CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Methyl-CpG –Binding Protein 2 (MeCP2)

1.1.1 MeCP2: Gene, transcript and protein

MECP2 is an X-linked gene encoding Methyl-CpG binding protein 2 located on the long arm of chromosome X (Xq 28). MeCP2 is the member of the methylated DNA-binding domain (MBD) gene family (Lewis et al., 1992). Mutation in MeCP2 gene leads to the neurodevelopmental disorder known as Rett syndrome (RTT) (Amir et al., 1999), an autism spectrum developmental disorder (Hagberg, 1985). Hundreds of diverse mutations of the MeCP2 gene have been linked with RTT and less frequently with other forms of intellectual disabilities, such as autism, schizophrenia, mental retardation and Angelman-like syndrome (Chahrour and Zoghbi, 2007). Later, duplication and triplication of this gene have also been identified as the genetic cause of the MECP2 duplication syndrome that usually affects boys which lead to neonatal encephalopathy and death during the first year of life (Van Esch, 2011).

Structurally there are 6 distinct domains of MeCP2 (Adams et al., 2007). Listed from amino to carboxy termini, these are the

1. N-terminal Domain (NTD)
2. Methyl Binding Domain (MBD)
3. Intervening Domain (ID)
4. Transcription Repression Domain (TRD)
5. Carboxyl terminal domain (CTD)-α
6. CTD-β

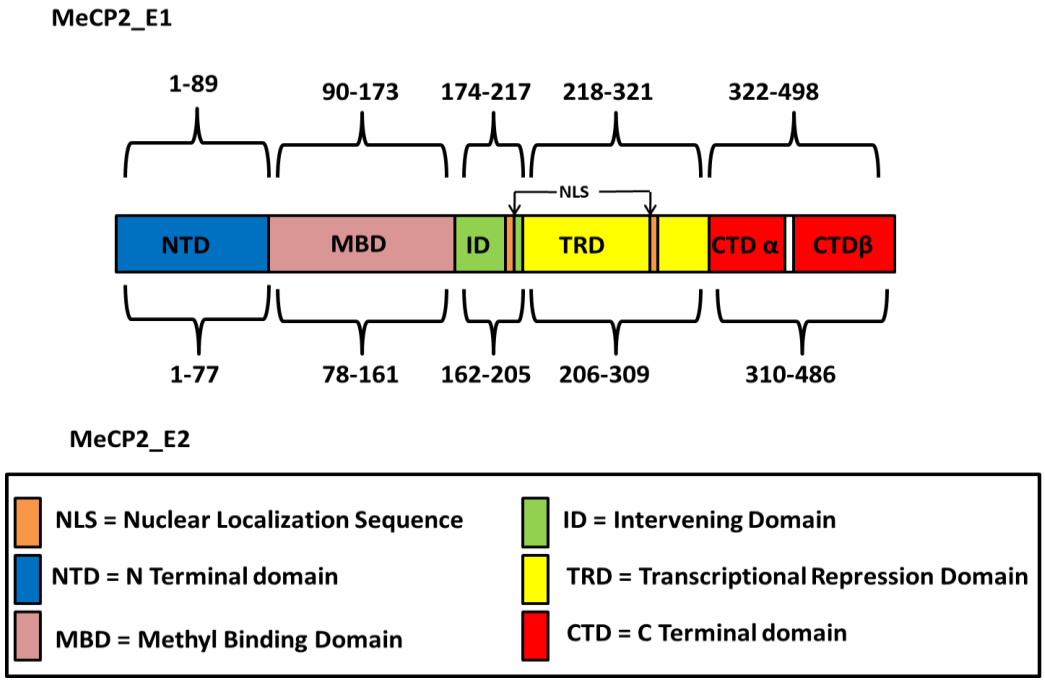

Fig-1.1: Schematic illustration of MeCP2 domains and isoforms: *MeCP2 domains are NTD: N-terminal domain, MBD: Methyl Binding Domain, ID: Intervening Domain, TRD: Transcription Repression Domain and CTD is C-Terminal Domain. Amino acid counts for each domain of MeCP2_E1 were based on that for a previously described MeCP2_E2 domain structure.*

The two well-characterized functional domains of MeCP2 are MBD and TRD (Fig-1.1). MBD domain is responsible for binding to the methylated CpGs. Recently, it was reported that MeCP2 not only binds to CpGs but also to hydroxyl methylated cytosines (Kinde et al., 2015). The MBD has also been shown to be important for binding of MeCP2 to heterochromatin (Nan et al., 1996), as well as to the unmethylated four-way junction DNA (Galvao and Thomas, 2005). The TRD domain mediates its link with the histone deacetylases (HDAC1 and HDAC2) and transcriptional co-repressors mSin3A, c-Ski and N-CoR. HDAC1 and HDAC2 combine with mSin3A to form a co-repressor complex (Jones et al., 1998; Kokura et al., 2001; Nan et al., 1998).The TRD interacts with the transcription factor IIB (TFIIB), a component of the basal transcriptional machinery to stabilize the repressed state of the DNA bound MeCP2 on methylated DNA

(Kaludov and Wolffe, 2000). The C-terminal domain is involved in the interactions with DNA and its protein partners (Bienvenu and Chelly, 2006). CTDα may contribute in recognizing the methylated DNA of chromatin (Nikitina et al., 2007) while CTDβ encodes the WW domain-binding region (Buschdorf and Strätling, 2004). WW domains are characterized by two tryptophan (W) residues separated by 20–22 amino acids and facilitate protein–protein interactions by recognizing proline-rich motifs (Sudol et al., 1995). The amino acid composition of the residues on the N-terminal side of the MBD has a very similar composition to that of the high mobility group proteins (HMG) proteins. On the basis of this structure, NTD refer to as HMGD1 (Adams et al., 2007). The N-terminal region has been shown to interact with the heterochromatin protein 1 (HP1) which is a repressive chromatin regulator. All the three domains including HMGD1, MBD and the N-terminal portion have been shown to have association with histone H3 methyl transferase activity (Fuks et al., 2003). One of the nuclear localization signal (NLS) is embedded within the TRD (Nan et al., 1996) and the other is located in HMGD2, between the MBD and TRD, which was identified by expressing human MeCP2 in *Drosophila melanogaster* cells, and has not been shown to be functional in mammalian cells (Kudo, 1998). HMGD2 is the alternate name of ID domain (Jeffery and Nakielny, 2004). The group also identified an RG (arginine-glycine) repeat region in HMGD2 which are known to mediate RNA-protein interactions. The CTD is required for chromatin condensation into higher-order structure (Chandler et al., 1999; Nikitina et al., 2007). MeCP2 has a higher level of complexity than principally believed. MeCP2 gene contains 3′ untranslated region (3'UTR) and evidence suggests that the 3′UTR is an important contributor in the fine-tuning of homeostatic MeCP2 expression levels throughout development (McGowan and Pang, 2015). Moreover, it also contains several polyadenylation signals that in turn give rise to transcripts of different lengths with different expression patterns (Coy et al., 1999; D'esposito et al., 1996; Pelka et al., 2006; Reichwald et al., 2000).

