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Regulation and function of stimulus-induced phosphorylation of MeCP2

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Abstract

DNA methylation-dependent epigenetic regulation plays important roles in the development and function of the mammalian nervous system. MeCP2 is a key player in recognizing methylated DNA and interpreting the epigenetic information encoded in different DNA methylation patterns. Mutations in the *MECP2* gene cause Rett syndrome, a devastating neurological disease that shares many features with autism. One interesting aspect of MeCP2 function is that it can be phosphorylated in response to diverse stimuli. Insights into the regulation and function of MeCP2 phosphorylation will help improve our understanding of how MeCP2 integrates environmental stimuli in neuronal nuclei to generate adaptive responses and may eventually lead to treatments for patients.

Keywords

MeCP2; phosphorylation; Rett syndrome

Introduction

DNA methylation and histone tail modifications are the two most extensively studied epigenetic marks. Methyl-DNA binding proteins can act as a molecular linker between these two epigenetic marks. As the founding member of the methyl- DNA binding domain containing protein (MBD) gene family (Lewis et al., 1992), the methyl-CpG binding protein 2 gene (*MECP2*) has been linked to Rett syndrome (RTT) (Amir et al., 1999), an autism spectrum developmental disorder (Hagberg, 1985). Moreover, several recent studies have found that *MECP2* is altered at both the genomic level and the expression level in many autism patients (Nagarajan et al., 2006; Xi et al., 2007; Nagarajan et al., 2008; Ramocki et al., 2009). Thus, studying the function of MeCP2 will not only advance our understanding of

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Compliance with ethics guidelines

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RTT, but may also provide insights into the mechanisms underlying a broad spectrum of neurological diseases.

The MeCP2 protein specifically binds to methylated DNA (Lewis et al., 1992; Nan et al., 1997). Earlier studies are mostly consistent with MeCP2 acting as a transcription repressor through its interaction with a core repressor complex containing mSin3A and histone deacetylases (Jones et al., 1998; Nan et al., 1998). However, recent evidence suggests MeCP2 can also activate gene transcription through its interaction with CREB and co-activators (Chahrour et al., 2008). MeCP2 protein is almost as abundant as the histone octamers in the mouse brain, and is widely distributed across the entire genome tracking the density of 5-methylcytosine (Skene et al., 2010). Similar to histones, MeCP2 is subject to posttranslational modifications, such as phosphorylation (Chen et al., 2003). Thus, MeCP2 appears to have the necessary molecular properties in serving as a master molecular switch on the chromatin to integrate diverse extracellular signals and generate adaptive transcriptional/functional outputs. To test this hypothesis, several key questions need to be addressed. First, how many of these potential sites get phosphorylated in neurons *in vivo*? Second, what stimulus induces phosphorylation at which site in what type of neurons? Third, what is the *in vivo* function of any such phosphorylation? Fourth, does any such phosphorylation change the ability of MeCP2 to bind to either methyl-CpG or MeCP2-interacting proteins? Here, we will review the recent advances in studying MeCP2 phosphorylation, focusing on the mechanisms of how MeCP2 phosphorylation is regulated and how phosphorylation fine-tunes MeCP2 function. We will also summarize the results from mouse models in understanding the *in vivo* roles of MeCP2 phosphorylation in the development and function of the mammalian brain.

MeCP2 phosphorylation

MeCP2 phosphorylation was initially discovered by the Greenberg group in a study aimed to identify the role of MeCP2 in neuronal activity-dependent transcription regulation (Chen et al., 2003). A previously unknown slow-migrating form of MeCP2 was observed from protein lysate of membrane-depolarized cortical neurons in SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Alkaline phosphatase treatment of the lysate led to the disappearance of this slow-migrating form of MeCP2, suggesting this MeCP2 species is a result of phosphorylation (Chen et al., 2003). This phosphorylation site was later identified as serine 421 (S421), because S421 to alanine mutation abolished this neuronal activity-induced MeCP2 mobility shift (Zhou et al., 2006). However, S421 is not the only site of MeCP2 that can be phosphorylated, as mass spectrometry analysis of immuno-precipitated MeCP2 from normal and epileptic rodent brains identified 8 potential phosphorylation sites, including S80, T148/S149, S164, S229, S399, S421 and S424 (Tao et al., 2009). Interestingly, phosphorylation of S421 and S424 is only present in the slow-migrating form of MeCP2 purified from the epileptic brain, whereas phosphorylation of other sites exists in both the basal and slow-migrating forms of MeCP2 (Tao et al., 2009). Most recently, three additional MeCP2 phosphorylation sites (S86, S274 and T308) have been identified by phosphotryptic mapping (Ebert et al., 2013). MeCP2 phosphorylation at S86, S274 and T308 is detectable under basal condition, but is greatly induced by neuronal activity in both cultured cortical neurons and intact brains. Many of the phosphorylation sites identified so

