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Multifaceted roles of MeCP2 in cellular regulation and phase separation: implications for neurodevelopmental disorders, depression, and oxidative stress

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Abstract

Methyl CpG binding protein 2 (MeCP2) is a chromatin-associated protein that remains enigmatic despite more than 30 years of research, primarily due to the ever-growing list of its molecular functions, and, consequently, its related pathologies. Loss of function *MECP2* mutations cause the neurodevelopmental disorder Rett syndrome (RTT); in addition, dysregulation of MeCP2 expression and/ or function are involved in numerous other pathologies, but the mechanisms of MeCP2 regulation are unclear. Advancing technologies and burgeoning mechanistic theories assist our understanding of the complexity of MeCP2 but may inadvertently cloud it if not rigorously tested. Here, rather than focus on RTT, we examine relatively underexplored aspects of MeCP2, such as its dosage homeostasis at the gene and protein levels, its controversial participation in phase separation, and its overlooked role in depression and oxidative stress. All these factors may be essential to understanding the full scope of MeCP2 function in healthy and diseased states, but are relatively infrequently studied and require further criticism. The aim of this review is to discuss the esoteric facets of MeCP2 at the molecular and pathological levels and to consider to what extent they may be necessary for general MeCP2 function.

Key words: MeCP2, LLPS, homeostasis, regulation, oxidative stress, MDD

Introduction

Methyl CpG Binding Protein 2 (MeCP2) is best known for causing Rett Syndrome (RTT) and MeCP2 duplication syndrome, for neither of which there is a cure. In recent years, growing interest has been directed towards understanding the diverse molecular functions of MeCP2, with particular attention to its involvement in phase separation and its implications for neurological disorders. While all these data are essential for understanding the full scope of MeCP2 function, the degree of molecular and clinical importance of certain functionalities or psychiatric phenotypes is unclear. Here, we discuss recent developments in the characterization of the nuclear protein MeCP2. We first discuss molecular mechanisms behind functional regulation at the protein level and homeostasis. Focus will be given to liquid–liquid phase separation (LLPS), which has emerged as a compelling biophysical mechanism in regulating diverse biological processes, especially in the nucleus, but for which biologists should be careful in applying the definition, as will be discussed. Intriguingly, three separate groups published research in short succession in 2020 suggesting that MeCP2 undergoes LLPS, and that RTT-causing mutations disrupt its liquid behaviour (Fan et al. 2020; Li et al. 2020; Wang et al. 2020). The sudden surge of MeCP2 LLPS data suggests a potential missing link in understanding MeCP2 function in cells, but it has similarly already garnered criticism (Pantier et al. 2024). The discussion here will consolidate the current data on MeCP2 dosage and biophysical properties, and provide the required nuance in this conversation, which has many MeCP2 researchers excited. We then explore MeCP2 implications on major depressive disorder (MDD) and mitochondrial dysfunction with mutated MeCP2. MeCP2 dysregulation is linked to impaired expression of genes involved in stress response and synaptic plasticity, such as reelin (RELN) and brain-derived neurotrophic factor (BDNF) (Zhubi et al. 2014; Cao et al. 2022; Sanchez-Lafuente et al. 2022). These genes, negatively regulated by MeCP2, are crucial for neuronal development and maintenance, potentially governed by LLPS. Exploring LLPS in the context of MeCP2 function may shed light on its molecular interactions and their implications in conditions like RTT and MDD.

Table 1. Gene and protein regulators of MeCP2 dosage.

Regulation stage	miRNA/protein regulators	Type of regulation (±)	Mechanism of action	References
Gene -level	miR-132/212	–	Binds to the longer 3′UTR transcripts of MeCP2 (brain specific)	Klein et al. 2007
	miR-7b	–	Binds to 3′UTR—bi-directional feedback regulation	Chen et al. 2014
	HMGN1	–	Binds MeCP2 promoter; in brain of DS patients HMGN1 is overexpressed, which results in lower MeCP2 levels	Abuhatzira et al. 2011
	MEF2C	+	Mutation of MEF2C resulted in diminished MeCP2 and CDKL5 expression; details of mechanism unknown	Zweier et al. 2010
MeCP2-E1/E2 self regulation	E1 and E2 isoforms	–	*Mecp2* promoter was inhibited by both isoforms detected by eGFP signal and flow cytometry	Lockman et al. 2024
Protein-level	*N*-methionine excision	–	N-end rule describes that the destabilization of Nt-amino acids resulting in shorter half life. MeCP2 isoforms, which only differ in their Nt-sequence have significantly different half-lives	Bachmair et al. 1986; Bachmair and Varshavsky 1989; Sheikh et al. 2017; Martinez de Paz et al. 2019
	PEST-mediated degradation	–	MeCP2 PEST domains trigger proteolysis upon phosphorylation of a serine next to a proline, which is followed by the ubiquitination of a flanking lysine and proteasomal degradation	Rogers et al. 1986; Singh et al. 2006; Thambirajah et al. 2009; Kalani et al. 2024

MeCP2 dosage regulation at the DNA and mRNA level

MeCP2 protein levels oscillate by approximately 30% in a circadian cycle-dependent manner (Martinez de Paz et al. 2015); however, deviations from its specific range are deleterious. DNA methylation of *Mecp2* regulatory elements correlates with its transcript levels in a sex-specific manner in primary neurons and astrocytes (Liyanage et al. 2019). MeCP2 is regulated at the mRNA and protein level by several mechanisms, organized in Table 1 and discussed in detail below.