Based on alternative splicing of MeCP2 transcript there are 2 isoforms MeCP2e1 and MeCP2e2 (Kriaucionis and Bird, 2004; Mnatzakanian et al., 2004). These isoforms, MeCP2e1 (MECP2B or MeCP2a) and MeCP2e2 (MECP2A or MeCP2b), differ only at their N-terminal regions even though they include both the two main domains MBD and TRD. Isoform E1 is 498 amino acids long and is translated from exons 1, 3, and 4,

whereas isoform E2 is 486 amino acids and translated from exons 2, 3, and 4 (Fig. 1.1).These isoforms are also spatio-temporally regulated and have differential distribution in developing postnatal mouse brains (Dragich et al., 2007).The e1 isoform is more abundant in the brains of mice and humans but the e2 isoform was the first identified variant of MeCP2 and therefore the best characterized (Mnatzakanian et al. 2004). MeCP2-e1 is the most abundant isoform and demonstrates more widespread expression throughout development and thus considered as the most relevant isoform in RTT pathology (Yasui et al., 2014). However several studies have reported that altered expression of both MeCP2-e1 and MeCP2-e2 and disruption in RTT patients along with disruption in the MeCP2 alternate splicing (Petel-Galil et al., 2006; Saxena et al., 2006; Sheikh et al., 2013). Moreover, there are reports showing both of these isoforms co-localize to heterochromatic regions in murine fibroblast cells (Jiang et al., 2008; Kumar et al., 2008). Although both the MeCP2 isoforms have difference in structure but both the isoforms can rescue RTT phenotypes in mice to different extents (Jugloff et al., 2008). All these reports suggest that both MeCP2 isoforms are important in maintaining normal brain function and altered expression of both isoforms may lead to neurological complications.

1.1.2 Multifaceted Functions of MeCP2

Since decades people are trying to investigate the function of MeCP2 and surprisingly the studies have proved it to be a multifunctional protein having a diverse and potential role in the central nervous system of brain. MeCP2 is required for correct brain function and development. Loss of MeCP2 has been shown to delay neuronal maturation and synaptogenesis, and cause Rett syndrome (Diaz de Leon-Guerrero et al., 2011).

MeCP2 is known to perform many diverse functions (Fig 1.2) in the CNS such as transcriptional repression, activation, chromatin condensation, RNA splicing and chromatin compaction with its capability of binding to different protein partners reading an epigenetic signature (Bedogni et al., 2014). Initially, MeCP2 was discovered as a member of methyl binding protein family that preferentially binds to the methylated region of DNA (Wade, 2001). MeCP2 is known to repress many genes through its TRD

domain and for this MeCP2 also recruits the additional co-repressor complexes such as HDACs, Sin3a, Ski and NCoR, which in turn remodel the chromatin into a repressive state (Jones et al., 1998; Kokura et al., 2001; Nan et al., 1998).

Reports have shown that MeCP2 null mice have unexpectedly mild gene expression changes, thus, strengthening the relevance of other MeCP2 chromatin functions (Tudor et al., 2002). Moreover, related results were reported in the Rett Patients (Mencarelli et al., 2010; Urdinguio et al., 2009). In connection to this, some studies have shown that MeCP2 mutations affect the DNA binding and its capacity to induce compaction of nucleosomal arrays thereby affecting its chromatin binding kinetics. This led to the discovery of MeCP2 to have a function in chromatin compaction (Georgel et al., 2003; Kumar et al., 2008; Marchi et al., 2007; Nikitina et al., 2007). The relationship between MeCP2 and chromatin structure was further revealed by identification of its interacting partners, such as the chromatin remodeling complexes Brahma and ATRX, the corepressors c-Ski, CoREST and LANA and a H3K9 histone methyl transferase (Chahrour and Zoghbi, 2007; Guy et al., 2011). This was supported by a study reporting MeCP2 at a high molar ratio to nucleosomes, thus, leading to the formation of a novel highly compacted chromatin structure (Georgel et al., 2003). Accumulated evidence suggests that MeCP2 was able to compete with histone H1 and the displacement of histone H1 might represent a mechanism whereby MeCP2 modulates higher-order chromatin architecture, perhaps in order to control gene expression (Ghosh et al., 2010; Nan et al., 1997). Though, the mechanism and regulation of the MeCP2 induced higher order heterochromatin organization is still uncertain.

Later, studies proved that MeCP2 not only functions as transcriptional repressor but also works as a transcriptional activator (Fig-1.2). This function was inferred from transcriptional profiling studies of RNA purified from hypothalamic and cerebella of RTT mice (Chahrour et al., 2008). Further, the data was supported by the identification of MeCP2 on the promoters of activated genes and its interaction between MeCP2 and the transcriptional activator CREB1 (cAMP responsive element binding protein 1) on an active gene (Chahrour et al., 2008). In another study, it was shown that AKT/mTOR signaling is reduced in both Mecp2-null mice and heterozygous females and protein synthesis was significantly impaired in these mice (Ricciardi et al., 2011).

Additionally, it has been shown that *invitro* MeCP2 can bind to RNA (Jeffery and Nakielny, 2004) and regulates alternative splicing (Maunakea et al., 2013; Cheng et al., 2014b; Young et al., 2005). It has been reported that MeCP2 interact with a Y-box transcription factor (YB1) to regulate the alternative splicing (Young et al., 2005). In the brain, MeCP2 has been shown to be a part of a multiprotein complex containing Prpf3,a major component of the spliceosome, and Sdccag1, a mediator of nuclear export (Long et al., 2011). RTT transgenic mouse lines showed aberrant alternative splicing events (Young et al., 2005). Thus, MeCP2 might therefore also serve to regulate gene