far are located in important functional domains of the MeCP2 protein (Figure 1), suggesting that the precise regulation of the phosphorylation state at these sites may significantly influence the molecular function of MeCP2.

Regulation of MeCP2 phosphorylation

In cultured cortical neurons, membrane depolarization-induced MeCP2 S421 phosphorylation can be detected as early as 5 min after stimulation, and gradually reaches its maximal level in 30–60 min after depolarization (Chen et al., 2003; Zhou et al., 2006). Neurotransmission stimulation with glutamate, NMDA or GABAA-receptor antagonist bicuculline and neurotrophin treatment with BDNF, NT3, or NT4 are also sufficient to induce MeCP2 S421 phosphorylation in cortical neurons (Zhou et al., 2006). The induction of MeCP2 S421 phosphorylation requires calcium influx through the L-type voltage-gated calcium channels (L-VSCCs), as LVSCCs antagonist nimodipine blocks neuronal activity-dependent MeCP2 S421 phosphorylation (Chen et al., 2003; Zhou et al., 2006).

Consistent with the involvement of calcium signaling pathway, two calcium/calmodulin-dependent protein (CaM) kinases, CamKII and CamKIV, have been implicated in mediating MeCP2 S421 phosphorylation. Zhou et al. showed that specific inhibitor of CamKII abolishes membrane depolarization-induced MeCP2 S421 phosphorylation, and recombinant constitutively active CamKII is able to phosphorylate MeCP2 *in vitro* (Zhou et al., 2006). Buchthal et al. further confirmed that CamKII, generally considered as a cytosolic enzyme, is also present in the nucleus, and overexpression of a constitutively active form of CamKII in cultured hippocampal neurons is sufficient to induce MeCP2 S421 phosphorylation (Buchthal et al., 2012). Tao and colleagues, however, showed that siRNA knockdown of CamKIV greatly attenuates membrane depolarization-induced MeCP2 S421 phosphorylation (Tao et al., 2009). No direct binding has been established between MeCP2 and either kinase. Whether CamKII/IV indeed directly phosphorylates MeCP2 *in vivo* or actually acts as an upstream intermediate for the induction of S421 phosphorylation remains unknown.

Although MeCP2 is ubiquitously expressed in many mammalian tissues, MeCP2 S421 phosphorylation is only detected in the brain (Zhou et al., 2006). Stimulus-induced MeCP2 S421 phosphorylation has been reported in specific neuronal subtypes in various brain regions. The first reported case is light-induced S421 phosphorylation in neurons of the suprachiasmatic nucleus (SCN) (Zhou et al., 2006). In the hypothalamus, early life stress induces S421 phosphorylation specifically in neurons in the parvocellular division of the hypothalamic paraventricular nucleus (Murgatroyd et al., 2009). In the striatum, antidepressant drugs, such as amphetamine (AMPH) and imipramine, have been shown to induce S421 phosphorylation selectively in GABAergic interneurons of the ventral structure of nucleus accumbens (NAc) (Deng et al., 2010; Hutchinson et al., 2012b), while cocaine has been shown to induce S421 phosphorylation in both ventral (NAc) and dorsal (caudate putamen) structures (Mao et al., 2011). In the hippocampus, both fear conditioning and Morris water maze training induce S421 phosphorylation in CA1–3 neurons (Li et al., 2011). In the peripheral nervous system (PNS), S421 phosphorylation was first observed in the superficial dorsal horn neurons in the spinal cord after induction of peripheral joint

inflammation (Geranton et al., 2007). Later work from the same group suggested S421 phosphorylation in the superficial dorsal horn neurons is regulated by the convergence of the descending serotonergic inputs and the ascending primary afferent stimulation (Géranton et al., 2008), which provides the first example of integration of synaptic inputs through MeCP2 phosphorylation. Such type of integration also appears to be responsible for the neuronal subtype specificity of AMPH-induced S421 phosphorylation in the NAc. AMPH activates multiple monoamine neurotransmitter systems, among which activation of the dopamine (DA) and serotonin (5-HT) systems, but not the norepinephrine (NE) system, are sufficient to induce S421 phosphorylation (Hutchinson et al., 2012a). A separate study by Mao et al. (2011) has implicated the NMDA receptor as the mediator of cocaine-induced S421 phosphorylation in the dorsal, but not the ventral striatum. To date, S421 phosphorylation has been observed only in postmitotic neurons.