The nucleosomal and mRNA binding protein, high mobility group N1 (HMGN1), and several miRNAs bind the *Mecp2*/*MECP2* promoter and mRNA, respectively, (Abuhatzira et al. 2011), negatively regulating MeCP2 expression (Fig. 1A). Only one positive regulator of MeCP2 has been described so far: the myocyte enhancer factor 2 C (MEF2C) (Li et al. 2008; Meur et al. 2010).

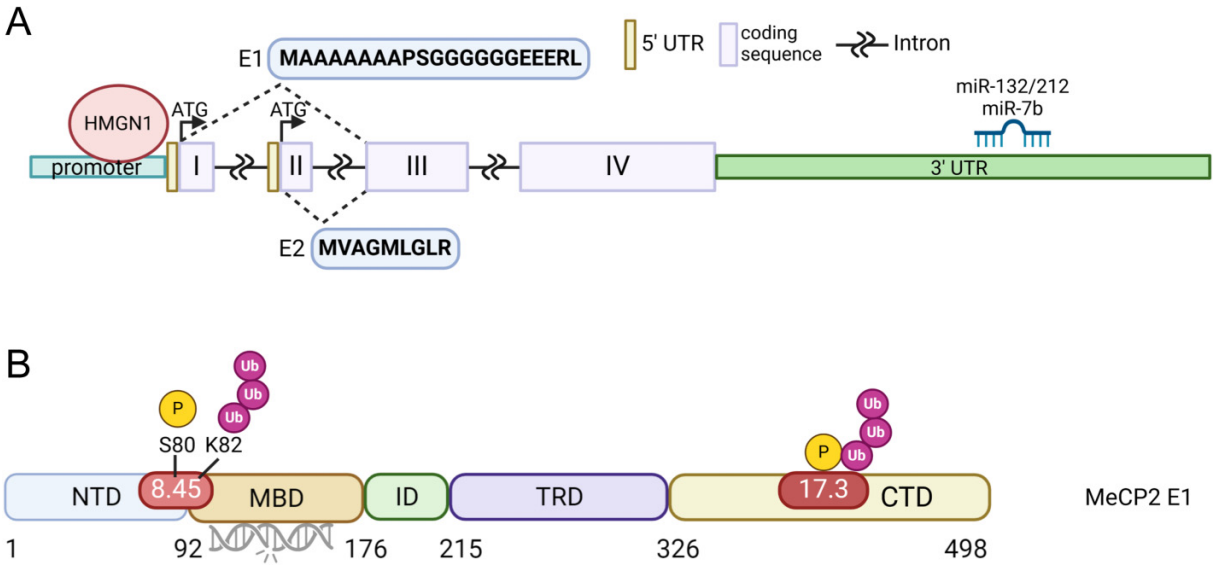
The original observation resulting in the finding that HMGN1 negatively regulates MeCP2 expression at the promoter level was due to its reduced expression in Down Syndrome (DS) patients. This finding substantiates the importance of studying MeCP2 in less common contexts. The miRNAs that bind to the miRNA recognition elementswithin the *MECP2* 3′UTR include CREB-induced miRNA 132/212 (Klein et al. 2007) and miRNA-7b (Chen et al. 2014) (Fig. 2A). *MECP2* has multiple polyadenylation sites, which result in several 3′UTRs ranging between ∼2 and 8.6 kilobases (Kbp). Longer transcripts found in the brain are highly conserved across mammals (Rodrigues et al. 2020); the miR-132 binding site is only available in the longer 3′UTR transcripts in the brain, suggesting that tissue-specific negative regulation by miR-132 is limited to the brain. The autoregulatory feedback loop between MECP2/BDNF/miR132 was originally identified in animal models of MDD, where miR-132 inhibits *MECP2* by 3′UTR binding, and MeCP2 binding to *BDNF* promoter III (Chen et al. 2003) reduces BDNF levels (Su et al. 2015). Recent findings from the postmortem human brains of control and RTT patients corroborated this autoregulation, though this mechanism may not be applied to the cerebellum (Pejhan et al. 2020).

Bi-directional feedback autoregulation exists between MeCP2 and miRNA-7b. The mouse miRNA-7b gene can become hypermethylated at its 5′-flanking region. MeCP2 recruitment to methylated CpG islands was shown to diminish the expression of miRNA-7b. Conversely, the binding of miRNA-7b to the 3′UTR of MeCP2 inhibits its protein levels (Fig. 1A). This autoregulation between MeCP2 and miRNA-7b occurs during postnatal murine neuronal maturation when MeCP2 levels increase (Chen et al. 2014).