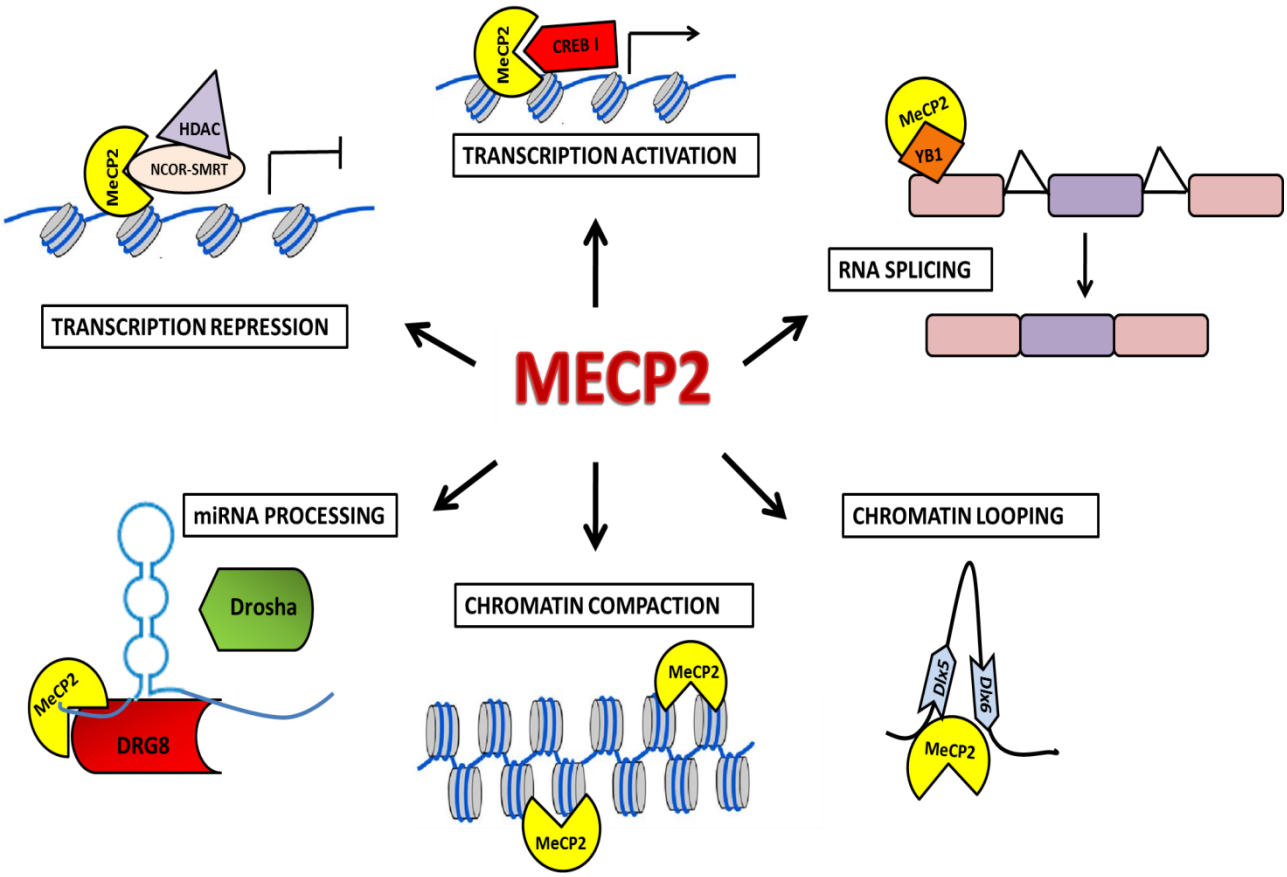

Fig-1.2: Schematic representation of molecular functions of MeCP2. *Clockwise from top left corner. MeCP2 is known to function both as a transcriptional repressor by recruiting co-repressors HDAC and NCOR-SMRT at its binding site while can recruit CREB I and act as an transcriptional activator. MeCP2 mediates RNA splicing by interacting with YB1, chromatin looping by interacting with Dlx5 and Dlx6, chromatin compaction and also mi-RNA processing along with DRG8.*

expression at a post-transcriptional level. Additionally, various miRNAs were found to be upregulated in the hippocampus of Mecp2-null mice compared with wild-type mice, which suggested that MeCP2 negatively regulates the miRNA processing by interacting with DGCR8 and interfering with the assembly of the DGCR8–Drosha complex (Cheng et al., 2014b). Thus, MeCP2 is a multifunctional protein which is known to play diverse set of functions by binding to its distinct partners. However, the physiological roles of many of these interactions and their relevance to RTT pathology remain to be established.

1.1.3 Post-Translational Modifications of MeCP2

The structure of MeCP2 together along with various post-translational modifications (PTMs) might justify the functional versatility, possibly occurring through the capacity of the methyl-binding protein to interact with several diverse protein partners (Klose and Bird, 2004). Various PTMs like phosphorylation, methylation, sumoylation, acetylation ubiquitination and ADP-ribosylation are seen in MeCP2 but so far not much information is available regarding their site specific functionality (Fig-1.3). The best studied PTM of MeCP2 is phosphorylation, details of which will be discussed in the later part of this chapter (Refer section 1.2). However, Gonzales et al. 2012 found new ubiquitination and acetylation sites by expressing epitope-tagged MeCP2-e1 in SH-SY5Y cells to analyze its PTMs using tandem mass spectrometry. However, the functional relevance of these sites has not yet been studied in detail.

Another study by Cheng et al., 2014a showed that MeCP2 is also modified by the covalent linkage of small ubiquitin-like modifier (SUMO) to several lysines. Sumoylation particularly K223 (Lysine 223) is necessary for transcriptional repression function of MeCP2. Moreover, they demonstrated that sumoylation at K223 is also necessary for the HDAC1/2 complex recruitment. This was proved by mutating the lysine 223 site to arginine which further abolished the suppression of gene expression function in mouse primary cortical neurons. This mutation significantly led to alterations in the excitatory synaptogenesis in vitro and in vivo. There are many lysine residues on

MeCP2 like K363 and K412 which are sumoylated (Tai et al., 2016). This study reported that MeCP2 SUMOylation rescues the behavioral and synaptic deficits in Mecp2

conditional knockout (cKO) mice (Tai et al., 2016). Moreover, MeCP2 could be SUMO-modified by PIAS1 at K412. MeCP2 SUMOylation is neuronal activity-dependent and interestingly MeCP2 phosphorylation (S421 and T308) facilitates MeCP2 SUMOylation (Tai et al., 2016).

In addition, MeCP2 has been found acetylated in several residues (Choudhary et al., 2009; Gonzales et al., 2012; Pandey et al., 2015; Zocchi and Sassone-Corsi, 2012). One of the study showed that acetylation of K464 of Mecp2has been identified in cultured cortical neurons. This study demonstrated that acetylation of K464 is mediated by p300 and deacetylation was carried out bySIRT1, a NAD+-dependent histone deacetylase.