Opposite to the dynamics of S421 phosphorylation, S80 is the most constitutively phosphorylated residue in resting neurons and undergoes dephosphorylation upon membrane depolarization (Tao et al., 2009). S80 dephosphorylation also requires calcium influx through L-VSCCs, as nimodipine effectively blocks membrane depolarization-induced S80 dephosphorylation (Tao et al., 2009). Different from S421 phosphorylation, S80 phosphorylation has been observed in non-neuronal cells, such as Hela cells and human fibroblasts (Bracaglia et al., 2009; Tao et al., 2009). Homeodomain-interacting protein kinase 2 (HIPK2) binds to MeCP2 and is able to phosphorylate MeCP2 at S80 *in vitro* (Bracaglia et al., 2009). Furthermore, overexpression of HIPK2 in human fibroblasts results in a significant increase in S80 phosphorylation, and siRNA knockdown of HIPK2 in cortical neurons strongly reduces the level of S80 phosphorylation, further supporting the involvement of HIPK2 in mediating S80 phosphorylation (Bracaglia et al., 2009). The phosphatase responsible for the neuronal activity-dependent S80 dephosphorylation is still unknown.

In cortical neurons, MeCP2 phosphorylation at S86, S274 and T308 is also induced by membrane depolarization. Interestingly, stimulation with BDNF or with forskolin to activate protein kinase A (PKA) is able to induce S86 and S274 phosphorylation, but fails to activate T308 phosphorylation (Ebert et al., 2013).

A summary of our current knowledge on the extracellular signals and the intracellular signaling pathways leading to the phosphorylation/dephosphorylation of various residues on MeCP2 protein is shown in Figure 2.

Phosphorylation modifies the function of MeCP2

Phosphorylation is an important regulator of protein function. The addition of a phosphate group can modify the activity of the target protein by modifying protein electrostatics, by inducing protein conformational changes, and by altering protein–protein interactions. Here, we summarize recent findings in how phosphorylation at multiple residues modifies the function of MeCP2 in various ways, including DNA binding, transcription regulation, and protein–protein interactions.

The relative slow kinetics of MeCP2 S421 phosphorylation after membrane depolarization correlates with the slow induction of a subgroup of neuronal activity-regulated genes, such as *Bdnf* and *Narp* (Chen et al., 2003; Zhou et al., 2006), raising the possibility that the phosphorylation of MeCP2 S421 plays a role in the activation of this class of genes. Consistent with this hypothesis, Chen et al. showed that membrane depolarization induces the release of MeCP2 from *Bdnf* promoter III in rat cortical neurons, and the slow-migrating phosphorylated form of MeCP2 binds poorly to methylated *Bdnf* promoter *in vitro* (Chen et al., 2003). Zhou et al. (2006) further presented that neuronal activity-dependent *Bdnf* induction is attenuated in cortical neurons overexpressing the non-phosphorylatable MeCP2^{S421A} mutant protein, compared to neurons overexpressing wild type (WT) MeCP2. However, these results were challenged by a recent study, in which Cohen et al. (2011) showed that membrane depolarization does not alter the binding of MeCP2 to the promoters of multiple neuronal activity-dependent genes, and the MeCP2 occupancy at *Bdnf* promoter is indistinguishable between cortical neurons cultured from WT and *Mecp2*^{S421A} knockin mice. The authors reasoned that the inconsistent results among multiple studies are possibly due to the fact that the chromatin immunoprecipitation (ChIP) assays performed in the earlier work were semiquantitative and more subject to error. In addition, the extent and time course of *Bdnf* induction upon membrane depolarization is not significantly different between WT and *Mecp2*^{S421A} cortical neurons (Cohen et al., 2011). But it remains possible that phosphorylation of MeCP2 at additional sites are involved in regulating *Bdnf* expression.

