Transcription regulation in time and space: Engineered cell systems to modulate the epigenetic chromatin structure: The role of Methyl-CpG-binding protein 2

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Publication date
2016

Document Version
Final published version

Chapter 3

The balancing act of MeCP2: A multi-tasking protein

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Abstract

Methyl CpG binding protein 2 (MeCP2) contains several binding domains and it is known to exhibit chromatin remodeling activities. Depending on its folding the MeCP2 protein binds to specific DNA target sites and attracts several different proteins triggering a variety of functions in the nucleus. MeCP2 does not contain a catalytic domain but it typically is able to guide regulatory proteins to its DNA target sites. Genome-wide transcription is coordinated by the complex interplay of proteins that directly or indirectly interact with DNA to form the chromatin fiber. This network of interactions underlies the cell type specific epigenetic chromatin composition of the genome. MeCP2 plays an important role as epigenetic 'reader protein' in chromatin-related gene expression regulation having a strong affinity for methylated CG dinucleotides and MeCP2 exhibits chromatin remodeling activities via MeCP2 bound epigenetic regulatory proteins. Mutations in the MeCP2 coding sequence or MeCP2 duplication as observed in neurodevelopmental disorders such as Rett syndrome and Xq28 duplication syndrome affect the network of chromatin-related interactions thereby altering gene expression regulation.

MeCP2 is ubiquitously expressed in somatic cells and particularly enriched in the brain. Throughout the years many different functions of MeCP2 have been discovered, illustrating the functioning of MeCP2 in the nucleus specifically in brain cells. Here, we provide a chronological overview of the discoveries regarding MeCP2 molecular (dys)functioning. Moreover, we discuss steady state MeCP2 binding to DNA or regulatory proteins as a result of MeCP2 mutations and duplication considering an altered MeCP2 binding or MeCP2 cellular concentration.

Introduction

Folding of the eukaryotic genome into higher-order chromatin structures and gene expression patterns are tightly related with each other (Li et al., 2007). The genome is partitioned into distinct functional chromatin domains delineated by regulatory elements that help to assure cell type-specific gene expression (Bodnar and Spector, 2013; Ernst

and Kellis, 2010; Gierman et al., 2007; Noordermeer and Duboule, 2013; van Steensel, 2011; Yadon et al., 2013). Gene expression is coordinated by the complex interplay of proteins that modulate the epigenetic chromatin composition (e.g. DNA methylation and histone modifications). The affinity of chromatin binding proteins is determined by their interactions with specific histone modifications, DNA binding proteins and co-regulators. Vice versa, the location of histone modifications is modulated by the DNA sequence and the presence of chromatin binding proteins. CpG binding proteins such as methyl CpG binding protein 2 (MeCP2) are typical proteins that provide a link between histone modifications and DNA methylation (Nan et al., 1998b).

MeCP2 is a multifunctional protein exhibiting chromatin remodeling properties. MeCP2 is ubiquitously expressed and abundantly present in mature neuronal brain cells (Skene et al., 2010; Song et al., 2014). Moreover, MeCP2 is present in normal brain in all types of glia, including astrocytes, oligodendrocyte progenitor cells and oligodendrocytes. Mutations in the MeCP2 gene located on the X chromosome give rise to the postnatal neurodevelopmental disorder known as *Rett syndrome*. Rett syndrome is a rare genetic neurological disorder almost exclusively affecting girls. Rett syndrome at first does not show large phenotypic changes in affected children, but 6 - 12 months after birth the second stage of the disorder manifests itself through rapid regression of neuronal and motor functioning which stabilizes when children are around 4 years old. After a long plateau phase of the disease the final disease phase is recognized by severe motor dysfunctioning (Neul and Zoghbi, 2004). Duplication of the *MeCP2* gene inducing upregulation of cellular MeCP2 levels causes another neurodevelopmental disorder, named *Xq28 duplication syndrome* showing a disease outcome that is very similar to Rett syndrome. Rett syndrome symptoms starting gradually during childhood include loss of purposeful hand use, slowed brain and head growth, movement and breathing problems, seizures, and intellectual disability (Anderson et al., 2014; Neul and Zoghbi, 2004). Xq28 duplication syndrome distinguishes itself from Rett syndrome by the prompt development and the recurring infections. Similarities in Rett and Xq28 duplication syndrome symptoms include aberrant breathing, gastroesophageal reflux, hypotonia, epilepsia, speech problems and disability to walk (Anderson et al., 2014; Van Esch, 2012).

MeCP2 is expressed in brain cells, both in neuronal cells and also in glial cells that support and exhibit protection for the nervous sytem. Recent studies indicate that in MeCP2 deficient cells showing a Rett syndrome phenotype, MeCP2 is not expressed in astrocytes, in contrast to its expression in wild type mice. It is noted that neuronal growth is compromised and that the morhopholy and density of neurons is decreased, when astrocytes from MeCP2 deficient mice are co-cultured with hippocampal neurons from wild type mice (Ballas et al., 2009). The authors showed that upon restoring the expression of MeCP2 in astrocytes from MeCP2 deficient mice caused a non-cell-autonomous growth inducing effect on neurons from MeCP2 deficient mice thereby re-establishing the dendritic morphology and reducing Rett syndrome symptoms of the mice such as abbarent breathing (Lioy et al., 2011).

The history of observations regarding MeCP2-related disorders started in the early 50's last century when the Austrian physician Andreas Rett (1924-1997) first encountered patients with a not yet described postnatal developmental disorder. It took at least a decade before Andreas Rett published his findings. The first molecular biological questions regarding Rett syndrome were not answered until Adrian Bird discovered the MeCP2 protein in 1992 (Meehan et al., 1992; Nan et al., 1993), although the link between MeCP2 and Rett syndrome was discovered in 1999 (Amir et al., 1999). Since this discovery alterations in MeCP2 functioning as a chromatin remodeling protein were reflected against downstream neuronal and muscle-related Rett syndrome symptoms.

In this review, we provide an overview of research findings related to the functioning of MeCP2 and MeCP2 involved disorders (see Table 1). We outline the DNA binding sites and protein binding partners of MeCP2 indicating their relationship with molecular effects of MeCP2 (dys)regulation within MeCP2-related neurological disorders. In this context, we provide a minimal model-representation showing the affinity of MeCP2 binding and its bound functional fraction (Figure 1).