MeCP2 dosage regulation at the protein level

At the protein level, two modes of regulation have been described. Both are specific to short-lived proteins (0.5 h < t < 4 h) and involve the adenosine triphosphate (ATP)-dependant covalent binding of ubiquitin to select MeCP2 lysine residues. The two models are the N-end rule, postulated initially by Alexandre Varshavsky, which describes how destabilizing N-terminal (Nt) residues determine protein

Fig. 1. MeCP2 regulation at the gene, transcript, and protein level. (A) *Mecp2*/*MECP2* is negatively regulated by HMGN1 binding to its core promoter and preventing its transcription. At the mRNA level, miR-132/212 and miR-7b bind to its 3′UTR and repress its translation into either the E1 (exon II excluded) or E2 (exon I excluded) isoforms, which differ only at their N-termini. (B) MeCP2 regulation by its PEST motifs, shown in red with their PEST scores. MeCP2 with deleterious mutations within its MBD is degraded at higher rates than WT. In PEST-mediated degradation, MeCP2 loses its stability by phosphorylation at a serine near a lysine, two of which have been identified (S80 and K82).



half-life (Bachmair and Varshavsky 1989), and proline/glutamic acid/serine/threonine (PEST)-mediated degradation, involving proteasome signalling by targeted post-translational modifications (PTMs), which is so far only defined in the context of MeCP2 with deleterious methyl DNA binding domain (MBD) mutations (Fig. 1B) (Kalani et al. 2024). The four domains surrounding the structured MBD are highly disordered, where the N-terminal domain (NTD) and intervening domain (ID), flanking that the MBD are associated with protein half life and facilitating MBD–DNA binding, whereas the transcriptional repression domain (TRD) and C-terminal domain (CTD) are generally known for MeCP2–protein interactions (Tillotson and Bird 2019). Both N-end rule and PEST-mediated degradation require proteosome machinery; evidence of MeCP2 degradation via the proteosome has been shown in vivo and in vitro by several groups (Lamonica et al. 2017; Buist et al. 2022; Chai et al. 2023), where the proteosome inhibitor MG132 restored MeCP2 levels, especially that of the MeCP2-E1 isoform (Buist et al. 2022).

Nearly all polypeptides translated by the ribosome begin with a methionine (Met), marking translation initiation. The Nt methionine is co-translationally excised (NME) by Met–aminopeptidases bound to the ribosome if the penultimate residue is small enough, such as Ala, Gly, Val, Ser, Thr, Cys, and Pro. In ∼80% of nascent human proteins, the Nt–Met or the penultimate residue after NME undergoes Nαt-acetylation (Ree et al. 2018). Nt-acetylation acts as a degradation signal (degron) targeted by ubiquitin ligases for proteolysis; however, there are records of numerous long-lived stable proteins that become co-translationally acetylated at the second position after NME; a plausible explanation for this contrast has been the inaccessibility of the NTD to the proteasomal machinery created by higher order structures (Nguyen et al. 2018). It has been postulated that destabilizing Nt residues reduce the half-life of given proteins, and this phenomenon is referred to as the N-end rule (Gonda et al. 1989; Hwang et al. 2010; Varshavsky 2011). In *Saccharomyces cerevisiae*, the E3 ubiquitin ligase, Doa10, adds a polyubiquitin chain to Nt-acetylated Met, Ala, Gly, Ser, Thr, and Cys (Hwang et al. 2010). Our lab previously analyzed the Nt-co and PTM of the NTD–MBD of the MeCP2 E1 and E2 isoforms in HEK293T cells (Martinez de Paz et al. 2019). Mass spectrometry (MS) analysis of MeCP2-E1 showed no Nt–Met, indicating complete NME; in addition, the Ala that became exposed following NME was acetylated. Several MS reads exhibited the cleavage of the first two, three, five, and six Nt–Ala (Fig. 1A) following NME and the acetylation of the following exposed Nt residue (Sheikh et al. 2017). For the shorter MeCP2 E2 isoform, MS analysis revealed the retention and acetylation of Nt–Met and acetylation of Val at the penultimate position P′1 (Fig. 1A) (Martinez de Paz et al. 2019). At the same time, a few peptide reads showed NME and acetylation of the P′1 Val. NME and acetylation of the penultimate residue are found in more peptide reads in the E1 relative to E2. According to the N-end rule, the E1 isoform would be more destabilized by its acetylated Nt-residue than the shorter E2 isoform, which retains Nt–Met (Varshavsky 2011). Cycloheximide chase assays of MeCP2 isoforms in transiently transfected SH–SY5Y neuroblastoma cells showed that after 4 h, the E1 isoform was ∼30% less than the E2 (Martinez de Paz et al. 2019). Moreover, the lower folding stability of the two NTDs of MeCP2-E1, NTD–MBD, and its lower affinity for DNA suggests a higher presence of the E1 isoform in solution, expediting its degradation by

means of exposing it to proteolytic machinery (Suskiewicz et al. 2011; Martinez de Paz et al. 2019). The higher degradation rate of the E1 isoform is thought to be essential for its involvement in dynamic processes in neurons throughout development.

PEST-mediated degradation

PEST domains were first described by Rogers et al. in 1986 as protein regions enriched in proline (P), glutamic acid (E), serine (S), and threonine (T), flanked by positively charged residues such as lysine (K) or arginine (R), that target the protein for degradation (Rogers et al. 1986). PEST domains have marginal stability due to secondary structures such as polyproline type II (PPII) and alpha helices. Upon being phosphorylated at a serine or threonine adjacent to a proline, the conformational stability of the PEST domain is impaired and proteolysis follows (Rogers et al. 1986; García-Alai MM 2006). MeCP2 has two strong PEST domains near the N- and C-termini. The C-terminal PEST is the stronger candidate with a score of 17.29, generated by the PESTfind algorithm (Schuster and Graber 2002) (Fig. 1B). Proteasomal degradation of the MeCP2 T158M missense mutant observed by Lamonica et al. (2017) conforms with its PEST-mediated degradation. Our lab postulated that the PTMs of this motif may control MeCP2 proteolysis (Thambirajah et al. 2009; Kalani et al. 2023). We recently tested this hypothesis by deleting the Nt- and Ct-PEST domains separately in MeCP2 T158M transiently expressed in C2C12 mouse myoblast cells. We noted a significant increase in protein amounts, suggesting that PEST-mediated degradation is one of the mechanisms responsible for MeCP2 turnover with Rett-causing mutations within the MBD (Kalani et al. 2024). However, the increase in protein levels was insufficent to fully rescue the protein to WT levels, implying the complexity of mechanisms regulating MeCP2.