In fact, S424 phosphorylation was observed together with S421 phosphorylation in slow-migrating form of MeCP2 induced by neuronal activity (Tao et al., 2009). We reported that *Mecp2*$^{S421A;S424A}$ knockin mice exhibit increased *Bdnf* transcript level in the hippocampus, and the MeCP2$^{S421A;S424A}$ protein binds more tightly to *Bdnf* promoters than the wild type MeCP2 does, suggesting S424 phosphorylation may collaborate with S421 phosphorylation in regulating gene transcription (Li et al., 2011). In addition to its direct effect on gene transcription, increased chromatin binding by the phosphor mutant MeCP2 protein may also influence the modification of DNA by further protecting the methylated CpG dinucleotides. This is possible because the loss of MeCP2 has been shown to correlate with an increase in hydroxymethylation at CpG sites (Szulwach et al., 2011), which may have been a result of loss of protection of the those methylated CpGs.

S80 is located at the N-terminal of the MBD domain of MeCP2, thus phosphorylation of S80 was proposed to affect the DNA binding affinity of MeCP2. Tao et al. demonstrated that mutating S80 to alanine leads to decreased binding of MeCP2 at the promoters of multiple genes, including *Rab3d, Vamp3* and *Igsf4b*. They also showed that cortical neurons overexpressing MeCP2^{S80A} exhibit increased *Rab3d, Vamp3* and *Igsf4b* expression compared with neurons overexpressing WT MeCP2 (Tao et al., 2009). 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., 2014). Cheng at al. reported that the N terminus and the C terminus of MeCP2 form an intramolecular interaction. In resting neurons, phosphorylation at S80 blocks this intramolecular interaction, making the C terminus of MeCP2 accessible for its association

with DGCR8. In response to neuronal activity, S80 undergoes dephosphorylation and the intramolecular interaction of MeCP2 is reestablished, leading to the release of DGCR8 for regulating neuronal activity-dependent micro-RNA processing (Cheng et al., 2014). Furthermore, Gonzales et al. suggested that phosphorylation of MeCP2 at S80 may influence the association of MeCP2 to transcription repressor Sin3A, heterochromatin protein 1 (HP1), cohesin complex member SMC3, and RNA binding protein YB1 (Gonzales et al., 2012). In addition, Rexach et al. demonstrated a reversible yin-yang relationship between S80 phosphorylation and MeCP2 glycosylation (Rexach et al., 2010), which was the first report suggesting a crosstalk between MeCP2 phosphorylation and other posttranslational modifications.

The phosphorylation site T308 is proximal to a common RTT missense mutation R306C, a mutation that disrupts the ability of MeCP2 to interact with the nuclear receptor co-repressor (NCoR) complex (Lyst et al., 2013). Ebert et al. demonstrated that phosphorylation of T308 abolishes the interaction between MeCP2 and NCoR complex, and suppresses MeCP2-NCoR-mediated transcription repression (Ebert et al., 2013). In cortical neurons isolated from T308A knockin mice, the induction of a subset of neuronal activity-regulated genes, including *Npas4* and *Bdnf*, is reduced compared to the WT counterpart (Ebert et al., 2013).

In an early report, Miyake at al. suggested that phosphorylation of MeCP2 regulates the intracellular localization of MeCP2 during neuronal cell differentiation (Miyake and Nagai, 2007). However, multiple studies using phospho-specific antibodies of MeCP2 showed that the subcellular distribution of MeCP2 with phosphorylation at S80, S229, or S421 is indistinguishable from that of total MeCP2, and mutating serine to alanine or aspartic acid at these residues does not affect the subcellular localization of MeCP2 (Zhou et al., 2006; Tao et al., 2009; Gonzales et al., 2012).

A summary of our current knowledge on how phosphorylation at several sites may regulate the molecular function of MeCP2 is shown in Figure 2.

In vivo function of MeCP2 phosphorylation

Among all the phosphorylation sites of MeCP2, S421 has been the most extensively studied. *In vitro* results have suggested that neuronal activity-induced S421 phosphorylation may regulate MeCP2 binding to the *Bdnf* promoter (Chen et al., 2003), *Bdnf* transcription, dendritic growth and spine maturation (Zhou et al., 2006). Several correlative *in vivo* studies have implicated S421 phosphorylation in regulating pain sensitivity (Géranton et al., 2007, 2008), encoding early life stress (Murgatroyd et al., 2009), and modulating behavioral responses to psychostimulants (Deng et al., 2010). To directly study the *in vivo* function of S421 phosphorylation, two knockin mice were independently generated. The Greenberg group made a $Mecp2^{S421A}$ allele. Our group made a $Mecp2^{S421A;S424A}$ allele. We decided to make the S421A;S424A double knockin allele because our mass spectrometry study identified both serines potentially phosphorylated in response to neuronal activity (Tao et al., 2009).