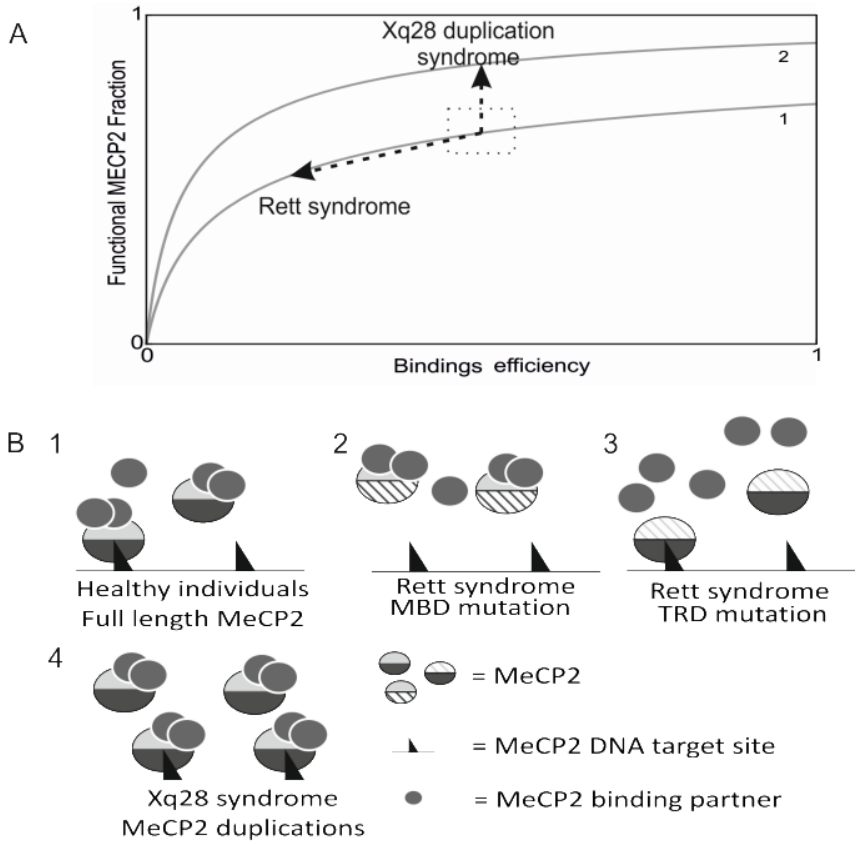

Figure 1: Effects of MeCP2 binding properties.

A) This figure provides the model-representation of the steady-state behavior of MeCP2 binding as a result of MeCP2 binding parameters and MeCP2 concentration. In Rett syndrome the MeCP2 functional bound fraction is decreased due to the lower binding efficiency (lower k_b) of MeCP2 at DNA target sites or at MeCP2 protein binding partners. In Xq28 duplication syndrome an increase in the amount of MeCP2 (relative to the amount of target DNA), causes an increase in the MeCP2 functional bound fraction.

B) The cartoon illustrates the MeCP2 bound fraction for different MeCP2 binding efficiencies. 1. In individuals without Rett or Xq28 syndrome, full length MeCP2 binds its DNA targets and MeCP2 binding proteins allowing the formation of a functional MeCP2 bound fraction. Since protein-protein and protein-DNA binding is a dynamic process not all sites are continuously occupied. 2. In Rett syndrome MBD or TRD mutations both interfere with the formation of a functional MeCP2 bound fraction (i.e. binding of MeCP2 to DNA target sites and to protein binding partners) compared to the situation in individuals carrying full length MeCP2. 3. In Xq28 syndrome, MeCP2 duplication induces an increase in the amount of MeCP2 (relative to the amount of target DNA), thereby causing an increase in the MeCP2 functional bound fraction.

MeCP2 is a multifunctional protein with multiple binding sites

Nowadays MeCP2 is accepted to represent a multifunctional protein that can exhibit different functions e.g. the ability to repress as well as to activate gene expression (Chao and Zoghbi, 2009). MeCP2 is a protein that can bind to DNA and that is able to bind a variety of regulatory proteins. MeCP2 is reported to represent a rather unstructured protein, implicating that a large part of MeCP2 can adapt a different 3D protein structure depending on its binding partners. Only 35-40% of the MeCP2 protein represents a structured part i.e. parts of the methyl binding domain (MBD) and an AT-hook domain in the transcription repression domain (TRD) (Adams et al., 2007; Martı et al., 2014). The rest of the MeCP2 protein including the C-terminal part are proposed to represent the 'disordered' part of the protein exhibiting structural folding depending on the association with defined proteins or posttranslational protein modifications (Adams et al., 2007). MeCP2 binds with its MBD to DNA (Nan et al., 1993), having affinity for both methylated and non-methylated CG dinucleotides (CpGs) and DNA in general. The MeCP2 MBD largely overlaps with a nuclear domain that is suggested to represent a matrix attachment binding region (MAR) that is supposed to be part of a nuclear cytoskeleton or scaffold. MeCP2 binds with its TRD to proteins involved in transcription regulation thereby either turning genes on or off. The C-terminus of MeCP2 is theoretically shown to cover several binding sites of which at least two have been proven experimentally (see Table 1) (Buschdorf and Strätling, 2004; Dintilhac and Bernués, 2002). The C-terminal domain is known to facilitate both binding of MeCP2 to DNA and to contain a WW domain that is predicted to play a role in protein-protein interactions (Buschdorf and Strätling, 2004).

MeCP2 has increased affinity for methylated compared to non-methylated C nucleotides (Ghosh et al., 2010b). Moreover, the enrichment in A and T nucleotides adjacent to methylated CpGs is known to involve high-affinity binding of MeCP2 to DNA target sites (Klose et al., 2005). The binding of MeCP2 to hydroxymethylated C nucleotides includes another level of complexity in MeCP2 functioning. Several assays showed that the affinity of MeCP2 for hydroxylated and non-methylated C nucleotides is similar. Moreover, an A nucleotide following a hydroxymethylated C nucleotide has been noted to provide high

affinity MeCP2 binding (Kinde et al., 2015; Mellén et al., 2012). Hydroxymethylation is often a result of oxidative damage, possibly hampering the binding of MeCP2 (Kinde et al., 2015; Valinluck et al., 2004). Kinde et al. showed that MeCP2 also binds to both hydroxymethylated and methylated CA dinucleotides. The genomic profile of methylated CG, AC or hydroxymethylated CG dinucleotides has important implications to understand the functional role of MeCP2 at methyl and hydroxymethyl-specific binding sites. Methylated CA dinucleotides largely parallel the profile of methylated CG dinucleotides across genomic elements, including intergenic regions, regulatory elements of inactive genes, promoters and gene bodies of lowly expressed genes and repetitive DNA sequences,. Hydroxymethylated C nucleotides typically demarcate active regulatory elements, i.e. hydroxymethylated CG dinucleotides are preferentially enriched throughout gene bodies of highly expressed genes and are depleted from transcriptional start sites (Lister et al., 2013). In neuronal cells hydroxymethylation is suggested to represent an intermediate epigenetic state marking sites of subsequent CG demethylation (Guo et al., 2011; Lister et al., 2013). Hydroxymethylation is considered to also denote a stable epigenetic state, playing a role in learning, memory, neurogenesis and neuronal activity-regulated gene expression.

