* mRNA were detected, but FMRP protein levels were reduced due to less efficient mRNA translation as a result of the expanded CGG repeat. These findings were complemented by pharmacological treatments with 5-aza-deoxycytidine, which reverses DNA methylation, and their effect on the epigenetic markers at *FMR1* (Tabolacci 2005). These studies demonstrate that H3K4 methylation as well as DNA methylation have key roles in activating the expression of *FMR1* gene, while histone acetylation plays a less important role. An adapted model was given wherein methylated H3K9 recruits HMTs associated with HDAC1 via the heterochromatin protein 1 (HP1), resulting in histone deacetylation, DNA methylation and H3K4 demethylation.

Dissecting and understanding the epigenetic modifications at the *FMR1* locus may allow additional ways for pharmacological intervention and rescue of fragile X syndrome phenotypes. Until now, MPEP, an antagonist of metabotrophic glutamate receptor 5 (mGluR5) has been effective to reverse sensitivity to audiogenic seizures and abnormal open field exploratory behaviour in a fragile X syndrome mouse model [122]. Similarly, use of mGluR antagonists or lithium in a *Drosophila* model of fragile X restores some memory defects [123]. It is tempting to speculate that disruption of a key epigenetic modification at the *FMR1* locus could also contribute to (partial) rescue of some fragile X syndrome phenotypes in human.

Rett syndrome

Mutations and gross deletions of the methyl CpG-binding protein 2 (*MECP2*) gene at Xq28 are associated in about 80% of patients with Rett syndrome, a progressive neurodegenerative disorder affecting almost exclusively females [124, 125]. Mutations in males result in a broad spectrum of phenotypes ranging from nonsyndromic MR to very severe neurological symptoms [126]. Interestingly, not only loss-of-function mutations but also a 2-fold increase of the MECP2 dosage results in a severe neurological phenotype. This was first observed in mice with artificial overexpression of *MECP2* [127, 128], and later in male MR patients due to a duplication of a small region at Xq28 including the *MECP2* gene [114]. *MECP2* is expressed in many tissues but shows highest levels in postmitotic neurons. It specifically binds to methylated CpG dinucleotides at promoters, where it forms a complex that includes SIN3A, HDAC1 and HDAC2 [6]. This co-repressor complex is thought to reorganize the adjacent chromatin environment with subsequent repression of transcription. Although MeCP2 can interact with several other components of HDAC complexes, the link between histone deacetylation and MeCP2-mediated transcriptional repression is not yet clear. Similarly, the function of MeCP2 in establishment of histone methylation and its role in HMT complexes remains unknown. In any case, several studies suggest that MeCP2 contributes to the establishment of various epigenetic marks and subsequently, a role in transcriptional repression (reviewed in [129])

A growing number of genes are being identified for which the expression is altered in brains of *Mecp2* knockout mice or Rett patients. These are *BDNF* [130, 131], *UBE3A* and *GABRB3* [132, 133], *Sgk1* and *Fkbp5* [134], the *DLX5/DLX6* imprinted gene cluster [135], and very recently, overexpression of *Uqcrc1* [136] as well as all four inhibitors of differentiation (*ID1–ID4*) [137] have been reported in *MECP2* deficient mice. Moreover, lower levels of several dendrite-specific and synaptic proteins have been demonstrated in brain extracts of Rett patients [138, 139]. The contribution of each of these alterations to the disease state however, has yet to be established.

ATRX syndrome

Mutations in the *ATRX* (alias *XNP*) gene at Xq21.1 are rather frequent and can result in a broad spectrum of clinical phenotypes of which most include severe mental retardation and a variable degree of α-thalassaemia. ATRX is a large nuclear protein that contains an N-terminal zinc

finger domain and seven helicase motifs at its C-terminus. These motifs resemble the helicases found in the SWI/SNF subgroup of the Tritorax group of proteins that are involved in chromatin remodeling [140]. Therefore, it is not surprising that ATRX interacts with other proteins involved in this process such as heterochromatin protein 1 (HP1), the Polycomb group protein Enhancer of Zeste 2 (EZH2) [141–143], and the transcription cofactor DAXX, for which the complex displays chromatin remodeling activities [143]. ATRX localizes at heterochromatic regions at the pericentromeres and the short arm of acrocentric chromosomes, supporting a role in chromatin condensation. Finally, methylation differences have been reported in patients with ATRX syndrome. These differences might disturb the normal chromatin organization near these loci [144]. In *Atrx* knockout mice, changes in DNA methylation patterns are also reflected in the presence of an abnormal X-inactivation pattern in extra-embryonic tissue [145].

