TITLE: Diverse roles of MeCP2 in the specification and maintenance of midbrain dopamine neuronal phenotype.

Running title: MeCP2 in midbrain dopamine neuron development.

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Abstract

Midbrain dopamine (DA) neurons are associated with locomotor and psychiatric disorders. DA neuronal phenotype is specified in ancestral progenitors and maintained throughout differentiation. Here we demonstrate that premature MeCP2 expression prevents DA progenitors from acquiring DA phenotype through interfering NURR1 transactivation. By contrast, the maintenance of DA phenotype is not affected by MeCP2 overexpression in DA neurons. By analyzing the DNA methylation and MeCP2 binding to the promoter of DA phenotype gene tyrosine hydroxylase (*Th*) along differentiation, we show that *Th* expression is determined by TET1-mediated de-methylation of NURR1 binding sites within *Th* promoter. Premature MeCP2 dominates the DNA binding of these sites thereby blocking TET1 function in DA progenitors, whereas TET1 prevents excessive MeCP2 binding in DA neurons. Finally, we show that targeted de-methylation in DA progenitors protects phenotype specification from premature MeCP2 expression, whereas targeted methylation disturbs phenotype maintenance in MeCP2-overexpressed DA neurons. These findings demonstrate MeCP2 as a novel determining factor for DA neuronal phenotype and function.

Introduction

Neurotransmitter phenotypes define the molecular, cellular and functional characteristics of different neurons, which are specified during neuronal differentiation and maintained throughout life. These programs are achieved by orchestration of complex signaling networks and a variety of transcription factors and epigenetic modifications. Midbrain dopamine (DA) neurons are transcriptionally characterized by constitutive expression of DA phenotype genes such as *Th* encoding the DA synthesis rate limiting enzyme tyrosine hydroxylase and *Aadc* encoding aromatic L-amino acid decarboxylase, both of which are responsible for the conversion of L-tyrosine into dopamine. *Th* is also one of the earliest expressed DA phenotype genes that is maintained throughout DA neuron lifespan. The specification of DA phenotype marks the fate commitment of neural progenitors into DA lineage in embryonic brains, and the maintenance of DA phenotype is essential for mature DA neuronal functions, loss of which in adult midbrains is associated with movement and emotional disorders such as Parkinson's disease and schizophrenia (Hynes and Rosenthal, 1999; Goridis and Rohrer, 2002; Arenas et al., 2015).

In mouse, DA neurons arise from nuclear receptor related 1 protein (NURR1)-expressing DA progenitors residing in the ventral-most region of the neural tube at embryonic day E10.5. DA phenotype specification initiated in DA progenitors is under precise spatio-temporal control of numerous intrinsic factors and extrinsic cues (Ang, 2006). Continuous activation of *Nurr1* is implicated in the specification as

well as the maintenance of DA phenotype. Genetic ablation of *Nurr1* in transgenic mice resulted in absence of DA phenotype (Zetterström, 1997; Saucedo-Cardenas et al., 1998). Conditional ablation of *Nurr1* caused loss of DA phenotype in maturing DA neurons (Kadkhodaei et al., 2009). A large number of studies including ours have demonstrated that NURR1 serves as a transcription activator by directly activating the promoter activity of DA phenotype genes such that its ectopic expression was able to force the expression of DA phenotype markers in non-DA cells (Sakurada et al., 1999; Kim et al., 2003b; He et al., 2011; Yi et al., 2014; He et al., 2015). Based on this well-elucidated mechanism, various transcription factors and epigenetic factors have been identified in DA phenotype specification (van Heesbeen et al., 2013), including DNA hydroxymethylase ten-eleven translocation 1 (TET1) and TET1-induced DNA de-methylation of DA phenotype genes (He et al., 2015).

Genetic deficiency of X-linked gene Methyl-CpG binding protein 2 (*Mecp2*) is associated with neurological and developmental disorder Rett syndrome. Evidence exists that have associated MeCP2 with DA-related disorders. For instance, deficit of MeCP2 expression in the brain is associated with DA metabolites abnormality in patients and animal models of Rett syndrome (Samaco et al., 2009; Kao et al., 2015; Panayotis et al., 2011a; Gantz et al., 2011; Ide et al., 2005, 2; Zhao et al., 2013; Panayotis et al., 2011b). Underlying mechanisms include autonomous deficits of key DA synthesizing enzymes including TH in DA neurons of substantia nigra (SN) and

striatum, and non-autonomous effects derived from serotonin neurons. MeCP2 duplication syndrome, a neurodevelopmental disorder shares many similar behavioral and molecular deficits with Rett syndrome, including abnormal DA levels and motor function (Na et al., 2012, 2; Peters et al., 2013). The expression pattern of MeCP2 in developing brain highly correlates with neurogenesis (Tate et al., 1996; Cassel et al., 2004; Jung et al., 2003), suggesting the significance of its proper expression for the development of neuronal lineages. Although the roles of MeCP2 for general neuronal maturation and functions have been widely revealed, little is known about the contribution of MeCP2 to the development and maintenance of DA neurons.