MeCP2 is a protein that has a large set of binding partners. In Figure 2 we highlight MeCP2 DNA and protein binding partners indicating the multifunctional role of MeCP2. Identifying downstream molecular and cellular targets that are affected by MeCP2 (dys)functioning will help to interpret the role of MeCP2 in brain development and maintenance of brain function.

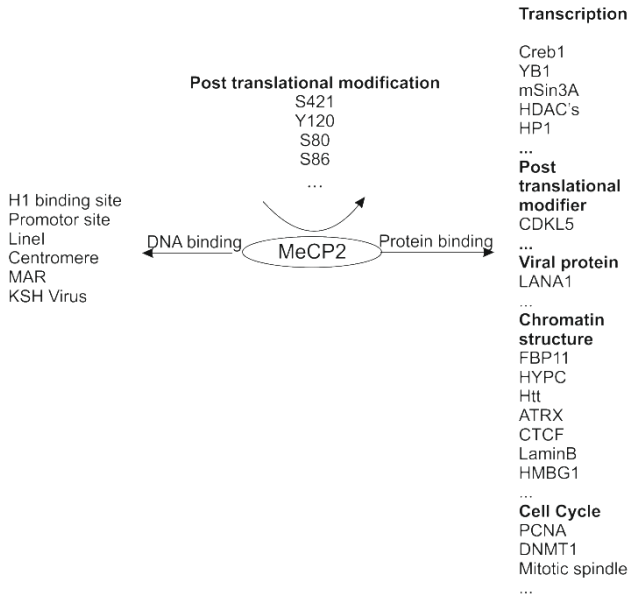

Figure 2. MeCP2 is a multi-functional protein.

MeCP2 has a directing functionality on regulation of the chromatin structure, it binds various DNA targets with its MBD and recruits with its TRD regulatory proteins towards MeCP2 bound DNA target sites. MeCP2 binds to proteins that involve various functionalities, i.e. transcription regulation, chromatin structural changes, cell cycle regulation and even viral activities. MeCP2 is a disordered protein that gains its functionality upon posttranslational modifications and protein binding.

MeCP2 dysfunctioning: an altered balance

A large body of evidence indicates that mutations in chromatin remodeling or chromatin structural proteins are related to neurological disorders (Lv et al., 2013). Examples of such neurological disorders are mutations in ATRX causing *Alpha-thalassemia mental retardation syndrome* (Ratnakumar and Bernstein, 2013), mutations in cohesion proteins such as SMC leading to *Cornelia the Lange syndrome* (Revenkova et al., 2009), CDKL5 mutations giving rise to clinical features similar to those noted in Rett syndrome, also being diagnosed as *West syndrome* and *X-linked infantile spasms* (Kato, 2006)**.** Moreover, several other duplicated loci show a correlation with chromatin structural alterations and neurological-related disorders such as *autism spectrum disorders* (ASD) and/or *schizophrenia* (Nomura and Takumi, 2012). Mutations and amplification in the coding sequence of MeCP2 give rise to Rett syndrome and Xq28 duplication syndrome,

respectively. A wide variety of MeCP2 mutations causes a more severe or milder Rett syndrome disease outcome largely depending on the effect of the mutation on the MeCP2 protein structure. Table 1 shows a historical overview of the research related to an (altered) functioning of MeCP2 involving Rett syndrome, Xq28 duplication syndrome and other MeCP2-related disorders. Note that some recent studies envision a role for MeCP2 that is contradicting MeCP2-related findings of earlier days.

More than 2000 MeCP2 mutations have been reported in females with Rett syndrome. Eight common MeCP2 mutations are known to result in loss of function due to truncated, unstable or abnormally folded MeCP2, whereas more recently, large MeCP2 rearrangements including deletions were reported (Amir et al., 1999; Bienvenu and Chelly, 2006). MeCP2 mutations affect the affinity of MeCP2 for its binding partners. Structural analysis of MeCP2 mutations at defined sites of the TRD show an improper formation of the AT-hook of the TRD. These AT-hook TRD MeCP2 mutations can still bind DNA, although transcriptional patterns are changed, indicating that the MeCP2 protein is not properly functioning. These TRD MeCP2 mutations are known to produce a severe Rett phenotype and to disrupt a large part of the structural ordered part of MeCP2. MeCP2 mutations just outside the AT-hook TRD are known to cause a mild Rett phenotype suggesting that TRD mutation-induced alterations in MeCP2 folding play a role in the disease outcome (Baker et al., 2013). It has been noted that the R113C MeCP2 mutation in the MBD disrupts H-bonds required for MBD DNA target binding. This R113C MeCP2 mutation is known to induce only a mild form of Rett syndrome (Kucukkal and Alexov, 2015), whereas MeCP2 gene duplication as noted in Xq28 duplication syndrome will cause a general increase in the cellular MeCP2 concentration thereby affecting saturation of MeCP2 binding to DNA or regulatory proteins, or possibly inducing a competition with other chromatin binding proteins (Baker et al., 2013; Christodoulou et al., 2003; Fyfe et al., 2003). In the brain MeCP2 is suggested to be required for the reactivity of neuronal responses. In Rett syndrome, MeCP2 is associated with a low density of dendritic spine (Na et al., 2013). In Xq28 duplication syndrome dendritic spine occurs in larger numbers after birth and spine numbers drop a few weeks after birth, although the size of the dendritic spine is smaller compared to the size in healthy mice

(Jiang et al., 2013). Of importance, it is known that introduction of full length MeCP2 in a knock-out Rett syndrome mouse model induces partial recovery from the Rett syndrome symptoms (Guy et al., 2007). This indicates that development of the Rett syndrome phenotype most likely occurs post developmentally, which is of interest for treatment strategies.