Despite the known importance of MeCP2 dosage to healthy brain function, we continue learning what drives its homeostasis, requiring further study to adequately target the subtle differences in MeCP2 expression between RTT, DS, and MDD, for example.

MeCP2 functional regulation

Here, MeCP2 functional regulation broadly refers to the mechanisms guiding chromatin and protein interactions under different contexts. Our perception of MeCP2–DNA preference has shifted over the years, beginning with methylated CpG (mCpG) dinucleotides (Lewis et al. 1992; Meehan et al. 1992) then mCH (H = A/C/T) (Chen et al. 2015; Connelly et al. 2020), more specifically mCA (Gabel et al. 2015), hydroxymethylated CpG (Mellén et al. 2012; Kinde et al. 2015), and, more recently, unmethylated and hydroxymethylated CA tandem repeats (Ibrahim et al. 2021). Recent biophysical characterizations of MeCP2 binding nucleosomal DNA (Chua et al. 2024) and canonical histones (Ortega-Alarcon et al. 2024) suggest another level of MeCP2 regulation, through combinatorial interactions with histones themselves. The local ratio of given DNA targets plays a role in the competition, as it were, for MeCP2 binding, such as aberrant MeCP2 binding to the hypermethylated claudin 6 gene (*CLDN6*) (Liu et al. 2016) in breast cancer, or MeCP2 binding to mCA in neurons, in which it is relatively abundant compared to other cell types (Lister et al. 2013). Owing to the intrinsically disordered nature of the majority of MeCP2, much of which is known to influence MBD–DNA binding (Claveria-Gimeno et al. 2017; Sheikh et al. 2018; Martinez de Paz et al. 2019), MeCP2 likely participates in myriad site-specific interactions depending on cellular context, underscoring the importance of determining MeCP2 function in the most pathologically relevant model systems possible.

MeCP2 PTMs can also regulate binding to DNA and protein partners. For example, neuronal activity-dependent gain or loss of phosphorylation at S421 (pS421) and pS80, respectively—as discussed in the context of depression below—is necessary for MeCP2 occupancy at the *Bdnf* promoter (Bellini et al. 2014), and pS164, which is developmentally regulated in mouse neurons, reduces MeCP2–DNA binding in vitro and in vivo (Stefanelli et al. 2016). Activity-dependent pT308 in the TRD, abrogates binding to the nuclear co-repressor (NCoR) protein complex (Ebert et al. 2013); thus, phosphorylation represents a means of regulating MeCP2 recruitment to gene targets as well as the downstream effect at those targets. Furthermore, Ser/Thr phosphorylation was shown to be involved in translocating MeCP2 from the cytosol to the nucleus during differentiation of mouse and human neurons (Miyake and Nagai 2007). In addition to phosphorylation, MeCP2 methylation, acetylation, and sumoylation have been shown to alter MeCP2–protein and chromatin interactions (Pandey et al. 2015; Good et al. 2017; Schmidt et al. 2022; Kalani et al. 2023). Monoubiquitination, O-GlcNAcylation, and lactylation are known MeCP2 PTMs with pathogenic effects but relatively unknown molecular regulatory mechanisms (Cheng et al. 2022; Wang et al. 2023).

Regulatory long non-coding RNAs (lncRNAs) are another layer of MeCP2 spatiotemporal regulation. The known MeCP2–lncRNA interactions with regulatory implications are reviewed in detail in Good et al. (2021). lncRNA expression is dynamic during development and is species and cell type specific (Nie et al. 2019; Good et al. 2021); thus, identifying additional regulatory lncRNA binding partners could bridge our understanding of context-dependent MeCP2 function.

Likely, a combination of local interactome abundance, context-dependent PTMs, and ncRNA expression facilitate disparate MeCP2 spatiotemporal function. For example, MeCP2 phosphorylation regulates recruitment to and repression of the methylated *BDNF* promoter (Chen et al. 2003), whereas MeCP2 interaction with hypomethylated transcriptional start sites is associated with direct RNA polymerase II binding and activation of genes related to synaptic and axon functionality, with strong correlations to autism spectrum disorder (ASD) (Liu et al. 2024). The precise network of mechanisms leading to opposing MeCP2–protein recruitment to genes or transcripts under various contexts is yet to be elucidated. Adding to this complexity is the continual addition of possible regulatory mechanisms that require investigation, such as LLPS.

Liquid–liquid phase separation: protein regulation of unknown significance

Recently, many cases of biomolecular condensation in the nucleus were shown to occur through LLPS, a passive process in which molecules engage in weak multivalent interactions with each other and "de-mix" from the surrounding solution. Increasing the local concentration of these biomolecules causes self-association into dynamic, liquid-like droplets, which is thought to increase the efficiency of molecular processes such as transcription, RNA splicing, and chromatin compaction (Wagh et al. 2021). MeCP2 LLPS was recently shown in multiple papers published in short succession, to such a degree that the concept of MeCP2 LLPS has already been used to describe phenotypic changes upon treatment of RTT-mutant MeCP2 with pre-clinical small compounds in a proof of principle cellular assay (Lata et al. 2024). Still, there is some legitimate debate surrounding to what degree MeCP2 LLPS occurs in cells and its biological importance. Thus, before elaborating on MeCP2 LLPS, we will first define protein LLPS, the debate surrounding its conceptual understanding, and how to best characterize the phenomenon currently.