Neural plasticity and cognitive function

Rett syndrome features mental retardation and loss of acquired language and motor skills (Chahrour and Zoghbi, 2007). *Mecp2* deficient mice display impaired learning and memory (Moretti et al., 2006), attenuated LTP and LTD (Asaka et al., 2006; Moretti et al., 2006). Furthermore, neurons from *Mecp2* deficient mice present decreased dendritic complexity and reduced excitatory synapse formation (Chao et al., 2007). Elucidating the role of MeCP2 S421 phosphorylation in brain development and plasticity is a key step to understand how S421 phosphorylation modifies MeCP2 function and how S421 phosphorylation might contribute to Rett syndrome.

Because both increased and decreased expression of MeCP2 leads to deficits in neuronal function, we first confirmed that the level of MeCP2$^{S421A;S424A}$ is comparable to that of the wild type MeCP2, and that S421 phosphorylation is abolished in the *Mecp2*$^{S421A;S424A/y}$ mice (Li et al., 2011). While we made multiple attempts to generate an antibody that specifically recognizes phosphorylated S424, we have yet to succeed. The lack of a phosphor-S424 antibody prevents us from directly assess the status S424 phosphorylation *in vivo*. Nonetheless, we performed detailed analysis of the *Mecp2*$^{S421A;S424A/y}$ mice, and found that, comparing to WT mice, they perform better in two hippocampus-dependent spatial learning/memory assays (fear conditioning and Morris water maze), have enhanced long-term potentiation at two hippocampal synapses (the Schaffer collateral-CA1 synapse and the dentate-CA3 synapse), have increased number of excitatory synapses on hippocampal and cortical neurons, and show related gene expression changes in the adult hippocampus (Li et al., 2011). It is worth noting that these phenotypes of *Mecp2*$^{S421A;S424A/y}$ mice are remarkably similar to what was observed in mice with MeCP2 overexpression, and opposite to that of *Mecp2* deficient mice (Collins et al., 2004; Chao et al., 2007; Chahrour et al., 2008). In addition, the MeCP2$^{S421A;S424A}$ protein appears to bind more tightly to gene promoters than the wild type MeCP2 does (Li et al., 2011). These results indicate that loss of S421/S424 phosphorylation might turn MeCP2 to a hypermorph.

MeCP2 has also been implicated in homeostatic synaptic scaling, a non-Hebbian form of synaptic plasticity (Qiu et al., 2012). MeCP2 S421 phosphorylation is dynamically regulated during the process of synaptic scaling (Zhou et al., 2006; Tao et al., 2009; Qiu et al., 2012). Our group tested whether S421 and/or S424 phosphorylation is required for homeostatic synaptic scaling. We demonstrated that hippocampal neurons cultured from *Mecp2*$^{S421A;S424A/y}$ mice present normal tetrodotoxin (TTX)-induced synaptic scaling up, but exhibit impaired bicuculline-induced synaptic scaling down (Zhong et al., 2012). In addition, neurons from *Mecp2*$^{S421A;S424A/y}$ mice showed reduced metabotropic glutamate receptor 5 (mGluR5) at both mRNA and protein levels than neurons from WT mice. Pretreatment with group I mGluR agonist or overexpression of GluR5 in *Mecp2*$^{S421A;S424A/y}$ neurons is sufficient to restore the bicuculline-induced synaptic scaling down to normal level (Zhong et al., 2012).