Molecular behavior of MeCP2 functional binding

We created a simplified model-representation illustrating the impact of MeCP2 mutations and MeCP2 duplication on the amount of functional MeCP2, i.e. the MeCP2 bound fraction (Figure 1). In this model-representation we simplified the cellular complexity of MeCP2 binding, the cellular MeCP2 distribution, the MeCP2 target site distribution and the MeCP2 binding kinetics, whereas we take into account the concentration of MeCP2, MeCP2 binding sites and two parameters for respectively the binding and dissociation of MeCP2. The ordinary differential equations associated with this model are the following:

$$\frac{dCpG}{dt} = \frac{dMeCP2}{dt} = -MeCP2 \cdot \ CpG \cdot k_a + k_d \cdot MC$$

$$\frac{dMC}{dt} = MeCP2 \cdot \ C \cdot k_a - k_d \cdot MC$$

MC represents the concentration of MeCP2 bound to MeCP2 binding sites, i.e. (non)methylated dinucleotides, hydroxymethylated cytosines, any DNA or MeCP2 binding proteins. K represents the MeCP2 binding constant and k_d the MeCP2 dissociation constant. Since the ratio between the number of MeCP2 proteins and the number of MeCP2 binding sites is unknown, we assume that both variables are equimolar. Different MC ratios do not alter the curve of the plot. In Xq28 duplication syndrome it is expected that somatic and neuronal cells exhibit elevated cellular MeCP2 levels and hence an increased pool of MeCP2 is able to bind DNA target sites or proteins binding sites. In contrast, in Rett syndrome the amount of MeCP2 target binding (either the DNA target site or MeCP2 binding proteins) is lower due to mutations in the MBD, TRD or other parts of the MeCP2 coding sequence. We only take into account MeCP2 point mutations that

affect a change in the MeCP2 coding sequence. Decreased binding affinity of MeCP2 for DNA or MeCP2 binding proteins will induce a drop in the overall amount of functional MeCP2. The model-representation illustrates the impact of an altered MeCP2 binding and an altered MeCP2 concentration on the MeCP2 functional fraction, plotted as the MeCP2 bound fraction. This model-representation indicates that the molecular behavior of the two MeCP2-related neurodevelopmental syndromes are strikingly different although the phenotypic outcome of these disorders shows some overlap.

MeCP2 downstream functioning

As described in Table1 various observations have been made in relation to MeCP2 functioning and MeCP2-related disorders. Here we illustrate some of the MeCP2 downstream functioning involved phenomena and their relationship with MeCP2 related disorders.

MeCP2 related to genome stability, immunity and circadian rhythms

The ability of MeCP2 to bind hydroxmethylated and methylated dinucleotides as well as other genomic sites illustrates the complexity of MeCP2 functioning for neuronal gene expression and hence neuronal development, plasticity and disease. Specially mutations in the MeCP2 MBD affecting the ability of MeCP2 to bind methylated and hydroxymethylated sites will alter gene expression patterning and genomic stability. During replication MeCP2 associates with the replication fork and binds to DNA methyltransferase DNMT1 thereby restoring the DNA methylation pattern after replication (Kimura and Shiota, 2003). Moreover, MeCP2 is known to exhibit DNA methylation-induced silencing of retro-transposable elements such as Line-1 (Muotri et al., 2010), thereby providing genomic stability preventing retro-transposable elements to cause insertional mutagenesis. Of interest, MeCP2 is upregulated in virally infected cells, for instance infected by John Cunningham Virus (Shirai et al., 2011). In this context, MeCP2 is known to upregulate IFNγ cytokine levels in infected cells thereby supporting

innate and adaptive immunity (Yang et al., 2012). MeCP2 has been shown to interact with viral proteins involved in viral latency (Chiang et al., 2013; Matsumura et al., 2010). In this context, HIV-1 can regulate expression of miRNA132 which downregulates the expression of MeCP2 allowing HIV to become viral (Chiang et al., 2013). MiRNA132 also regulates circadian rhythm involved proteins, i.e. histon acetylase activity of CLOCK, and binding of CLOCK to PERIOD genes Per1 and Per2. Moreover, miRNA132 is known to regulate MeCP2 expression and hence expression of Per1 and Per2 via a CREB dependent pathway. Interestingly, MeCP2 is circadianly regulated and circadian MeCP2 levels are noted to have important consequences on the chromatin structure and transcription of MeCP2 dependent genes. It is tempting to propose that MeCP2 is able to facilitate a light-induced CREB-dependent circadian transcription regulation. Circadian alterations in MeCP2 levels could be responsible for sleep disturbances as noted in autism and Rett syndrome (Alvarez-Saavedra et al., 2011; Martínez de Paz et al., 2015). In conclusion, the MeCP2 domain that binds to DNA affects a wide variety of effects ranging from genome stability, DNA replication, immunity to circadian rythm regulation. It is intruiging to connect these different functional effects of MeCP2 in one regulatory layer.

Posttranslational modifications of MeCP2 as basis for multifunctionality

Although MeCP2 does not explicitly contains a catalytic domain, MeCP2 is demonstrated to contribute to binding and recruitment of other, catalytically active protein complexes. MeCP2 can exhibit both gene activation and repression depending on its genomic and protein binding profile (Tao et al., 2009). MeCP2 posttranslational modifications (e.g. phosphorylation at S80 and de-phosphorylation at S421) largely influence the genomic and protein binding profile of MeCP2 and thereby its biological functioning (Bellini et al., 2014). S421 phosphorylation occurs in response to neuronal activity, mutations at S421 influence dendritic growth and regulation of genes involved in neuronal connectivity. MeCP2 S421 phosphorylation is suggested to be specifically connected to neurodegenerative features of Rett syndrome and Xq28 duplication syndrome (Zhou et al., 2006). Moreover, S80 phosphorylation is noticed to be associated with an increased

MeCP2 binding at promoters of a set of genes, e.g. Rab3d, Vamp3 and Igsf4b, with a reduced transcriptional activity of these genes and with a rested neuronal state. Deficient S80 phosphorylation exhibits reduced MeCP2 binding and increased transcription of Rab3d, Vamp3 and Igsf4b. SCDKL5 is a protein that is known to phosphorylate MeCP2. Intriguingly, mutations in CDKL5 lead to a syndrome (West syndrome) often diagnosed as Rett syndrome showing disease symptoms that are very comparable to Rett syndrome symptoms (Weaving et al., 2004). Alteration in posttranslational modification states of MeCP2 has been connected with MeCP2 nuclear location and functioning (Bergo et al., 2014). It has been noticed that phosphorylation of Y120 is associated with an accumulation of MeCP2 at the centrosome and the mitotic spindle regulating cell growth and cytoskeleton stability. Y120 is located within the MeCP2 MBD domain and it is a highly conserved site in mammals. A patient affected by a variant form of Rett syndrome was found to harbor a MECP2 mutation, possibly mimicking a constitutively phosphorylated Y120 state. This mutation was shown to cause decreased affinity of mutated MeCP2 at compact chromatin domains causing deficient spindle morphology, microtubule nucleation and prolonged mitosis. These studies indicate that posttranslational MeCP2 modifications play a major role in the ability of MeCP2 to interact with its DNA and protein partners thereby regulating functional activities of MeCP2.