A protein undergoing LLPS should at least meet the following criteria: (1) Having a defined saturation concentration, C_{sat}, above which liquid droplets form. The environment, such as temperature, salt, pH, or surrounding biomolecules affects the concentration at which the protein phase separates. Importantly, as total LLPS protein concentration increases, droplet size increases with a relatively stable concentration within the droplet, and protein concentration outside of the condensate (the bulk phase) remains at C_{sat}, as shown in the case of the nucleolus (Weber and Brangwynne 2015; Banani et al. 2017; Soding et al. 2020). (2) Liquid droplets are spherical (round) in shape, and (3) fusion of droplets is observable. In vitro, these three parameters can be determined using controlled amounts of untagged or fluorescently tagged purified protein and high-resolution microscopy. Like structural biologists' ability to induce protein crystallization for X-ray crystallography, most proteins can theoretically undergo LLPS in vitro under appropriate conditions (Poudyal et al. 2023), exposing the importance of in vivo corroboration. However, protein concentration in vivo is more difficult to control and measure. Perhaps equally important is verifying whether endogenous protein concentration drives LLPS, and thus an overexpression model alone is insufficient to confirm a biological role for LLPS.

Fluorescence recovery after photobleaching (FRAP) is a gold standard for characterizing proteins' liquid properties. Recent criticisms found that FRAP is not diagnostic of LLPS over other mobile proteins that condense through other means, like specific high affinity binding (Alberti et al. 2019; McSwiggen et al. 2019). Photobleaching half of a labelled condensate and combining data from the unbleached and bleached halves can differentiate those formed through LLPS or low-valency interactions with spatially clustered sites (ICBS) (Muzzopappa et al. 2022). Increasingly powerful single-molecule imaging methods to detect and characterize liquid properties in vivo (Mekonnen et al. 2023), such as atomic force microscopy, single particle tracking (SPT), and fluorescence correlation spectroscopy (Wang et al. 2022), improves the precision of nuanced materials science terms applied to observations. For example, chromatinized DNA under 1 Kbp length condenses into droplets formed through LLPS—or perhaps polymer–polymer phase separation (also known as bridging induced phase separation) (Michieletto and Marenda 2022)—then, increasing DNA length results in solid-like structures in vitro (Muzzopappa et al. 2021), which is corroborated by the solid behaviour of chromatin at the mesoscale in vivo (Strickfaden et al. 2020). Condensates may be described as a liquid, hydrogel, liquid crystal, or solid/amyloid-like based on particle viscoelasticity, mobility in and outside the condensate, and the reversibility of condensate formation (Wang et al. 2022).

MeCP2 LLPS: evidence for, against, and biological implications

Before the LLPS model, MeCP2–chromatin interaction was described as cooperative oligomerization in the presence of nucleosomal arrays (Horowitz-Scherer et al. 2010), despite being monomeric when alone over a range of salt and MeCP2 concentrations (Adams et al. 2007). This aligns with the lack of MeCP2 droplets observed in vitro when alone (Fan et al. 2020; Li et al. 2020; Wang et al. 2020; Zhang et al. 2022); however, cooperativity does not fit into the canonical definition of LLPS (Peng and Weber 2019), but it may play a part in the ordered cooperative assembly of not necessarily liquid protein assemblies coined pleomorphic ensembles (PEs) (Korkmazhan et al. 2021). MeCP2-methyl CpG DNA binding is achieved electrostatically (Ho et al. 2008), and non-specific DNA binding was shown by other MeCP2 domains, including the ID, TRD, and CTD, contributing to weak interactions thought to allow "hopping" along a chromatin fibre, but perhaps also to the multivalent avidity observed in LLPS (Halford and Marko 2004; Adams et al. 2007; Ghosh et al. 2010; Krishnan et al. 2022). MeCP2 was previously shown to compact nucleosome arrays in vitro (Georgel et al. 2003; Nikitina et al. 2007), which was recapitulated by Wang et al. (2020) and for which LLPS was then suggested to drive MeCP2–chromatin assembly. In vivo, MeCP2 assembles on pericentric heterochromatin (PCH) into visible punta in murine cell lines such as NIH3T3 fibroblasts, C2C12 myoblasts, and mouse embryonic stem cells (mESCs), which have become models to study MeCP2–chromatin interaction, dynamics, and now, LLPS (Agarwal et al. 2007; Sheikh et al. 2016; Marano et al. 2019; Ito-Ishida et al. 2020; Lata et al. 2024). However, as will be discussed below, this may not be a suitable model to study MeCP2 LLPS in vivo.