We previously have shown that MeCP2 is involved in the developmental repression of DA phenotype genes in differentiating DA progenitors (He et al., 2011). This finding was extended in this study to provide a comprehensive understanding for distinct roles of MeCP2 in the specification and maintenance of DA phenotype along with midbrain DA neuron differentiation.

Results

MeCP2 overexpression abolishes specification but not maintenance of DA phenotype.

DA phenotype is the key signature identifying the maturation as well as the neuronal subtype of midbrain DA neurons, which is maintained throughout the lifespan of these cells. Mitotic DA progenitors extracted from rodent embryonic mesencephalon can be induced to differentiate into midbrain DA neurons within 6 days in vitro, serving as a well-defined primary cell model to study the mechanisms underlying DA phenotype specification and maintenance (Studer et al., 1998; He et al., 2011, 2015).

To understand the roles of MeCP2 in these two consecutive steps within DA neuronal differentiation, we infected cultured DA progenitors with a lentivirus carrying a sequence encoding a tetracycline-inducible MeCP2. Time- and dose-specific MeCP2 overexpression was induced by addition of 0.1-2 μM doxycycline at early or late periods of differentiation in vitro (DIV), corresponding to two critical time points determining the specification and maintenance of DA phenotype, respectively (Fig. 1A). We found that MeCP2 overexpression in early period of differentiation remarkably reduced the terminal yield of cells expressing DA phenotype marker TH (Fig. 1B). Interestingly, 0.1 μM doxycycline resulted in comparable yield reduction of TH+ cells as 2 μM doxycycline, suggesting no additional influence from dosage of ectopic MeCP2. By contrast, the yield of TH+ cells was not affected by MeCP2 overexpression in late period of differentiation. Molecular analysis demonstrated that

the mRNA levels of *Th* and *Aadc* were decreased only when MeCP2 was overexpressed in early but not late period of differentiation (Fig. 1C), suggesting that the specification of DA phenotype in DA progenitors was transcriptionally regulated by MeCP2. Collectively, these results indicate differential roles of MeCP2 in the specification and maintenance of DA phenotype.

Protein structural studies have demonstrated that MeCP2 consists of two functional domains, namely DNA binding domain (DBD) and transcriptional repressing domain (TRD). To further dissect the domain functionality of MeCP2 in DA phenotype specification, primary DA progenitors were infected with lentiviruses carrying sequences encoding full-length MeCP2 or R168X, a truncated mutation containing intact DBD but not TRD. A sequence encoding ZsGreen1 fluorescent protein (GFP) was incorporated to label the infected cells. We found that overexpression of MeCP2 from DIV 0 significantly reduced the yield of TH+ cells at DIV 6, verifying the results observed from doxycycline-induced MeCP2 overexpression. However, overexpression of R168X did not alter the yield of TH+ cells (Fig. 1D), indicating that the TRD of MeCP2 is responsible for the suppression of DA phenotype genes in DA progenitors. Noticeably, TH and GFP double positive cells were found in cells infected by empty vector or R168X, but not in cells infected by MeCP2 (Fig. 1E), indicating that MeCP2 suppress DA phenotype specification in an autonomous manner.

Endogenous MeCP2 is not expressed in DA progenitors but highly expressed in DA neurons.

The wide range of doxycycline effect suggested that cultured DA progenitors were extremely sensitive to the level of MeCP2 when they were committing DA phenotype, leading us to ask how endogenous MeCP2 was expressed in embryonic midbrain dopamine neuron development. To address this question, we examined the MeCP2 expression pattern by immunostaining in the ventral regions of embryonic day 10 (E10), E13 and P28 mouse midbrains, where reside emerging DA progenitors, maturing DA neurons and functional mature DA neurons, respectively (Arenas et al., 2015). Given its significance in DA phenotype specification and maintenance, NURR1 was co-stained to mark DA progenitors at E10 and DA neurons at E13 and P28. TH was also co-stained to mark DA neurons whose phenotype have already been specified. We found that, the NURR1+ DA progenitors residing in the ventral and intermediate zones at E10 were negative for MeCP2, whereas the NURR1+ maturing DA neurons in the marginal zone at E13, which were already expressing TH, were positive for MeCP2. Consistently, NURR1+TH+ DA neurons in ventral tegmental area (VTA) and SN at P28 were highly expressing MeCP2 (Fig. 2A). This result demonstrates that NURR1 and MeCP2 expressions are highly correlated but MeCP2 expression is temporarily preceded by NURR1 at the just beginning of DA phenotype specification.