MeCP2 and its opposite functionality: transcriptional activity versus repression

MeCP2 is described to be able to recruit transcription regulatory proteins that are associated with opposite effects. The MeCP2 TRD recruits proteins that exhibit histone deacetylation transcription repressive activities such as NCor/HDAC3 and Sin3a corepressor complexes (Nan et al., 1997, 1998b). On the other hand, the MeCP2 TRD recruits Creb1 protein and Creb1 binding partners causing transcriptional activation instead of repression (Tao et al., 2009). Creb1 is a protein that binds a cAMP response element, a nucleotide sequence present in many viral and cellular promoters causing gene activity (Chao and Zoghbi, 2009). MeCP2 has been shown to interact with YB1, a

protein involved in alternative RNA splicing (Young et al., 2005). An interaction of MeCP2 with gene body DNA methylation is shown to induce alternative splicing thereby excluding methylated exons from the synthesized transcripts (Lev Maor et al., 2015). This abillity of MeCP2 to cause splicing of methylated exons might represent a 'repressive' action on actively transcribed genes. Moreover, MeCP2 is proposed to play a role in chromatin folding as it binds chromatin remodeling proteins such as Brahma and ATRX. The best evidence of the involvement of MeCP2 in chromatin structural changes is based on chromatin capture analysis of the imprinted Dlx5/Dlx6 locus implicating a role for MeCP2 in silent chromatin loop formation. The N-terminal domain of MeCP2 has been shown to bind Heterochromatin protein 1 (HP1) (Agarwal et al., 2007) a protein that is typically associated with the formation of transcriptionally silent chromatin as it binds with its chromodomain to histone H3 lysine 9 tri-methylation (H3K9me3) and recruits with its chromoshadow domain histone methyltransferase enzymes (Verschure et al., 2005). During mouse myogenesis cellular MeCP2 levels are largely increased and an abundant colocalization of MeCP2 together with HP1 (especially HP1γ) is noted at largely clustered and compact MeCP2 stained chromatin, i.e. chromocenters (Agarwal et al., 2007). We recently showed that MeCP2 targeting induced chromatin unfolding and that this chromatin unfolding is related with displacement of HP1γ from compact chromatin (Brink et al., 2013). These examples illustrate that MeCP2 can evoke an opposite effect on the chromatin composition and the transcriptional state. In a recent study a novel high-resolution microscopy imaging approach is used to compare the chromatin structure in neuronal tissues from MeCP2 null mice compared with tissues in wild type mice (Linhoff et al., 2015). It was noted that the chromatin of MeCP2 null cells represents a more compacted chromatin structure and a redistribution of various histone modifications related to pericentromeric heterochromatin was noted in cells of MeCP2 null cells. The chromatin structural changes in cells from MeCP2 null versus wild type MeCP2 mice is not observed in all neuronal cells, suggesting cell-type specific chromatin structures. For instance during myogenesis it was noted that MeCP2 caused compaction of chromocentres, suggesting that these events enable these cells to fullfill their specific tasks during development. MeCP2 functionality might act as a key facilitator to ensure

correct chromatin structural functionalities that are needed for cell-specific and moment speciic neuronal tasks, i.e. tasks during neuronal development or neuronal maintenance provided by proper interactions between neuronal and glia cells.

Competition of MeCP2 with nuclear binding proteins impacts nuclear integrity

The increased cellular MeCP2 levels noted in Xq28 duplication syndrome are expected to largely affect the binding efficiency of chromatin binding proteins that are seemingly unrelated to the functioning of MeCP2. For instance it has been shown that the naturally observed binding competition between MeCP2 and histone H1 at MeCP2 binding sites is lost in MeCP2 knockout mice causing a general upregulation of histone H1 levels (Skene et al., 2010). In principle histone H1 upregulation might represent a compensation for the lack of functional MeCP2 to evoke a total collapse of the nucleosomal chromatin structure.

Incorrect assembly of the nuclear envelope together with a decreased proliferation and cellular viability represents another cellular phenotype of cells in which MeCP2 is knocked down (Babbio et al., 2012). This fits well with the role of MeCP2 to interact via its MBD with Lamin B receptor (LBR) located at the nuclear periphery (Guarda et al., 2009; Nan et al., 1993; Weitzel et al., 1997). Nuclear lamins are major components of the lamina playing a role in maintaining nuclear envelope integrity, orchestrating mitosis and apoptosis, regulating DNA replication and transcription and providing chromatin domain anchoring sites.

Summary

MeCP2 is a multifunctional protein consisting of at least 3 binding domains without a typical catalytic domain. MeCP2 plays an important role in altering the binding affinity of regulatory proteins for their chromatin or DNA binding sites. Moreover MeCP2 plays an important role in maintaining a correct DNA methylation profile of importance for proper chromatin folding, transcription patterning and genomic stability. MeCP2 is able to facilitate gene expression programs at two levels. MeCP2 acts as a key regulator of

chromatin structure by preventing the formation of compact chromatin (Linhoff et al., 2015; Schotta et al., 2004) and by competing with binding of histone H1 at methylated nucleosomal linker chromatin (Ghosh et al., 2010a; Skene et al., 2010). Moreover, MeCP2 functions as a transcriptional regulator either by recruiting co-repressor (Lyst et al., 2013; Nan et al., 1998b) or co-activator proteins (Chahrour et al., 2008; Mellén et al., 2012) i.e. having an opposing 'Yin-Yang' regulatory effect, which is subject to the phosphorylated state of MeCP2. The type of functional activity of MeCP2 seems to depend on its protein structure that is for a large part intrinsically disordered. MeCP2 is involved in providing nuclear integrity and hence nuclear functionality such as replication, transcription, mitosis and genome stability. Disturbed binding of MeCP2 is proposed to have a crucial effect on the ability of MeCP2-bound catalytic proteins to find their correct target sites. MeCP2 plays an important role in facilitating genome structural functionalities. The dual functionality of MeCP2 helps to assure long-lived neurons to adapt their gene expression programs upon exposure to changed conditions such as an altered synaptic activity or injury.

Interestingly, Rett and Xq28 duplication syndrome show large similarities in their disease symptoms. We show with a model-representation that Rett and Xq28 duplication syndrome exhibit very different molecular behavior. In Xq28 duplication syndrome the relative increase in the amount of cellular MeCP2 levels compared to the amount of MeCP2 binding sites causes an increased MeCP2 functional bound fraction, whereas in Rett syndrome the MeCP2 functional bound fraction is lower due to a decrease in the binding efficiency of the mutated MeCP2 protein.