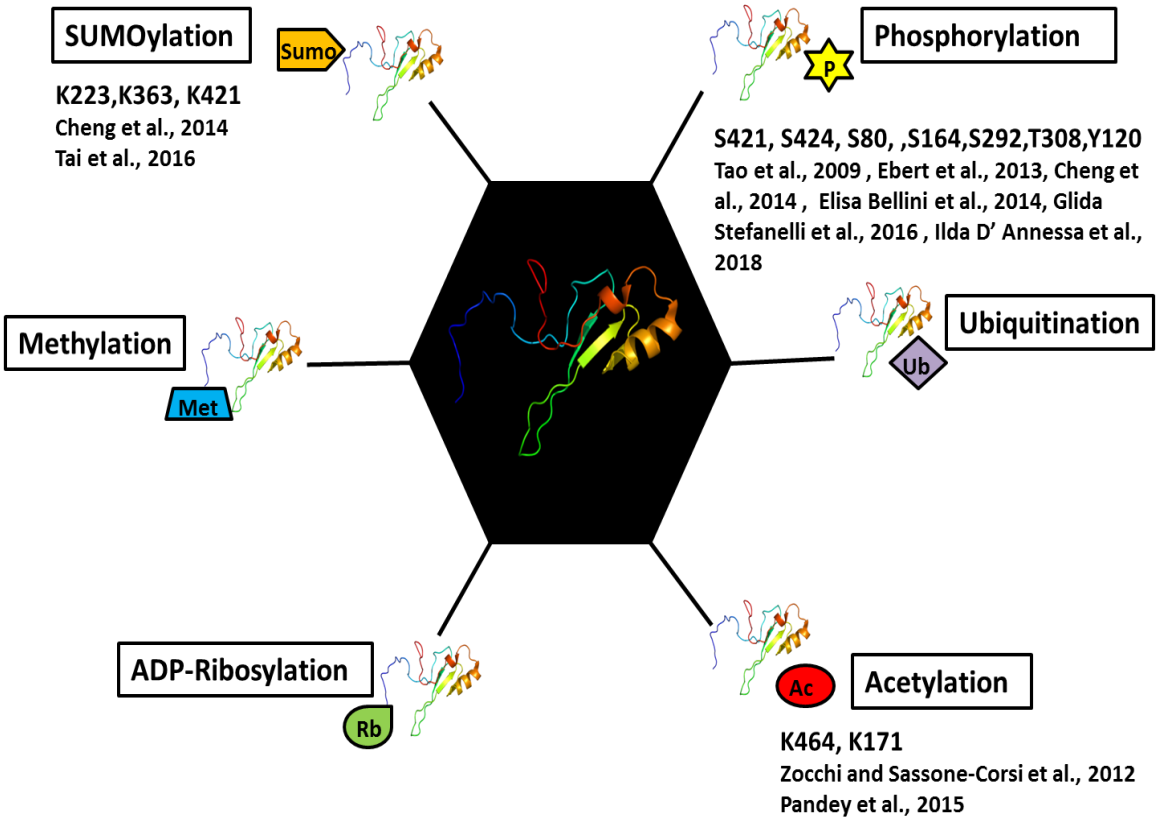

Fig-1.3: Schematic representation of the various post-translational modifications *that occur on MeCP2 along with functionally significant sites of specific post-translational modifications. Clockwise from left top corner, SUMOylation, Phosphorylation, Ubiquitination, Acetylation, ADP-Ribosylation and Methylation.*

Moreover, SIRT1-dependent deacetylation of MeCP2 could allow its release from the methylated CpG sites of the BDNF exon 4 promoter leading to increased BDNF transcription. Hence, they showed that K464 acetylation affects the DNA binding (Zocchi and Sassone-Corsi, 2012). The acetylation at K171 is important for MeCP2 interaction with at least two other chromatin remodeling enzymes ATRX and HDAC1 suggesting that acetylation may serve as a regulatory switch that could potentially modulate protein-protein interaction. This study was done using RKO colon and MCF-7 breast cancer cells in which they demonstrated that SIRT1 regulates MeCP2 acetylation levels and inhibition of SIRT1 results in a significant increase in the acetylation of MeCP2 protein without altering total MeCP2 protein levels (Pandey et al., 2015).

A recent study has come up with a new PTM of MeCP2 i.e. ADP-ribosylation. In this study, it has been shown that the endogenous MeCP2 from mouse brain tissue is poly (ADP-ribosyl)ated in vivo (Becker et al., 2016). Two distinct MeCP2 domains (ID and TRD) relevant for poly (ADP-ribosyl)ation were identified and showed that the deletion of these modifiable domains increased heterochromatin clustering. Furthermore, it was also found that PARP-1 deficiency increases the ability of MeCP2 to aggregate and to bind to pericentric heterochromatin. These findings unravel a novel mechanism modulating MeCP2-dependent chromatin organization (Becker et al., 2016). Lastly, MeCP2 has also been found methylated in 293T cells (Chahrour et al., 2008) and O-glycosylated in 293T cells and rat brain (Rexach et al., 2010).

1.2 MeCP2 phosphorylation

1.2.1 MeCP2 Phosphorylation Sites

In 2003, studies by Chen et al., and Martinowich et al., individually suggested for the first time that control of neuronal activity-dependent gene regulation of MeCP2 was through post-translational modifications. A phosphorylation site was then identified as serine 421 (S421) because S421 to alanine mutation abolished this neuronal activity-

induced MeCP2 mobility shift (Chen et al., 2003; Zhou et al., 2006). Later using mass spectrometry, multiple phosphorylation sites in MeCP2 under different conditions have been identified (Gonzales et al., 2012). These sites were mainly serine and threonine residues like S421, S424, S80, S86, S274, S229, S292, S164,S399,T148, S149,T308 (Fig-1.4) were found to be phosphorylated (Ebert and Greenberg, 2013; Gonzales et al., 2012; Tao et al., 2009). The two well documented sites of MeCP2 phosphorylation are S80 and S421. However, there are studies showing importance of other sites in RTT. The importance of S421 phosphorylation till now has been seen during circuit development (Cohen et al., 2011), drug sensitivity, mood regulation, chronic opoid consumption (Ciccarelli et al., 2013) and in integration of synaptic inputs (Géranton et al., 2008) mainly only in post-mitotic neurons and its role in other cell types needs to be established.

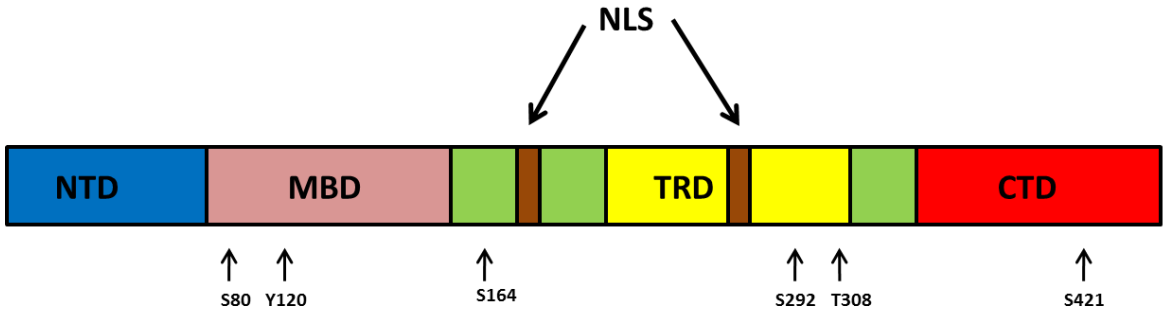

Fig1.4: Distribution of known phosphorylation sites on MeCP2 protein along its various structural domains.