In 2020, three separate groups published evidence for MeCP2 LLPS, and that its association with chromatin facilitates this process, followed by a fourth paper on this topic published in 2022 (Fan et al. 2020; Li et al. 2020; Wang et al. 2020; Zhang et al. 2022). In each case, MeCP2 required the addition of a crowder like polyethylene glycol or DNA to undergo LLPS, and methylated DNA followed by methylated nucleosomal arrays further decrease the

required MeCP2 concentration (C_{sat}) (i.e., increased the propensity) to form droplets in vitro. Several RTT-causing mutations in the MBD and TRD were tested and found to abrogate MeCP2 LLPS in vitro, including R106W, R133C, K144R, F155S, T158M, representing RTT-causing missense mutations in the MBD, P176R in the ID, and P225R, R306C, and R306H in the TRD. RTT-causing nonsense mutations R168X, R255X, R270X, and R294X had similar deleterious effect on MeCP2 LLPS (Fan et al. 2020; Li et al. 2020; Wang et al. 2020). Given that LLPS is a concentration-dependent phenomenon, its role in RTT pathology could be relevant in cases like common RTT-causing mutations, T158M or R106W, which, in addition to DNA binding inhibition, are known to result in reduced MeCP2 protein levels (Marchi et al. 2007; Johnson et al. 2017; Lamonica et al. 2017; Kalani et al. 2024). Rescuing MeCP2 T158M levels to those of WT nearly rescues the RTT phenotype in mice, indicating that protein level alone is partially responsible for the difference between a healthy and diseased state. An inability to reach a saturation concentration and coalesce into functional droplets due to decrease in protein level could explain this phenomenon. The functional regulatory role that LLPS may have on MeCP2 is unclear. Wang et al. (2020) found that MeCP2 and histone H1 condensates remain mutually exclusive from but adjacent to each other in vitro and in NIH3T3 nuclei, and the R106W MeCP2 mutation resulted in H1.4—an H1 subtype elevated in rat and human neuronal cells (Pina and Suau 1987; Siqueira et al. 2023)—forming large self-assemblies at heterochromatin where WT MeCP2 otherwise would have, suggesting that MeCP2 condensate formation facilitates the local concentration required for MeCP2's competitive binding to MeCP2/H1 chromatin targets. Li et al. (2020) showed that MeCP2 recruits the CTD of transducin-beta-like 1X-related protein 1 (TBRL1) of the NCoR repressor complex into protein condensates in vitro, and that MeCP2 condensates overlap with those of heterochromatin protein 1 alpha (HP1α) on heterochromatin in mESCs. If LLPS is responsible for the recruitment and (or) exclusion of important MeCP2 interaction partners, understanding this mechanism of MeCP2 condensation could carry clinical significance. However, while MeCP2 LLPS is appealing, whether the publications on the matter thus far have unequivocally shown this in vivo is dubious, as discussed below.

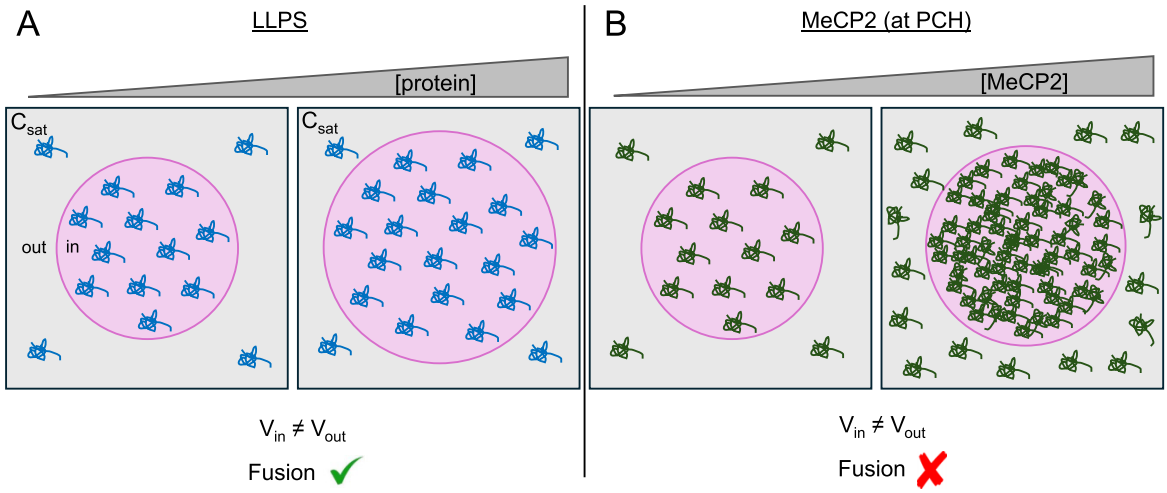
Using the outline earlier in this section, MeCP2 LLPS in vitro was well established. Phase diagrams were generated (Fan et al. 2020; Wang et al. 2020) of the concentration-dependent formation of MeCP2 droplets that are round and can fuse (Fan et al. 2020; Li et al. 2020; Wang et al. 2020). SPT was used to show (Zhang et al. 2022) that MeCP2–GFP was able to freely move within droplets but were excluded from the surrounding environment, a feature of surface tension. All authors showed that phase separation occurs within physiological salt and protein concentrations in vitro. Understanding in vivo MeCP2 LLPS is critical for pre-clinical developments. None of C_{sat}, roundness, nor fusion events in vivo were shown by Fan et al. (2020); Li et al. (2020), nor Wang et al. (2020). Zhang et al. (2022) estimated molar concentration of MeCP2–GFP transiently transfected C2C12 cells sorted into "high" and "low" protein levels and determined that the "low" population was near the nuclear physiological concentration. MeCP2–GFP concentration within heterochromatin condensates was found to be an order of magnitude greater in the "high" versus the "low" population, and in the surrounding nucleoplasm, it was also found to increase. A positive correlation between total protein concentration and that both within condensates and in the surrounding solution is *not* a physical characteristic of liquid phase separating molecules (Banani et al. 2017) (Fig. 2A). This point was corroborated by Pantier et al. (2024), wherein transfection of differing MeCP2–GFP amounts into *Mecp2*-KO mouse fibroblasts indicates a positive correlation between GFP fluorescence and that at heterochromatic foci as well as in the surrounding nucleoplasm (Pantier et al. 2024). The crux of this article's argument derives from the observation that round MeCP2 condensates in vivo are only seen in mouse cells with PCH rich in satellite DNA repeats, to which MeCP2 localizes, whereas MeCP2 condensation/punta formation was not seen in other cell types. FRAP half-bleaching, as described earlier, does not support liquid condensation of MeCP2 in immortalized mouse fibroblasts (Erdel et al. 2020), but rather an alternative mechanism of condensation such as ICBS, which is defined by low-valency binding to a polymer-like substrate, like chromatin, rather than circumambient multivalent interactions in solution (Muzzopappa et al. 2022). With all this is mind, using murine cell lines, while an excellent model historically used to study general MeCP2 MBD–DNA interaction (Kumar et al. 2008; Sheikh et al. 2016), is likely not an effective model to characterize MeCP2 LLPS, if it does occur in vivo. Figure 2 illustrates the differences and similarities between expected LLPS (Fig. 2A) and observed MeCP2 compartmentalization in murine cells (Fig. 2B).