MeCP2 facilitates chromatin functioning by its ability to bind to DNA target sites and to bind to catalytic active proteins. The dynamics of MeCP2 DNA or protein interactions has large impact on MeCP2 functioning. Almost all MeCP2 mutations lead to neurodegeneration, although only one phosphorylation site, up until now, is identified to be involved in neuronal connectivity. In general after depolarisation of the neuronal membrane, MeCP2 becomes phosphorylated at S421 and binds to its target genes such as BDNF in order to regulate dendritic spine density (Mellén et al., 2012; Zhou et al., 2006).

Following the model of functional binding as depicted in Figure 1B, a Rett syndrome mutation in the MBD will hamper binding of MeCP2 to its target genes and will therefore alter the MeCP2-induced target gene regulation. A Rett syndrome mutation in the TRD will prevent MeCP2 to bind to its protein binding partners required to change the transcriptional activity of its target gene, eventhough MeCP2 would have been phosphorylated upon the depolarisation. In principle a mutation in any of the MeCP2 domains will result in improper regulation of denditric spine density. In duplication syndrome, the binding affinity of MeCP2 for its target sites is expected to increase and this will result in more, but smaller dendritic spines. Although MeCP2 related diseases have a different molecular backgroundthe different MeCP2 mutations seem to induce the same kind of symptoms possibly all due to the changes in denditric spine density.

Clinical treatment of MeCP2-related disorders is not trivial since MeCP2 is involved in many different pathways. Treatment of MeCP2 related disorders typically concentrates on distant disease symptoms such as seizures and lung infections and also on treatment of symptoms related to deficiency in neuronal development such as dendritic spine formation and neuronal connectivity (Xu et al., 2014) for more details, see Gadalla et al (Gadalla et al., 2011). Regarding a more direct treatment of MeCP2, mice studies showed that replacing mutated MeCP2 with full-length MeCP2 is able to recover the Rett syndrome phenotype. Moreover, it was recently established that systemic delivery of MeCP2 cDNA in female mouse models rescues behavioral and Rett syndrome cellular symptoms (Garg et al., 2013). In this context, introduction of MeCP2 non-coding siRNA posttranslationally downregulating MeCP2 levels could provide a means to temper MeCP2 production in Xq28 duplication syndrome. Restoring correct MeCP2 functioning in glia could play an important role in ameliorating robust features Rett syndrome symptomes. Identifying key molecules to restore MeCP2 expression in glia may provide further clues in the mechanism of recovery, providing potential targets for therapeutic intervention. There is still a long road ahead to realize direct MeCP2 targeted treatments in humans and especially in diseased children.

Table 1: The history of MeCP2 functioning

Chronological overview of different findings on MeCP2 molecular functioning

Year and Reference	Description of findings
1966 (Rett, 1966)	Without any knowledge of the MeCP2 gene the Austrian physician Rett discovered a syndrome in girls. This syndrome is named **Rett syndrome**. Until today no cure for Rett syndrome has been discovered. Rett syndrome treatment includes treatment treatment of disease symptoms such as seizures and aberrant breathing.
1992 - 1993 (Meehan et al., 1992; Nan et al., 1993)	In the early 90's the research team of Adrian Bird discovered a new protein referred to as methyl-CpG-binding protein 2 (MeCP2). MeCP2 is characterized to **bind methylated CG dinucleotides** (CGs). MeCP2 contains a **methylated DNA binding domain** (MBD) being part of the MBD protein family. MeCP2 is described to bind in vitro to single methylated CpGs requiring symmetrical methylation. Moreover, MeCP2 is reported to exhibit no association with hemi-methylation or methylation at CpG islands. MeCP2 is also characterized to have a domain that binds the DNA helix in the large groove.
1997 (Weitzel et al., 1997)	The MeCP2 (rat) MDB is shown to exhibit a **matrix attachment region** (MAR). A homologue of MeCP2 has previously been described in chickens, the ARBP protein also having a MAR. MARs are supposed to attach to a nuclear matrix of scaffold region that would belong to a nuclear cytoskeleton. This MAR binding is the first indication of MeCP2s contribution to nuclear and chromatin structure as described later in more detail when studying MARs.
1997-98 (Nan et al., 1997, 1998b)	The **transcriptional repressive function** of MeCP2 is described to be located in the **transcription repression domain** (TRD). This TRD is shown to interact with Sin3a thereby forming a repressive complex attracting histone deacetylases (HDACs). Besides the attraction of a repressive complex, MeCP2 is noted to compete with histone H1, thereby interfering with the chromatin structure.
1999 (Lubs et al., 1999)	A disease characterized as **X-linked mental retardation** is shown to exhibit **duplication of the distal arm of the X chromosome.** The disease shows besides neurological deterioration an increase in pulmonary infections. Three months later the presence of MeCP2 at this locus was confirmed.

1999-2000 (Amir et al., 1999; Buyse et al., 2000)	**Genetic Rett syndrome mutations** are discovered for the first time linking MeCP2 to Rett Syndrome**.** A year later a new diagnostic tool is presented making use of DHPLC and direct sequencing. This tool allows **Rett syndrome genetic diagnosis** rather than diagnosing the phenotypic outcome.
2000-present (Christodoulou et al., 2003)	**MeCP2 mutations are not site specific**, i.e. a wide variety of genotypes and phenotypes exist. The international Rett syndrome foundation funded a database where (anonymously) single patient data are stored for open access named 'Rettbase'.
2001 (Guy et al., 2001)	**MeCP2 deficient mice** are developed showing a Rett syndrome phenotype. The first mouse strain containing mutated *MeCP2* under control of a lox-cre site shows the Rett phenotype when mutated MeCP2 is ubiquitously expressed. The original MeCP2 deficient mouse strain developed by the research team of Adrian Bird is named after the Bird-lab.
2001/2002 (Colantuoni et al., 2001; Tudor et al., 2002)	**mRNA profiling of MeCP2 deficient mouse strains** (expressing mutant MeCP2 or MeCP2 knock-out) shows that a large number of genes are affected. The noticed gene expression landscape in the MeCP2 mouse strains was rather unexpected. Since MeCP2 is shown to act mainly as a repressor, it was expected that MeCP2 mutations would mainly cause gene expression upregulation. It is concluded that MeCP2 affects several transcriptional regulators and not only the synaptic pathway that is regressing during late disease development.
2002 (Dintilhac and Bernués, 2002)	MeCP2 is noticed to bind high-mobility group B1 protein (HMGB1) based on a sequence in the MeCP2 C-terminal region. A pull-down assay proved that the **MeCP2 C-terminal domain binds to HMGB1** a protein that binds to histone proteins and recruits other protein complexes to chromatin. HMGB1 is able to bend DNA thereby enabling DNA-protein binding. HMGB1 is ubiquitously expressed in the nucleus and has a multifunctional role in chromatin organization.
2002 (Yntema et al., 2002)	Since the *MeCP2* gene is located on the X-chromosome (Xq28), it is speculated that Rett syndrome only occurs in females because in male MeCP2 mutations are not viable. MeCP2 mutations causing a mild or carrier phenotype in girls are noted to cause **Rett syndrome in males in a more severe phenotype.**
2003 (Kimura and Shiota, 2003)	**MeCP2 is shown to interact with DNMT1 as well as PCNA at the replication fork** during S phase. DNMT1, the maintenance DNA methyltransferase, follows the replication fork and methylates the hemi-methylated new strands immediately. The manner of recognition by