Tao et al., in 2009 found that S80 phosphorylation is the most conserved phosphorylation site of MeCP2 and is abundantly phosphorylated under resting conditions whereas neuronal activity induces its dephosphorylation. Mecp2S80A/y knock-in mice are found to be slightly overweight, had decreased locomotor activity (Tao et al 2009) and showed decreased binding of MeCP2 at the promoters of multiple genes, including Rab3d, Vamp3 and Igsf4b. Moreover, S80 phosphorylation has been recently identified as a crucial regulator of the interaction between MeCP2 and DGCR8 in modulating nuclear microRNA processing (Cheng et al., 2014b). Another MeCP2 phosphorylation site worked upon was phospho-serine292 (pS292) (Liu et al., 2014), where they revealed that overexpression of vascular endothelial growth factor (VEGF) enhances accumulation of

pS292MeCP2 in reactive astrocytes in ischemic injured rat striatum, implicating the role of pS292MeCP2 regarding epigenetic cross talk with VEGF. Later that same group in 2017, showed that MeCP2 and pS80 MeCP2 are concentrated in the nucleus, while pS421 MeCP2 and pS292 MeCP2 are detectable in cytoplasm of astrocytes at a relatively low level (Liu et al., 2017).

It is reported that T308 MeCP2 phosphorylation leads to the disruption of the interaction between MeCP2 and NCoR complex (Ebert et al., 2013) and Mecp2T308A/y mice indicates some types of RTT-like phenotype. Bergo et al. in 2015 reported that during neuronal maturation, S164 phosphorylation controls nuclei dimension and dendritic patterning in vitro and also largely affects its nucleosome binding and chromatin affinity in vivo. Though phosphorylation can occur at tyrosine residue, no reports on MeCP2 tyrosine phosphorylation have been recorded till now. However, a recent study showed that Y120 which was related to RTT syndrome in 2001 is phosphorylated and additionally reveal a novel localization of the protein to the centrosome, leading to a functional association of MeCP2 to cell growth, spindle geometry and microtubule nucleation. Moreover, a deep comprehension of the structural and dynamical properties of the MBD in the presence of the Y120D mutation or its phosphorylation is thus needed to reveal differences and similarities between the two events (D'Annessa et al., 2018).

1.2.2 Stimuli dependent regulation of MeCP2 phosphorylation

The understanding of the extracellular stimuli mediating intracellular signalling pathways leading to the phosphorylation/dephosphorylation at different residues of MeCP2 protein is still unfolding. In cultured cortical neurons, membrane depolarization-induced MeCP2 S421 phosphorylation due to calcium influx via L-type voltage-gated channels or by neurotransmission stimulus and neurotrophin treatment was sufficient for pMeCP2 S421 to express and that could be detected as early as 5 min after stimulation (Chen et al., 2003; Zhou et al., 2006). Later Tao et al., showed that CamKIV knockdown attenuates membrane depolarization-induced MeCP2 S421 phosphorylation (Tao et al., 2009) to which Buchthal et al. confirmed that CamKII overexpression in neurons is sufficient to induce MeCP2 S421 phosphorylation (Buchthal et al., 2012). Similarly other stimuli like early life stress, fear conditions, cocaine and antidepressant drug

consumption (amphetamine, imipamine), descending serotonergic inputs in neurons have shown to induce pMeCP2 S421(Deng et al., 2010; Géranton et al., 2008; Hutchinson et al., 2012; Li et al., 2014; Mao et al., 2011; Murgatroyd et al., 2009). Interestingly, unlike at T308, the other sites like S86, S274 and S421 were seen to get phosporylated by BDNF, KCl and forskolin stimulation via protein kinase A (Ebert et al., 2013). *In vitro* studies in human fibroblasts have revealed that Homeodomain-interacting protein kinase2 (HIPK2) could bind to MeCP2 and phosphorylate S80 while knockdown of HIPK2from cortical neurons strongly reduces the level of S80 phosphorylation (Bracaglia et al., 2009). The S421 phosphorylation in neural progenitor cells (NPCs) is linked to cell cycle and directly regulated by aurorakinase B, and plays a critical role in balancing aNPC proliferation/neural differentiation through the Notch signalling pathway (Li et al., 2014).

Table 1.1 Stimuli inducing MeCP2 Phosphorylation

SITE	STIMULI	REFERENCE
S421	•Ca+2 influx via L-type voltage-gated channel •Neurotransmission •Neurotrophins •CamKII •Early life stress and fear •Cocaine •Anti –depressant drugs •Growth factors FGF/EGF	Cheng et al., 2003 Zhou et al., 2006, Tao et al., 2009, Buchthal et al., 2012, Murgatroyd et al., 2009, Deng et al., 2010, Hutchinson et al., 2012, Mao et al., 2011, Li et al., 2014, Geranton et al.,2008
S80	•Ca+2 influx mediated dephosphorylation •Homeo domain-interacting protein kinase 2	Zhou et al., 2006 Bracaglia et al., 2009
T308, S86, S274 and S421	•BDNF, KCl and forskolin stimulation via protein kinase A	Ebert et al., 2013

Moreover, there are few recent studies on expression of pS292 MeCP2, a newly-reported phospho-MeCP2 which is induced in reactive astrocytes in the ischemic adult brain, but its function is not yet clear. Additionally, one report revealed the high levels of pS421

MeCP2 and its association with VEGF in proliferative diabetic retinopathy (PDR) membranes suggests that phospho-MeCP2-S421 might be involved in the pathogenesis

of PDR (Li et al., 2016). To summarize, MeCP2 phosphorylation is stimulated by diverse stimuli and thereby regulated by different signalling pathways which activate kinases and phosphatases which in turn regulate this phosphorylation of MeCP2 (Table-1.1).