Whether MeCP2 LLPS could occur in other relevant cells will require analysis on a case-by-case basis, given that LLPS largely depends on the molecular composition in the surrounding solution. For example, microscopy images taken from human MCF7 breast cancer cells (Koch and Stratling 2004) and adult cerebrum, cerebellum, and hippocampus brain tissue (LaSalle et al. 2001) appear in some cases to show MeCP2 puncta, despite not having the same satellite-rich DNA repeats as mouse cells (Fioriniello et al. 2020). These publications are now decades old, and techniques used to prepare cell sections, such as fixation (Irgen-Gioro et al. 2022), may affect apparent MeCP2 nuclear distribution. Nonetheless, these and the in vitro data suggest that MeCP2 LLPS is worth exploring in additional contexts. Despite the breadth of MeCP2 research, LLPS is relatively unexplored, and understanding the mechanism of MeCP2 distribution and (or) condensation within different cellular contexts will provide researchers with a biophysical framework of targeting MeP2 under various disease states.

Depression and impaired expression of MeCP2-related genes

Chronic stress and neuroinflammation decrease dendritic length and synapse density, which are hallmarks of MDD (Duman and Aghajanian 2012; Duman et al. 2016; Troubat et al. 2021). MeCP2 dysregulation, as observed in MDD

Fig. 2. Classic protein LLPS contrasted with observed MeCP2 compartmentalization at pericentric heterochromatin (PCH) in murine cell lines. (A) In LLPS, protein concentration in the bulk phase ("out") remains at C_{sat}, and the concentration within droplets ("in") remains stable, while droplet size increases proportionally with added total LLPS protein. Diffusion coefficient measurements in and outside of liquid condensates (V_{in} and V_{out}) indicate the presence of a surface tension boundary, and fusion of liquid droplets upon contact should be observable in vivo. (B) Differing diffusion coefficients inside and outside compartments indicates the presence of a boundary or preferred mixing inside PCH foci, but the complementary increase in MeCP2 concentration in and outside foci with increased total MeCP2, and the lack of observed fusion of MeCP2 condensates oppose the liquid model.



patients and animal models (Su et al. 2015; Zimmermann et al. 2015), is linked to impaired expression of genes involved in stress response and synaptic plasticity, such as RELN and BDNF. These genes, which are negatively regulated by MeCP2, are crucial for neuronal development and maintenance (Zhou et al. 2006; Zhubi et al. 2014). *Bdnf* was one of the first identified DNA binding partners of MeCP2 (Chen et al. 2003) and plays an essential role in development and plasticity of glutamatergic and GABAergic synapses (Colucci-D'Amato et al. 2020). RELN plays a critical role in the prenatal brain, especially in the cellular layer formation of brain and neuronal migration (Lambert de Rouvroit and Goffinet 1998). Postnatally, RELN promotes maturation of dendrites and synaptogenesis; these processes overlap with MeCP2's roles in dendritic arborization of maturing neurons (Kishi and Macklis 2004; Lee and D'Arcangelo 2016). This shared functionality raised the question of possible direct interaction and Zhubi et al. found that MeCP2 binds to both the promoter and gene body of *RELN* in the cerebellum. In postmortem brains of patients with ASD, there was significantly higher binding of MeCP2 to the *RELN* promoter and lower reelin expression, consequently, compared to controls (Zhubi et al. 2014). Similarly, in depressed mouse models, where repeated corticosterone (CORT) injections induced depression, there was significant (26%) decrease in reelin, especially in the subgranular zone of dentate gyrus (Caruncho et al. 2016). Although higher MeCP2 binding to *Reln* in the brain of depressed animal models has not yet been shown, dysregulation of MeCP2 homeostasis has been identified as one of the key molecular pathologies of depression (Ramocki et al. 2009; Ausio 2016). BDNF exhibits neuroprotective properties in adverse conditions such as acute hypoglycemia, neurotoxicity, and cerebral ischemia (Bathina and Das 2015). Chronic stress causes atrophy of neurons in the hippocampus and prefrontal cortex (McEwen et al. 2012); in parallel, exposure to chronic stress, including unpredicted and social defeat, decreased BDNF expression in the same brain regions of the hippocampus and PFC (Duman and Monteggia 2006). Constitutive or conditional deletion of *Bdnf* in the forebrain of heterozygous mice showed conflicting results in the animal's depressive symptoms (MacQueen et al. 2001; Duman and Monteggia 2006). However, these mice displayed a reduction in dendritic length and arborization and decreased synapse density (Chen et al. 2006; Liu et al. 2012). *Mecp2*-null mouse also displays synapse dysregulation and insufficient dendrite branching (Kishi and Macklis 2004; Smrt et al. 2007). Direct regulation of *BDNF*/*Bdnf* by MeCP2 is one of the first identified regulatory mechanisms of MeCP2. MeCP2 binds to *Bdnf* promoter IV, and upon neuronal stimulation and membrane depolarization, MeCP2 pS80, a constitutive phosphorylation site (Tao et al. 2009), is replaced by pS421 (Zhou et al. 2006), which releases MeCP2 from the promoter region and yields transcription of *Bdnf*. MeCP2 pS421 was also shown to be required for the long-term maintenance of effects by the fast-acting antidepressants ketamine and scopolamine (Kim et al. 2021). Finally, the regulatory loop between MeCP2, miR-132, and *Bdnf*/*BDNF*, discussed earlier, aligns with chronic depression's impact on BDNF also affecting MeCP2.