Coffin-Lowry syndrome

The Coffin-Lowry gene, *RSK2* (alias *RPS6KA3*) at Xp22.12, is a serine/threonine kinase of the 90 kDa ribosomal protein S6. Mutations in this gene mostly result in severe MR associated with other major characteristics like skeletal anomalies [146]. The severity seems to be correlated with specific mutations in this gene. Unlike most other X-linked disease genes, mutations in *RSK2* do not result in skewed X-inactivation leading to clinical features in female carriers too. The relation of RSK2 with chromatin remodeling is evident from its requirement for the EGF-mediated phosphorylation of histone H3 [147, 148] and its interaction with CBP [149]. The pleiotrophic role of the CREB/CBP pathway has been described earlier in this review. Finally, it was demonstrated that RSK2 is part of an active preinitiation complex at the collagenase promoter that also consists of the methyltransferase SET9 and the SWI/SNF protein Brg-1 [150].

Other XLMR genes

A presumed role in chromatin remodeling was suggested for other X-linked genes involved in cognitive functioning based on the similarity with other known proteins or the presence of specific domains known to be implicated in these processes. These include the *JARID1C* gene, which contains a plant homeodomain (PHD) also found in ATRX as well as several other DNA-binding motifs [151]. Several genes of the Krüppel-type zinc finger protein family (*ZNF41*, *ZNF81*, *ZNF674*) have been related to XLMR [152–154]. All members of this family contain a KRAB domain that binds KAP-1 and subsequently recruits HP1 in a complex including HDAC3 and SETDB1 [155, 156], which is highly suggestive for an epigenetic function. Finally, *BCOR*, mutated in a syndromic form of MR is a key regulator in transcription during embryogenesis and is thought to have a role in histone acetylation and chromatin remodeling [157].

An overview of cognitive disorders that result from epigenetic modifications is given in Table 1.

Epigenetic analysis to detect novel XLMR genes

As mentioned above, we believe that part of the unexplained cases of the XLMR population will result from modifications of the normal epigenetic profile at specific loci in the genome. The identification of these critical alterations can now be achieved through a variety of recent techniques. For a more comprehensive overview of the available methods in this field see other excellent reviews [3, 28, 158, 159].

These modifications should be a direct or indirect consequence of mutations that are present somewhere on the X chromosome but are difficult to trace because of current technical limitations. Such mutations might result in an altered expression of a known or unknown XLMR gene. Direct expression profiling of these genes in EBV-transformed peripheral blood lymphocytes (EBV-PBLs) by microarray however, has so far not been shown to be a reliable method to detect changes in mRNA levels (unpublished data) because of the very low to undetectable expression levels of many XLMR genes in this cell type. Therefore, an alternative indirect way to define expression changes could be to measure DNA methylation and histone modification [6, 160, 161] changes at promoter regions. We therefore hypothesize that

Table 1 Cognitive disorders that result from epigenetic modifications.