In a separate study, the Greenberg group reported their characterization of the *Mecp2*$^{S421A/y}$ mice (Cohen et al., 2011). Surprisingly, the *Mecp2*$^{S421A/y}$ mice only showed mild phenotypes, including increased dendritic complexity, increased inhibitory

neurotransmission, and a deficit in hippocampal learning/memory (novel object recognition behavior). Moreover, they reported no change in MeCP2 binding to DNA across the genome after neuronal activity-induced S421 phosphorylation (Cohen et al., 2011). It should be cautioned that it is difficult to achieve the necessary sequencing depth in ChIP-seq analysis to accurately quantify MeCP2 binding changes across the genome, because MeCP2 binds to so many places across the genome. Despite of this caveat, the *Mecp2*$^{S421A/y}$ mice appear to be phenotypically different from the *Mecp2*$^{S421A;S424A/y}$ mice. The obvious genetic difference (S424 vs. A424) between these two mice may help explain the phenotypic difference. First, S424 may be either phosphorylated or modified in some unknown ways in response to neuronal activity, which work together with S421 phosphorylation to ensure the correct function of MeCP2. Indeed, the MeCP2 sequence from S421 to S424, when S421 is phosphorylated, matches the consensus phosphorylation site of Casein kinase 1 (pS/T-X-X-S/T). The possible crosstalk between S421 and S424 phosphorylation requires further investigation. Second, the A424 mutation may have affected MeCP2 function independent of neuronal activity-induced S421 phosphorylation. Generation of the *Mecp2*$^{S424A/y}$ mouse and a phospho-S424 specific antibody will undoubtedly help distinguish the two possibilities.

Monoamine neurotransmission related mood regulation and drug addiction

Monoamine neurotransmission is essential in emotion regulation and has been implicated in mood disorders, such as depression and anxiety. RTT patients develop anxiety, aggression, and mood alterations (Chahrour and Zoghbi, 2007), accompanied with reduced levels of dopamine and serotonin (5-HT) metabolite (Fyffe et al., 2008). The activation of dopamine or 5-HT receptor is sufficient to induce MeCP2 S421 phosphorylation in NAc (Hutchinson et al., 2012a). Antidepressants, such as imipramine, increase the extracellular levels of monoamine neurotransmitters and induce S421 phosphorylation selectively in GABAergic interneurons of the ventral structure of nucleus accumbens (NAc) (Hutchinson et al., 2012b). Hunchinson et al. (2012b) showed that *Mecp2*$^{S421A/y}$ mice exhibit increased sensitivity to environmental stress in tail-suspension test and forced-swim test and do not generate the normal response to chronic imiparmine treatment in a chronic social defeat stress paradigm, suggesting a role of MeCP2 S421 phosphorylation in depression-like behavior and the behavioral response to antidepressant treatment.

Similar to antidepressants, psychostimulants, including cocaine and AMPH, activate monoamine receptors and are able to induce MeCP2 S421 phosphorylation in NAc (Deng et al., 2010; Mao et al., 2011). Deng at al. (2014) reported that *Mecp2*$^{S421A/y}$ mice display a reduced threshold for behavioral sensitization to experimenter-administered AMPH and increased sensitivity to chronically self-administered cocaine. At the cellular level, the authors observed a decrease in intrinsic excitability of the medium spiny neurons (MSNs) in the NAc shell of *Mecp2*$^{S421A/y}$ mice exposed to repetitive AMPH. At the molecular level, repetitive cocaine administration- induced CREB expression is impaired in the NAc of *Mecp2*$^{S421A/y}$ mice (Deng et al., 2014). These results indicated that S421 phosphorylation is required to induce both behavioral and cellular adaptations to psychostimulants.

The *in vivo* function of MeCP2 phosphorylation at other sites

Phosphorylation of MeCP2 at S80 and S421/S424 are dynamically regulated in opposite manners by neuronal activity (Tao et al., 2009). More interestingly, *Mecp2*$^{S80A/y}$ mice showed decreased locomotor activity, whereas *Mecp2*$^{S421A;S424A/y}$ mice presented increased locomotor activity (Tao et al., 2009), suggesting that S80 and S421/S424 phosphorylation might indeed play opposing roles in the brain. A more comprehensive behavioral analysis of *Mecp2*$^{S80A/y}$ will be needed to gain better understanding in this area. *In vitro* results also suggest that S80 phosphorylation play roles in regulating apoptosis (Bracaglia et al., 2009) and microRNA processing (Cheng et al., 2014), and it is necessary to examine whether loss of S80 phosphorylation in *Mecp2*$^{S80A/y}$ mice will lead to the corresponding alterations in such processes *in vivo*.