1.2.3 Functional significance of MeCP2 phosphorylation

In attempts of uncovering functional aspects of pMeCP2, independent studies by Greenberg et al. and Li et al. focused on generating knock-in mice models wherein the Greenberg group made a *Mecp2S421A* allele while Li et al., made a *Mecp2S421A;S424A* double knock-in mice. The latter group first confirmed S421 phosphorylation is abolished in the *Mecp2S421A;S424A/y* mice and that, compared to WT mice, they perform better during fear conditioning and Morris water maze, have enhanced long-term potentiation at two hippocampal synapses, show increased number of excitatory synapses on hippocampal and cortical neurons, binds more tightly to gene promoters and also show synaptic upscaling under tetrodotoxin but exhibit impaired synaptic down scaling under effects of bicuculline (Li et al., 2011; Zhong et al., 2012). Greenberg group reported their characterization of the *Mecp2S421A/y* mice only showed mild phenotypes, including increased dendritic complexity, increased inhibitory neurotransmission, and a deficit in hippocampal learning/memory with no change in MeCP2 binding to DNA across the genome after neuronal activity-induced S421 phosphorylation (Cohen et al., 2011). These reports suggest that the S424 site of MeCP2 might be phosphorylated in by some unknown mechanisms in neurons and in addition to that the mutations in S424 site might affect the MeCP2 functions which are independent of neuronal activity-induced S421 phosphorylation. In another study Li et al. proved that pMeCP2 plays an important role in regulating proliferation and neuronal differentiation of NPCs via Notch pathway and also identified growth factors involved in signalling upstream of S421 phosphorylation along showed that Aurora kinase B is an direct target of pMeCP2S421 (Li et al., 2014). Hunchinson et al. (2012b) through *Mecp2S421A/y* mice, suggests a role

of MeCP2 S421 phosphorylation in depression-like behaviour and the behavioural response to antidepressant treatment correlating works of Deng et al., 2010 and Mao et al., 2011. Considering other sites of MeCP2, *Mecp2S80A/y* mice showed decreased locomotor activity, whereas *Mecp2S421A;S424A/y* mice presented increased locomotor activity (Tao et al., 2009), suggesting that S80 and S421/S424 phosphorylation indeed play opposing roles in the brain. Moreover, *In vitro* results suggest that S80 phosphorylation play roles in regulating apoptosis (Bracaglia et al., 2009) and microRNA processing (Cheng et al., 2014b). T308, whose phosphorylation leads to the disruption of the interaction between MeCP2 and NCoR complex, while the *Mecp2T308A/y* mice also showed RTT-like phenotype along with slight decrease in brain weight, hind-limb clasping and showed motor coordination deficit in accelerating rotarod test, have a reduced seizure threshold compared to WT mice, suggests that disruption of T308 phosphorylation and disregulation of MeCP2/NcoR interaction may contribute to RTT pathogenesis (Ebert et al., 2013). Recently reported Y120 site of MeCP2 phosphorylation was studied and revealed that addition of a phosphate group to Y120 or mutation in aspartic acid affect domain mobility that samples an alternative conformational space with respect to the WT, leading to impaired ability to interact with DNA (D'Annessa et al., 2018).

1.3 Glial Cells of CNS

1.3.1 Oligodendrocytes

Oligodendrocytes (OLGs) are glial cells that produce and maintain myelin, the lipid enriched axon-ensheathing membrane, which is essential for saltatory conduction of action potentials in the central nervous system (CNS) (Baumann and Pham-Dinh, 2001). There are five basic phases of the OL lineage: generation, migration, proliferation, differentiation and myelination. Furthermore, extensive studies have shown transcription factors essential for oligodendrocyte specification as well as growth factors that influence the oligodendrocyte proliferation and differentiation (Lu et al., 2002; Lu et al., 2000; Orentas and Miller, 1996; Takebayashi et al., 2000). OLGs arise as oligodendrocyte precursor cells (OPCs) which are primarily identified by the expression

of platelet-derived growth factor receptor alpha (PDGFRα) and NG2 which are the key markers of OPCs. In the CNS the OLG lineage occurs in three distinct waves through

time and space. It has been reported that in the mouse brain and spinal cord the first wave of OLGs are found as the migrating precursors at E12.5. The second wave, though much smaller, follows almost 2 days later, with a third wave, still smaller, originating postnatally at P0 (Goldman and Kuypers, 2015). These migrating OLGs are actually the OPCs which are very motile, dividing cell already committed to the oligodendrocyte lineage identified by its bipolar morphology and characterized by OPC markers like PDGFR-α, A2B5, NG2, Olig1/2 and Sox10 (Fancy et al., 2004; Pfeiffer et al., 1993). The CNS produces an overabundance of OPCs across all regions during development. A large percentage dies during the myelination process, as the cells compete for limited environmental growth factors (Barres et al., 1992; Barres and Raff, 1999; Hill et al., 2014; Trapp et al., 1997). Hill et al identified a critical temporal window after NG2 cell division during which the cells are susceptible to environmental factors that affect their

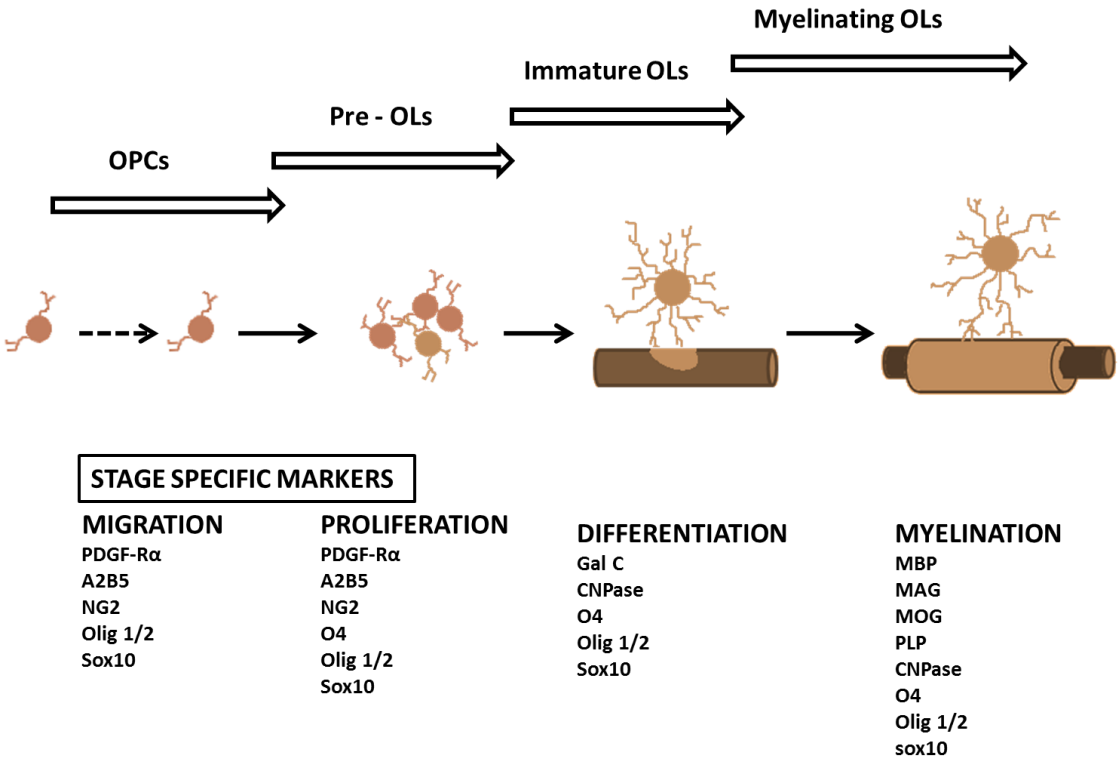

Fig1.5: Stage wise development of oligodendrocyte progenitor cell to mature myelinating oligodendrocyte. *These stages are identifiable according to their increasingly complex morphology along with expression of well-defined markers and their ability to migrate, proliferate, differentiate and perform myelination.*