In mouse studies that induce depression by social isolation, numerous changes in the epigenome were identified, such as decreased peroxisome proliferator-activated receptor alpha (PPAR-α) mRNA expression, which was the result of increased methylated cytosine (mC) of all three *Ppar-α* genes in the hippocampus relative to control. As a result of this

increased methylation status, both *Mecp2* and histone deacetylase 1 (*Hdac1*) mRNA levels were higher in the hippocampus of mice that experienced social isolation (Matrisciano and Pinna 2021). PARP-α is a ligand-activated nuclear receptor and recently identified target for behavioural regulation involving neuroinflammation, oxidative stress (OS), and mitochondrial and proteasomal function (Wahli and Michalik 2012). Increased methylation of *Ppar-α* was the upstream epigenetic factor observed in socially isolated mice, and the resulting decreased expression mediated by HDAC1 and MeCP2 correlated with aggressive behaviour in mice. Social isolation was the condition that induced *Ppar* methylation, and its repression was maintained by increased MeCP2 binding to its methylated promoter (Matrisciano and Pinna 2021).

Important MeCP2 role in oxidative stress and mitochondrial dysfunction

OS is a contributor to numerous neurological conditions, including depression (Teleanu et al. 2022), and MeCP2 was shown to regulate important proteins involved in redox balance inside the cell (Filosa et al. 2015). Mitochondria is the primary source of reactive oxygen species (ROS) production. The electron transport chain of mitochondria consists of five multimodular complexes: 1,4-dihydronicotinamide adenine dinucleotide dehydrogenase (complex I), succinate dehydrogenase (complex II), coenzyme Q-cytochrome c reductase (complex III), cytochrome C oxidase (complex IV), and ATP synthase (complex V) (Rani and Yadav 2015). ROS are produced in all cells that respirate aerobically, but chronic stress can dramatically increase ROS production. Just as chronic exposure to stressors can impair the cellular feedback mechanisms to revert to a steady state (Sanchez-Lafuente et al. 2022), chronic OS, involving expression of stress-related genes, may then revert cells to a new steady-state that is higher than before the stress, called a "quasi-stationary state" (Lushchak and Storey 2021). Systemic OS has been observed in Rett patients, strongly correlating with clinical severity (Filosa et al. 2015). Mitochondrial alterations have also been described in Rett patients and MeCP2 null mouse models (Kriaucionis et al. 2006; Belichenko et al. 2009; Gibson et al. 2010). RTT mutations have been shown to impact BDNF and proline dehydrogenase, which also play a part in cellular redox balance (Filosa et al. 2015); hence, these genes may be essential contributors to OS. In *Mecp2*-null RTT mouse models, increased expression of the mitochondrial respiratory complex subunit III protein ubiquinol–cytochrome c reductase core protein 1 (Uqcrc1) correlated with disease severity, and this increased expression of *Uqcrc1* coincides with increased mitochondrial respiration (Kriaucionis et al. 2006). Furthermore, the frontal cortex of RTT patients exhibits downregulation of the cytochrome c oxidase subunit 1 (*MTCO1*) gene, which encodes the primary subunit of the catalytic core of cytochrome C oxidase (COIV), consequently resulting in reduced COIV enzymatic activity (Gibson et al. 2010). Altogether, these observations emphasize the critical role of MeCP2 in regulating stress-related genes and that mitochondrial dysfunctions observed in RTT patients exacerbate the severity of phenotype due to imbalances in the oxidative vs. reductive states inside the cell.

Conclusion

With all of the above considered, the role of MeCP2 is undoubtedly crucial for numerous pathologies in different contexts, indicating an urgency to understand MeCP2 not just in the context of RTT, but the means of context-dependent regulation. On the contrary, while making strides, the research on MeCP2 dosage and functional regulation is far from being fully clarified. Future research should focus on elucidation MeCP2 regulation in different contexts, to apply this knowledge broadly.

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This manuscript does not report data.

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Competing interests

The Centre for Addiction and Mental Health (JBV) holds a patent and receives royalties related to diagnostic screening

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