Disease	OMIM	Gene	Chrom band	Function
Schizophrenia	181500	*RELN*	7q22	Extracellular matrix protein at synapses
Rubinstein-Taybi Syndrome	180849	*CBP*	16p13.3	Transcriptional co-activator
Alzheimer	104300	*APP*	21q21	Transmembrane protein in brain
CHARGE Syndrome	214800	*CHD7*	8q12.1	ATP-dependent chromatin remodeling
Fragile-X Syndrome	309550	*FMR1*	Xq27.3	Regulator of translation at synapses
Rett Syndrome	312750	*MECP2*	Xq28	Binds to methylated CpGs to repress genes
Coffin-Lowry Syndrome	303600	*RSK2*	Xq21.1	Serine-threonine protein kinase; interacts with CBP
ATRX Syndrome	301040	*ATRX*	Xp22.12	DNA-binding helicase; chromatin remodeling
Mental retardation	607001	*EHMT1*	9q34.3	Histone methyltransferase
Mental retardation	300534	*JARID1C*	Xp11.22	Role in chromatin remodeling (?)
Mental retardation	314995	*ZNF41*	Xp11.1	Transcriptional regulator (?)
Mental retardation	314998	*ZNF81*	Xp11.1	Transcriptional regulator (?)
Mental retardation	300573	*ZNF674*	Xp11.1	Transcriptional regulator (?)
Mental retardation	300485	*BCOR*	Xp11.4	Transcriptional regulator during embryogenesis

*OMIM: number in the 'Online Mendelian Inheritance in Man' database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM&cmd=Limits).

analysis of epigenetic alterations in EBV-PBLs can be representative for the situation in brain, but this still needs to be firmly validated. In this respect, only few relevant studies have been published. As discussed in the section 'Fragile X syndrome', these reports demonstrated that the epigenetic marks of DNA methylation as well as histone acetylation and methylation in EBV-PBLs from Fragile X patients are indeed representative for a strong reduction of *FMR1* mRNA expression in brain with a subsequent altered spine shape and density [118, 119, 121]. In Rett syndrome patients, distinctive profiles of histone acetylation and methylation were found in PBLs, compared to controls. Moreover, these alterations seemed to correlate better with the phenotypic outcome when compared to the location of the mutation in the *MECP2* gene [162].

An epigenomic profile of a patient can be investigated at specific loci by PCR-based approaches, or at the whole genome level when combined with microarray technologies (ChIP-on-chip). Towards this end, highly specific antibodies against methylated (Me) or acetylated (Ac) residues of histone H3 and H4 have been developed [163]. By generating high density arrays it is now possible to fine map methylation at high resolution (reviewed by [164–166]. With these profiles, bioinformatic tools should allow to deduce the underlying defective (X-linked) gene. It is anticipated that some of the epigenetic changes will affect known XLMR genes so a particular focus will have to be at promoters of these genes. The selection of patients with idiopathic MR will be complemented with MR patients with apparently balanced translocations or inversions for which no candidate MR gene seems to be present. Indeed, epigenetic effects on the regions flanking the chromosomal alteration might disturb the local epigenetic environment of genes.

Conclusions

Our current understanding on epigenetic mechanisms has resulted in the identification of several disease-associated modifications of the epigenome predominantly in DNA methylation, and histone acetylation and methylation, resulting in altered chromatin condensation and a subsequent altered transcription. Comparison of the epigenomes of normal individuals with those of MR patients will help to uncover novel genes or mechanisms that result in memory deficits. However, data should be interpreted with caution. As recently has been demonstrated for the large genetic and genomic variability in the normal population, epigenetic polymorphisms are expected to substantially extend this natural variability. Till recently, it was assumed that most polymorphisms were single nucleotide substitutions (SNPs) [167], but in addition to this many deletions, duplications, amplifications and inversions (5 to 500 kb) have been observed [168–171]. It is anticipated that variability at each layer of the epigenetic profile is present making the interpretation of data very difficult especially for modest changes. Epigenetically modified loci or regions that are also found in healthy individuals most likely represent epigenetic polymorphisms. No such modifications have been described so far but a database that catalogs this variability is a requirement for efficient identification of disease-related changes.

The few congenital diseases described here that have been associated with epigenetic modifications, directly or indirectly, most probably constitute only the tip of the iceberg. For example, several other histone modifications exist, which might equally affect recruitment or binding of chromatin structure regulating proteins. In the next decade, an increased knowledge in this field as well as the development of new techniques to study it will boost the detection of new epigenetic links with diseases. This knowledge might have a great impact on novel therapeutic drug discovery or strategies that might be able to significantly ameliorate, prevent or even reverse the clinical outcome of MR patients.

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