Besides S421 and S80, the other phosphorylation site that draws a lot of recent attention is T308, whose phosphorylation leads to the disruption of the interaction between MeCP2 and NCoR complex (Ebert et al., 2013). *Mecp2*$^{T308A/y}$ mice have a normal bodyweight, but show a mild decrease in brain weight. *Mecp2*$^{T308A/y}$ mice display hindlimb clasping and show motor coordination deficit in accelerating rotarod test. *Mecp2*$^{T308A/y}$ mice also have a reduced seizure threshold compared to WT mice (Ebert et al., 2013). Although more detailed behavioral characterization needs to be performed on *Mecp2*$^{T308A/y}$ mice, these initial results indicate that *Mecp2*$^{T308A/y}$ mice present some types of RTT-like phenotype, suggesting disruption of T308 phosphorylation and disregulation of MeCP2/NcoR interaction may contribute to RTT pathogenesis caused by mutations in the vicinity of T308.

Looking into the future

As discussed above, we have learned a great deal about MeCP2 S421 phosphorylation. Going forward, it will be important for the field to reconcile the difference between the *Mecp2*$^{S421A/y}$ mice and the *Mecp2*$^{S421A;S424A/y}$ mice, and confirm the identity of S421 kinase by direct binding. To fully understand how phosphorylation states are tightly controlled at S421, it will be important to identify the phosphatase that de-phosphorylate S421 and study the molecular connect between the phosphatase and the responsible extracellular signals. To unequivocally determine the *in vivo* function of S421 phosphorylation, *Mecp2*$^{S421E/y}$ and/or *Mecp2*$^{S421D/y}$ mice (serine to glutamic acid or aspartic acid to mimic phosphorylation) should be generated and analyzed. More importantly, the field needs to move downstream and investigate how S421 phosphorylation affects physical interaction between MeCP2 and its interacting partners, so that we can begin to understand how stimulus-induced S421 phosphorylation may change chromatin state and gene transcription across the neuronal genome. While most of the MeCP2 phosphorylation studies have focused on neurons, it has become increasingly clear that other cell types in the brain also express MeCP2 and play significant roles RTT pathogenesis (Ballas et al., 2009; Lioy et al., 2011; Derecki et al., 2012; Nguyen et al., 2013). Thus, it will be interesting to determine whether S421 phosphorylation is present in these other cell types, how it is regulated and what functions it may have. The same set of questions applies to S80, T308 and all other potential phosphorylation sites on the MeCP2 protein.

If phosphorylation/dephosphorylation can indeed be induced at multiple sites by distinct stimuli in the same neuron, it would suggest signal integration on MeCP2 might produce a combinatorial phosphorylation code, which can then be translated into adaptive transcriptional/functional outputs. MeCP2 is already expressed at the level of histones, and widely distributed across the genome. Why not acquire another key feature of the histone?

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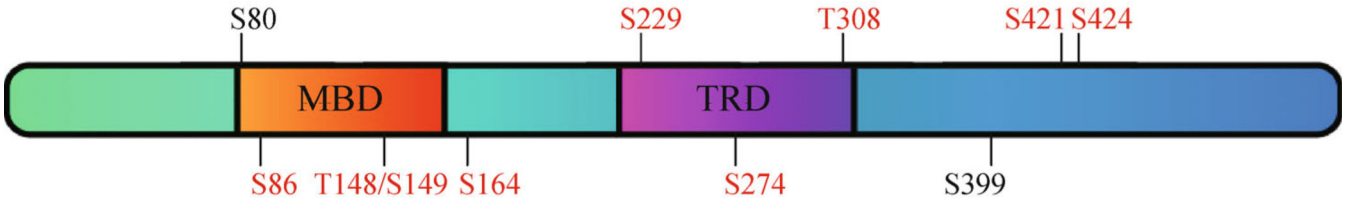

Figure 1.

Distribution of known phosphorylation sites on the MeCP2 protein. Neuronal activity-induced phosphorylation sites are marked in red. MBD, methyl-CpG binding domain; TRD, transcriptional repression domain.