We confirmed the expression pattern of MeCP2 in the primary DA progenitor

differentiation culture. At DIV 0, NURR1+TH- DA progenitors were not expressing MeCP2, whereas NURR1+TH+ DA neurons at DIV 3 and DIV 6 were expressing MeCP2 (Fig. 2B). These results collectively demonstrate a dynamic appearance of endogenous MeCP2 from the differentiation of DA progenitors towards DA neurons. In particular, in DA progenitors, MeCP2 was not expressed until DA phenotype was committed, suggesting a necessity to restrict MeCP2 expression in DA progenitors for the proper specification of DA phenotype.

MeCP2 impedes NURR1 transactivation of DA phenotype genes in DA progenitors.

Genetic gain-of-function studies have shown that NURR1 overexpression is sufficient to induce expression of DA phenotype genes especially *Th* in non-DA cells (Sakurada et al., 1999; Wagner et al., 1999; Kim et al., 2003a; b; Park et al., 2008; Lee et al., 2009). To investigate the association between MeCP2 and NURR1 in DA phenotype specification, neural precursor cells (NPCs) from rat embryonic cortices were co-infected with lentivirus encoding NURR1 and those encoding MeCP2 or R168X. About 50% of cells infected with NURR1 and control viruses started to express TH within 3 days, suggesting the success of forced TH expression by NURR1. MeCP2 overexpression greatly reduced this population to about 2% (Fig. 3A and 3B). Co-labeling of TH and GFP showed that the majority of cells overexpressing MeCP2 were negative for TH (Fig. 3C), indicating an autonomous effect of MeCP2. Consistent with DA progenitor results, overexpression of R168X did not affect the

yield of TH+ cells. Real-time PCR analysis demonstrated a similar transcriptional repression of *Aadc* as *Th* as in DA progenitors (Fig. 3D). As NPCs from embryonic cortices do not possess the majority of molecular machinery for DA neuron development, it appears safe to conclude that NURR1 is largely involved in MeCP2-mediated DA phenotype silencing.

A large number of studies including ours have reported that recruitment of NURR1 onto the promoters of DA phenotype genes as the molecular mechanism for the initiation of their transcription. In particular, NURR1 transactivates *Th* gene expression through binding to NURR1 binding motif namely NBRE on *Th* promoter. Therefore, we performed a series of ChIP experiments to examine how MeCP2 interacts with NURR1 on the promoter of *Th* gene in both DA progenitors and neurons. Within 1 kilo bp distance upstream to transcription starting site of mouse TH gene, there are three NBREs (Fig. 3E). When overexpressed in DA progenitors, MeCP2 occupancy of all three NBREs was remarkably increased (Fig. 3F), whereas the binding of NURR1 was reduced (Fig. 3G). Interestingly, the binding of R168X was also increased, but it did not decrease the binding of NURR1. The best explanation of this result is that the binding of NURR1 to NBRE sites might be interfered by the TRD of MeCP2, rather than a competitive binding for NURR1 and MeCP2.

By contrast, when overexpressed in DA neurons, enrichment of NURR1 onto NBREs

was not altered by overexpression of either MeCP2 or R168X (Fig. 3I). Importantly, the recruitment of MeCP2 onto NBREs was not increased by overexpression of MeCP2 or R168X (Fig. 3H), raising the possibility that the *Th* gene in DA neurons are not accessible to direct binding of MeCP2. Taken together, the data presented here demonstrate differential impact of MeCP2 overexpression on NURR1-mediated DA phenotype gene expression in DA progenitors and DA neurons.

MeCP2 competes TET1 to block DA phenotype specification in DA progenitors.