differentiation or survival (Hill et al., 2014). OLG differentiation is characterized by a rapid increase in the morphological complexity followed by expansion of myelin membrane thereby expressing differentiation protein markers such as Gal C, CNPase and O4 (Barateiro and Fernandes, 2014; Sommer and Schachner, 1981) (Fig-1.5). The process of differentiation starts as OLGs extend and multiple highly ramified processes that finally contact nude axons and trigger myelination. The process tips are simple dynamic filopodia like structures form a branched network (Czopka and Lyons, 2013; Fox et al., 2006; Sloane and Vartanian, 2007). These OLG process expands when come in contact with axon, wrap them in concentric layers of membrane. These myelinating OLGs express, in an orderly manner, myelin proteins like myelin basic protein (MBP), proteolipid protein (PLP), myelin associated glycoprotein (MAG), and finally myelin oligodendrocyte glycoprotein (MOG) (Reynolds and Wilkin, 1988; Scolding et al., 1989; Zhang et al., 2001a). This membrane compacts, expelling cytoplasm which leads to the formation of mature myelin (Snaidero and Simons, 2014).

1.3.2 Astrocytes:

Astrocytes are the largest class of glial cells that outnumber the neurons by over five fold in the mammalian CNS. Astrocytes are distinguished from other glial cell types by their stellate morphology, which is mainly divided into two subtypes. Protoplasmic astrocytes; morphologically have several stem branches which branch further into many fine processes and which reside in gray matter ; whereas, fibrous astrocytes which have long fiber-like processes and reside throughout the white matter (Cajal,1909). Astrocytes are found throughout the whole healthy CNS in a non-overlapping, highly well-organized manner (Bush et al., 1999). They play primary roles in upholding synaptic transmission, synaptogenesis and information processing by neural circuit functions (Allen, 2014; Araque et al., 2014; Chung et al., 2015) maintaining the blood–brain barrier (Sofroniew, 2009), providing trophic and metabolic support to neurons for neuronal survival and differentiation (Allaman et al., 2011). Consequently, astrocytes also express membrane

ion channels though the level of expression of these channels is relatively low and therefore they are not electrically excitable (Orkand et al., 1966; Seifert and Steinhäuser, 2001; Sontheimer, 1994; Verkhratsky and Steinhäuser, 2000). Thus, astrocytes play

pertinent roles in abundant processes of the physiology and development of the CNS without actively participating in the information processing by the nervous system (Kettenmann and Ransom 2004). Astrocytes are attributed with many essential and controlling functions in the healthy as well as in the injured brain. Moreover, astrocytes become activated in response to CNS lesion, stroke, inflammation, tumor or any such neurodegenerative disease. This is well characterized by alteration in gene expression, proliferation and hyper dystrophy (Ridet et al., 1997). These activated astrocytes can release a variety of immune mediators like chemokines, cytokines and other growth factors that may exhibit either neurotoxic effect or neuroprotective effects (Farina et al., 2007). Accumulating evidence suggests that the process of reactive astrogliosis contribute to CNS disorders through either the loss or gain of normal astrocyte function (Sofroniew, 2009).

1.3.3 Microglia:

Microglia are an abundant portion of the CNS cell population that reside within the parenchyma of the nervous system and possess properties of macrophages in other tissues. Microglia are involved in generation of significant innate and adaptive immune responses. With response to any CNS injury or insult, these microglial cells get converted to an active phenotype by releasing cytokines and reactive oxygen species (Liu et al., 1998). Moreover, these active microglial cells have increased rate of motility and proliferation and also show phagocytic activity (Carbonell et al., 2005a, b; Eliason et al., 2002; Schroeter et al., 1997; Zhang et al., 2001b). However, in their non-activated or resting state have a characteristic “ramified” morphology not seen in resident macrophages of other organ systems. These cells reflect three phases of plasticity an amoeboid phase found in the fetus, a ramified (resting) phase found in the nervous system framework, and a third phase of recovery of amoeboid properties and motility necessary for active discharge of their macrophagic function (del Río Hortega, 1932).

Amoeboid microglia are thought to have a crucial scavenger function in the developing brain by removing the large number of cells in the neocortex that die in the course of normal remodeling of the fetal brain (Lue et al., 2001). The state of microglial activation represents a continuum that is reflected by in vitro studies, with relatively minor changes being observed just in the process of preparing and culturing amoeboid microglia, which express CD14, a marker not found in ramified microglia. The findings showing activated microglia in areas of neuronal loss, such as in amyloid plaque deposits seen in the brains of patients with Alzheimer disease, does not necessarily indicate a causal role in the associated neurodegeneration. The biological and pathophysiological significance of the anatomical heterogeneity of microglia is still unknown.

1.4 MeCP2 in glial cell function:

Initially, neuronal MeCP2 was thought to be the main cause of RTT (Chen et al., 2001). However, after years of study, MeCP2 was recognized as a key regulator in various other cell types including macroglia (oligodendrocytes and astrocytes) and microglia taking an integral part of manifestation of RTT by affecting neuronal structure and function (Ballas et al., 2009; Derecki et al., 2012; Lioy et al., 2011; Maezawa and Jin, 2010; Nguyen et al., 2012). Initially a study revealed that the gene expression profiles of postmortem female RTT brain showed decreased levels in the expression of neuronal genes encoding synaptic markers and increased levels of expression of glial genes involved in neuropathological mechanisms (Colantuoni et al., 2001). With the help of brain magnetic resonance, a study in 2006 showed that there was a significant reduction in the thickness of the corpus callosum in Mecp2-/y mice which shows that white matter is affected in Rett syndrome (Saywell et al., 2006). In spite of these changes observed in the patient samples and MeCP2 null mice, glial degeneration had not been reported in Rett Syndrome (Jellinger, 1988) moreover the balance between neuronal and glial lineages produced from neural progenitors also appeared to be normal (Kishi and Macklis, 2004). In 2009 a report showed that MeCP2 is expressed in glial cells of CNS and astrocytes in the RTT brain carrying MeCP2 mutations have a non–cell autonomous effect on neuronal properties and thus cannot support neuronal growth, indicating a critical role of MeCP2 in glial function (Ballas et al., 2009). Interestingly, a transgenic mice was

generated in which MeCP2 was absent specifically in the oligodendrocyte lineage cells showed the locomotor deficits with severe hindlimb clasping phenotype (Hu et al., 2013). Also it has been shown that myelin gene expression are altered inMeCP2-null mice (Nguyen et al. 2013; Vora et al., 2010). Besides this it was reported that the protein levels of MBP, PLP, DM-20, and BDNF also significantly upregulated in MeCP2 knockdown oligodendrocytes (Sharma et al., 2015). A recent study was done from the cortical tissues obtained from the murine model of RTT by using RNA-Seq, proteomics and have compared their data using available databases of cellular brain gene expression patterns to better understand RTT pathophysiology. This study revealed new glial genes, proteins, and pathways which seem to suggest abnormal glial morphology as well as aberrant myelination functions in RTT (Pacheco et al., 2017).