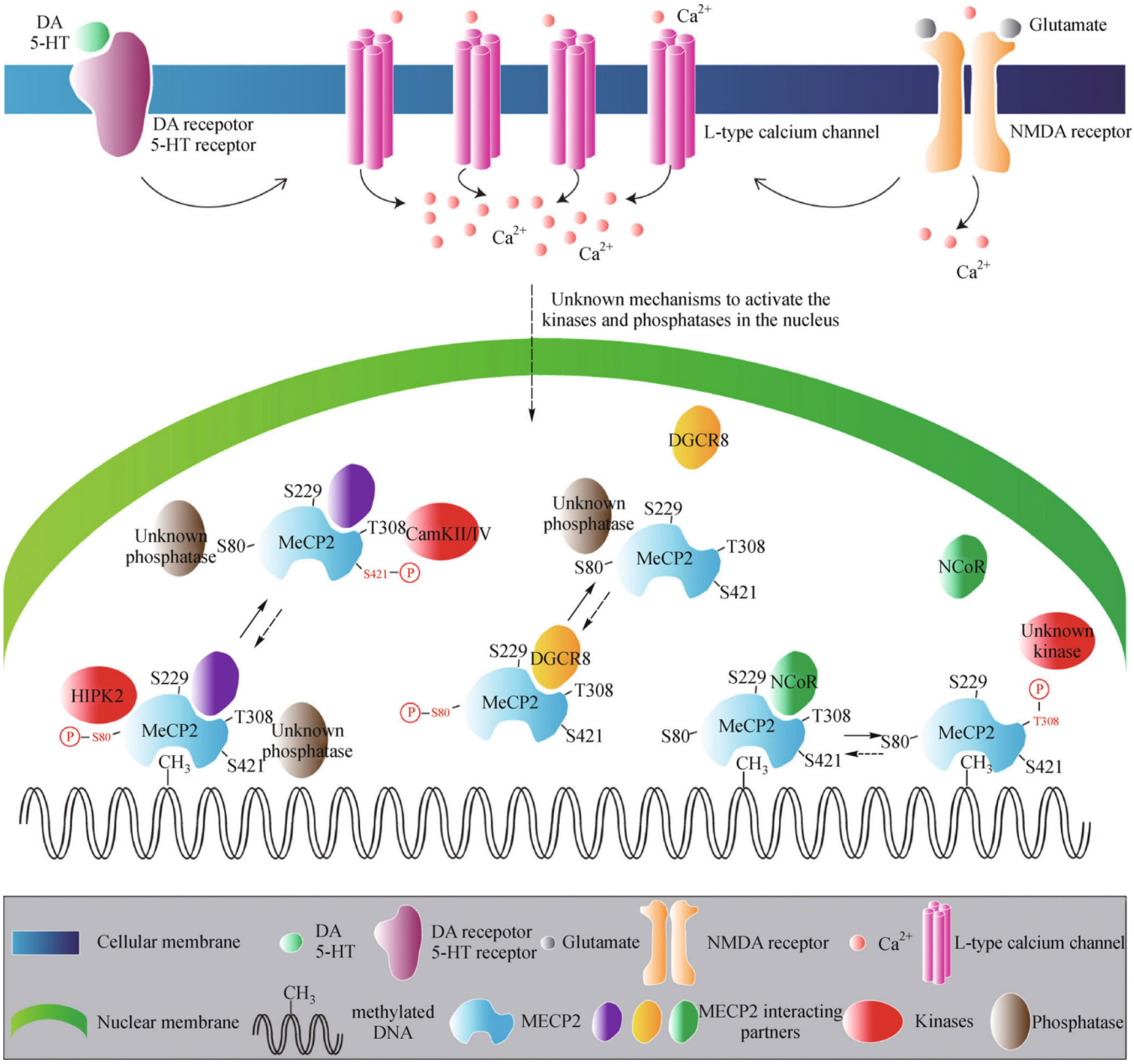

Figure 2.

Neuronal activity-induced phosphorylation and dephosphorylation modify MeCP2 function. Stimulation of NMDA receptors, DA receptors and 5-HT receptors, as well as membrane depolarization, activates L-type calcium channels on the cellular membrane and induces calcium influx. Specific kinases and phosphatases in the nucleus are subsequently activated and modify the phosphorylation status of MeCP2. Phosphoprylation of S421, together with dephosphorylation of S80, may modulate the binding of MeCP2 to the promoters of specific genes. Dephosphorylation of S80 releases DGCR8 from MeCP2 to regulate microRNA processing. Phosphorylation of T308 interrupts the association between MeCP2 and NCoR

co-repressor complex. NMDA receptors, N-methyl-D-aspartate receptors; DA receptors, Dopamine receptors; 5-HT receptors, 5-hydroxytryptamine receptors or serotonin receptors.