	DNMT1 with the hemi-methylated DNA is not explained.
2004 (Kriaucionis and Bird, 2004; Mnatzakanian et al., 2004)	**A second splice variant of MeCP2, MeCP2a** is identified which contains an extra sequence at the N-terminal of exon 1 and it contains no part of exon 2. This new variant is noted to be expressed in elevated amounts in the brain compared to the earlier described MeCP2. This new variant is named MeCP2a, because it turned out to be more abundant in the brain than the previously described isoform.
2004 (Weaving et al., 2004)	***Cyclin dependent kinase like 5 (CDKL5)* mutations** are described to be **associated with Rett syndrome.** Mutations in CDKL5 involve other autism related disorders.
2004 (Valinluck et al., 2004)	**MeCP2 is found to bind hydroxymethylated CGs**. The affinity of MeCP2 for hydroxymethylated CGs is shown to be lower than the affinity for methylated CGs. Affinity of MeCP2 for unmethylated CGs and hydroxymethylated CGs are reported to be similar.
2004 (Luikenhuis et al., 2004)	**A non-mutated *MeCP2* gene is introduced in the MeCP2 null mice** to recover the MeCP2 depleted phenotype. After this experiment using knock-in techniques, several studies have been conducted using strategies of importance for Rett syndrome clinical treatment.
2004 (Buschdorf and Strätling, 2004)	Annotation based on sequences revealed a **WW group II domain in the C-terminal region of MeCP2**. The WW group II domain is shown to bind FBP11 and HYPC RNA splicing involved proteins in vivo. C terminal WW group II domain mutations that are known to exist in Rett syndrome patients are identified to influence MeCP2 binding properties. This WW group II domain is not exactly the same C-terminal MeCP2 domain that was previously described to bind to HMGB1. The WW group II domain and the C-terminal domain that binds HMBG1 overlap and differ in only a few amino acids.
2004 (Collins et al., 2004)	**Mild over expression of MeCP2 in mice resembles the Rett disease phenotype.** Both MeCP2 overexpression and MeCP2 mutations cause postnatal neurological disorders, indicating a tight regulation of MeCP2 expression in mature neurons.
2005 (Mari et al., 2005)	**CDKL5** is shown to phosphorylate itself and **to mediate MeCP2 posttranslational modifications**. The expression patterns of CDKL5 and MeCP2 are shown to overlap in similar tissues.
2005	**Duplication of the Xq28 (*MeCP2*) locus is noted to cause a neurodegenerative disease in boys**. The onset of the Xq28 duplication syndrome is progressive and not delayed as noted in Rett syndrome. A very

(Meins et al., 2005)	notable difference between Rett and Xq28 duplication syndrome is the high susceptibility of Xq28 duplication syndrome for pneumonia. These phenotypic differences were first explained by the duplication of a neighboring gene Interleukin-1 receptor-associated kinase 1 (IRAK-1). Later it was noticed that patients with no IRAK-1 duplication also show immune deficiency. It is noted that *IFNy* is a target gene of MeCP2 and aberrant regulation of this protein is also involved in the recurring lung infections.
2005 (Young et al., 2005)	MeCP2 is shown to **interact with the RNA splicing factor YB-1.** In the brain, many genes undergo alternative splicing. YB-1 has several functions in chromatin and RNA regulation and binds to Y boxes in promoter regions.
2006 (Zhou et al., 2006)	**MeCP2 S421 phosphorylation** in response to neuronal activity is required for transcription of the brain derived neurotrophic factor BDNF, for dendritic growth and for the maturation of spine. It is noted that MeCP2 is involved in neuronal activity and required for the connectivity of neurons, and is likely connected to the neurodegeneration observed in Rett syndrome and duplication syndrome.,
2007 (Guy et al., 2007)	**The Rett syndrome phenotype can be rescued in MeCP2 knock-out mice by re-introducing full length MeCP2**. This suggests that the effect of MeCP2 deficiency in Rett syndrome is post-developmental. For this experiment Lox-Cre mice were created, thus this treatment is not yet applicable in humans. Only one mouse in this experiment did not recover from the treatment, possibly due to a recombination in the locus resulting in a skewed MeCP2 protein.
2007 (Adams et al., 2007)	MeCP2 is shown to represent an **intrinsically disordered protein** having only 35-40% ordered structure i.e. MeCP2 requires dimerization, complex formation or posttranslational modifications to stabilize the protein structure. The disordered protein organization is expected to give rise to a lot of other possible functional folding states of the MeCP2 protein. Of interest, the earlier described WW II domain is shown to be part of the disordered C-terminal of MeCP2, therefore only functioning in combination with other regulatory proteins.
2008 (Chahrour et al., 2008)	Microarray gene expression analysis of MeCP2 mice is performed by several groups, most notable in this study is the fact that **MeCP2 binds to promoter regions of genes that are actively expressed**.
2009 (Tao et al., 2009)	It is noted that **MeCP2 S86 phosphorylation** is associated with the functioning of **MeCP2 as an activator** instead of a repressor thereby inducing the binding of MeCP2 with Creb1 at promoters of target genes. The MeCP2 phosphorylated or non-phosphorylated state inducing the