1.5 Therapeutic Implications of MeCP2 phosphorylation in Rett Syndrome

The mutations found in RTT extent the complete MeCP2 protein which includes missense, nonsense, insertion, deletion, frame shift mutation and splice-site mutation (Bienvenu and Chelly, 2006; Kriaucionis and Bird, 2003; Matijević et al., 2006). However there are reports suggesting that there is duplication of the entire MeCP2 locus in several patients of RTT which suggests that the overexpression of MeCP2 proteins also leads to RTT (Ariani et al., 2004; Lugtenberg et al., 2006; Meins et al., 2005; Ramocki et al., 2009; Van Esch et al., 2005). However the major unresolved issue is how the disruption of this ubiquitously expressed MeCP2 protein leads to pathogenesis of RTT.

The symptoms of RTT develop after 6 to 18 months of apparently normal development after which they show severe problems with language and communication skills, learning, coordination, and other brain functions such as microcephaly. However, the symptoms of RTT appear during early childhood when sensory involvement is driving the synaptic reorganization essential for the development of appropriately functioning circuits in the mature brain. This observation leads to the hypothesis that the underlying cause of RTT is inappropriate synaptic connectivity or plasticity, resulting from

abnormal experience-dependent synaptic maturation, refinement, and/or maintenance (Watase and Zoghbi, 2003). In 2006 Chen et al., showed that by triggering the MeCP2 phosphorylation, neuronal activity regulates a program of gene expression that mediates nervous system maturation and that disruption of this process in individuals with mutations in MeCP2 may underlie the neural-specific pathology of RTT. Another finding in agreement to this provides genetic evidence that neuronal activity-induced MeCP2 phosphorylation is required for the development, maintenance, and function of the adult mouse brains (Li et al., 2011). Additionally, these results suggest that the emerging picture of epigenetic regulation of nervous system function, which include not only changes in DNA methylation and histone modifications, but also a critical role for stimulus-dependent phosphorylation of a methyl-DNA binding protein (Li et al., 2011).

Unlike different histone modifications which correlate with independent and often opposing effects on gene expression, various MeCP2 modifications also influences the chromatin at sites where they occur. It is known that MeCP2 is a multifunctional protein which is due to the discovery of multiple, independently occurring phosphorylation events on MeCP2 (Huttlin et al., 2010; Tao et al., 2009; Zhou et al., 2006). However, by triggering MeCP2 phosphorylation in neurons, gene expression is regulated thereby maintaining the neuronal connectivity in the nervous system. The disturbance in this process in individuals with MeCP2 mutations may underlie the neural-specific pathology of RTT. These studies are important in the understanding of RTT and the disease mechanisms. Hence, these findings suggest that the regulation of MeCP2 phosphorylation represents a potential therapeutic approach in the treatment of RTT.

1.6 Rationale

MeCP2 has a multifunctional role; it not only acts as a transcriptional repressor but also as an activator. The recent evidences suggest that its function is controlled by certain post-translational modifications (PTMs) mainly phosphorylation and that may regulate its multifunctionality. Studies in phospho-defective Mecp2 knock-in mice have demonstrated that neuronal morphology and circuit formation are affected along with the changes in the mouse behavioral phenotypes with respect to specific phosphorylations.

Animal model studies have further shown that even the loss of a specific phosphorylation site of MeCP2 disturbs normal maturation of the mammalian brain. It has also been shown that some important genes are responsible for activity-dependent regulations are otherwise dependent on MeCP2 phosphorylation. MeCP2 expression was initially thought to be limited to neurons. However, few recent studies have shown the importance of MeCP2 in glial cells. But there is no information available so far on phosphorylation of glial MeCP2 and their regulation in response to different stimuli during early brain development which may play very significant roles in MeCP2 deficiency related pathogenesis.

1.7 Hypothesis

We hypothesize that MeCP2 in glial cells is functionally regulated by stimuli mediated phosphorylation which would allow MeCP2 to bind to distinct sites with an effect on chromatin affinity through sub-cellular localization thereby providing regulatory specificity during CNS myelination.

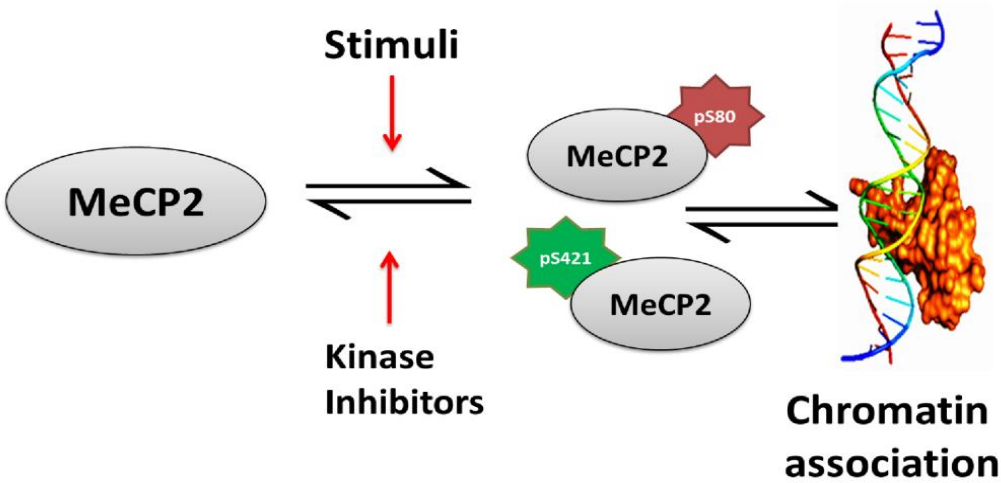

Fig 1.6 Hypothetical model of regulation of MeCP2 phosphorylation in glial cells

1.8 Key questions

1. What are the expression and distribution patterns of MeCP2 phosphorylation?

2. What are the factors regulating MeCP2 phosphorylation**?**
3. How MeCP2 phosphorylation regulates its chromatin affinity and association?

1.9 Significance

Elucidating how post-translational modifications may affect MeCP2 function in glial cells and will advance our understanding of MeCP2 normal function and the etiology of MeCP2 related disorders

1.10 Objectives

1. *To study the expression and sub-cellular localization of MeCP2 in glial cells*
2. *To study the factors regulating MeCP2 phosphorylation in glial cells*
3. *To study the underlying molecular mechanisms regulating MeCP2 phosphorylation and its chromatin association in glial cells.*