	activator versus repressor state is noted as the 'Yin-yang effect' of MeCP2.
2009 (Ballas et al., 2009)	**Astrocytes from MeCP2 knock-out mice (exhibiting Rett syndrome phenotype) do not support growth of neuronal cells.** In brains from wild type mice MeCP2 is expressed in both neurons and glial cells. Wild type neuronal cells, co-cultured with astrocytes from MeCP2 deficient mice show a compromised growth.
2009 (Guarda et al., 2009)	MeCP2 is shown to **colocalize with the Lamin B receptor** and to interact with LaminB by the MAR region. The inner membrane of the nucleus is described to provide a structural function as part of the nuclear cytoskeleton and chromatin close to the nuclear periphery and is described to represent silenced chromatin (Reddy et al., 2008).
2010 (Skene et al., 2010)	It is shown that **neuronal MeCP2 levels are similar to histone-octamer levels** and small changes in MeCP2 concentrations are noted to alter the chromatin structure. MeCP2 is noted to compete with the binding of H1 in neuronal cells, replacing almost half of them. In case of MeCP2 deficiency, H1 levels are noted to be upregulated to replace the missing MeCP2 as in other non-neuronal cells, expressing MeCP2 at a much lower level.
2010 (Ghosh et al., 2010b)	It is noted that in vivo the **binding affinity of MeCP2 at methylated CpGs versus non-methylated CpGs** differs only 3-fold.
2010 (Muotri et al., 2010)	**MeCP2 binding at retrotransposon Line-1 repetitive elements** is shown to **repress its replicative potential**, whereas Alu retrotransposons are unaffected by MeCP2 binding. An important role for MeCP2 in maintaining chromatin stability is proposed. Retrotransposons that replicate and integrate in the genome can cause mutagenesis.
2010 (Kernohan et al., 2010)	MeCP2 is noted to **interact with ATRX** (a protein associated with mental retardation alpha thalassemia syndrome) **and** with **cohesion at the maternal imprinted gene *H19***. The involvement of MeCP2 in transcription regulation of *H19* at sites distal or proximal from the gene promoter is suggested to occur via **chromatin looping**.
2011 (Lioy et al., 2011)	In MeCP2 deficient mice exhibiting a Rett syndrome phenotype, astrocytes do not express MeCP2. **Re-introduction of MeCP2 in astrocytes of MeCP2 deficient cells restores dendritic complexity in the brain** and reduces Rett symptoms such as abbarent breathing and hypoactivity and prevents premature lethality.
2011	**John Cunningham Virus (JCV)** is noted to trigger **transcriptional activation of MeCP2 in infected brain cells.** JCV is carried latent in brain cells in a

(Shirai et al., 2011)	large part of the world population. The presence of this virus can cause progressive multifocal leukoencephalopathy (PML) in immunodeficient individuals.
2011 (Agarwal et al., 2007)	MeCP2 is noted to be able to **induce the clustering of compact chromatin domains during myogenic differentiation** in mice. The N-terminal domain of MeCP2 and HP1β are noted to interact and induce clustering of compact chromatin domains, i.e. chromocenters.
2012 (Mellén et al., 2012)	The relative binding affinity of MeCP2 for methylated CGs, hydroxymethylated CGs en unmethylated CGs is described. The **affinity of MeCP2 for hydroxymethylated and methyldated CGs is similar**, in contrast to findings in earlier studies. This study takes in account possible nucleotide combinations in the neighborhood of the C nucleotide. MeCP2 Is shown to have a higher affinity for the CA combination.
2013 (Matsumura et al., 2010)	MeCP2 is noted to **bind with Latency Associated Nuclear Agent LANA-1, of the herpes virus KSHV**. This interaction is found to occur outside of the viral genome, indicating a collaboration between LANA-1 and MeCP2 to regulate gene expression of both the viral and endogenous genes.
2013 (Chiang et al., 2013)	It is noted that HIV infected CD4+ cells overexpress miRNA 132, which represses, among others, MeCP2. **HIV-induced MeCP2 repression is noted to enhance the HIV replication potential.**
2013 (Brink et al., 2013)	Targeting of **MeCP2 is noted to be able to induce significant chromatin decondensation** accompanied by the eviction of Heterochromatin protein 1 (HP1γ) as measured in cells carrying an amplified chromosomal domain. MeCP2-induced chromatin decondensation is noted to be comparable to targeting of a transcriptional activator VP16, although it does not alter the transcriptional activity of the reporter gene within the chromosomal domain.
2013 (Baker et al., 2013)	**Mutations in an AT-hook in the MeCP2 TRD domain** are noted to cause **a more severe Rett phenotype** than other TRD mutations. It is noted that this AT-hook has structural similarity with the high-mobility group AT-hook (HMGA). It is suggested that the chromatin structure plays an important role in this AT-hook related Rett phenotype through alteration of the local enrichment of nucleosomes.
2014 (McFarland et al., 2014)	**MeCP2 is shown to interact with Huntingtin protein (Htt).** In mutant Htt CAG repeats in the Htt protein encoding an enlarged polyQ tract are known to cause Huntington's disease. Htt is shown to bind to proteins that are also binding partners of MeCP2. Mutant Htt is noted to have an increased

	binding interaction with MeCP2.
2014 (Song et al., 2014)	**MeCP2 levels** are identified **in different cells of different tissues and at several stages of early life development**. MeCP2 is noted to be expressed in almost all body cells except the intestine and colon, microglial cells and mature gonads.
2014 (Khrapunov et al., 2014)	The **binding of MeCP2 with its MBD** is in vitro noted to be highly correlated with **salt concentrations in the nucleus**. The affinity of MeCP2 for either methylated or unmethylated CpGs is shown to be dependent on the nuclear salt concentration.
2014 (Bergo et al., 2014)	**Phosphorylation of MeCP2 at Y120** is noted to induce **colocalization at centromeres** and to play a role in **mitotic spindle formation**, **proper microtubule nucleation and progression through mitosis**.
2015 (Lev Maor et al., 2015)	An **interaction of MeCP2 with CTCF and HP1 is noted to direct RNA splicing**. CTCF and MeCP2 binding is noted to be regulated by DNA methylation facilitating exon exclusion or inclusion dependent on the DNA methylation levels, via the presence of RNA polymerase II and HP1 directed splicing factors.
2015 (Martínez de Paz et al., 2015)	MeCP2 is noted to be **circadianly regulated.** MeCP2 protein levels fluctuate with the rhythm of CLOCK genes and chromatin structure alterations are directly correlated with MeCP2 fluctuating levels. It is shown that when MeCP2 levels are increased the chromatin is in a more condensed state. miRNA 132 was reported to regulate MeCP2 as well as HAT activity of CLOCK proteins.
2015 (Linhoff et al., 2015)	A high resolution microscopy imaging approach (entitled chromATin) is used to examine the chromatin compaction and epigenetic chromatin composition in MeCP2-null mice brains compared to wild type mice. **MeCP2 null neurons showed an increased density of the compact chromatin** and a striking redistribution of various histone modifications related to pericentromeric heterochromatin (e.g. H4K20me3) compared to the chromatin of wild type neurons. The chromatin structure changes are not observed in all neuronal cell types illustrating the cell-type specific differences in chromatin structure in complex tissues.