We previously have revealed an essential role of TET1-mediated DNA de-methylation for NURR1 to transactivate the expression of DA phenotype genes (He et al., 2015). Therefore, we hypothesized that the DNA methylation status of DA phenotype genes distinguished the DNA binding and function of MeCP2 in DA progenitors and DA neurons. Consistent with the TET-assisted and bisulfite sequencing data in our previous study, hydroxymethylated/methylated DNA immunoprecipitation (hMeDIP/MeDIP) analyses confirmed that the 5mC level within 1 kilo bp of *Th* promoter was decreased along DA neuronal differentiation, in concert with the increase of 5hmC level (Fig. 4A). Accordingly, enrichment of TET1 on *Th* promoter, especially on the NBREs, was significantly increased (Fig. 4B), indicating that TET1 was responsible for the oxidation of 5mC of *Th* promoter along DA neuron differentiation. Interestingly, overexpression of MeCP2 in DA progenitors caused increased level of 5mC and decreased level of 5hmC after differentiation, suggesting that the natural alterations of these epigenetic marks along with differentiation were

prevented by premature MeCP2 expression (Fig. 4C). In addition, ChIP analysis demonstrated that TET1 was much less recruited onto *Th* promoter by MeCP2 overexpression in DA progenitors (Fig. 4D). These results suggested that MeCP2 overexpression in DA progenitors abolished DNA de-methylation of DA phenotype genes through interfering TET1 function.

To validate whether DNA methylation of *Th* promoter is necessary for MeCP2 binding in DA progenitors, vitamin C (VC) was added to MeCP2-overexpressed DA progenitors for the purpose of a global DNA de-methylation. As previously reported (He et al., 2015), VC significantly increased the yield of TH+ cells. As shown in Fig. 1D, in the absence of VC, overexpression of MeCP2 but not R168X decreased the yield of TH+ cells. However, addition of VC completely reversed the impact of MeCP2 overexpression (Fig. 4E). Furthermore, VC remarkably decreased the enrichment of MeCP2 on NBREs of *Th* promoter in MeCP2-overexpressed DA progenitors (Fig. 4F). These finding suggested direct associations between DNA de-methylation and *Th* gene expression with MeCP2 binding in the process of DA phenotype specification. To further improve the specificity of DNA methylation, we utilized a TET1 catalytic domain (TET1CD)-mediated targeted DNA demethylation lentivirus system which has efficiently converted NPCs fate into astrocytes by targeting the methylation status of GFAP promoter (Morita et al., 2016). Single guide RNAs (sgRNAs) targeting sequences near each of three NBREs were incorporated into the vector (Fig. 4G). MeDIP experiment confirmed that sgNBRE-1 was the most

potent guide RNA that was able to induce decreased levels of 5mC at and near the targeting region in DA progenitors (Fig. 4H). Co-introducing sgNBRE-1 with MeCP2 into DA progenitors attenuated the repression of *Th* expression (Fig. 4I). Furthermore, ChIP analysis demonstrated that sgNBRE-1 caused decreased MeCP2 binding and increased NURR1 binding on the first NBRE in the context of MeCP2 overexpression (Fig. 4J). Taken together, these results indicate hypermethylated promoters of DA phenotype genes in DA progenitors are targets for prematurely expressed MeCP2, thereby preventing DA phenotype specification through interfering TET1-mediated DNA de-methylation.

Tet1 counteracts MeCP2 to maintain DA phenotype in DA neurons.

As demonstrated above, *Th* promoter in DA neurons was hypomethylated in comparison to in DA progenitors, and it permitted no further enrichment of MeCP2 when overexpressed in DA neurons. These results appeared to suggest an association of DNA hypomethylation with the constitutive expression of DA phenotype genes in DA neurons. Opposite to its role as a global transcriptional repressor in NPCs, recent studies have revealed MeCP2 as a global activator in differentiated neurons. An intriguing question is whether MeCP2 function in the specification and the maintenance of DA phenotype is switched from repressor to activator or remained as repressor during DA neuronal differentiation. To validate this, we employed a well-defined shRNA-mediated TET1 inhibition (He et al., 2015) to prevent the transition of 5mC to 5hmC in differentiating DA neurons after the DA phenotype

specification (from DIV 4) . MeDIP analysis demonstrated that TET1 knockdown caused efficient DNA methylation on *Th* promoter (Fig. 5A), suggesting that TET1 was responsible for not only the conversion but also the maintenance of DNA hydroxymethylation. Concomitantly, MeCP2 enrichment was significantly increased at NBRE-l. Overexpression of MeCP2 further exacerbated this recruitment (Fig. 5B). As a consequence, enrichment of NURR1 was reduced (Fig. 5C), and gene expression of *Th* was compromised (Fig. 5D).

Using a luciferase reporter assay, we previously have shown that *Th* gene expression is extremely sensitive to CpG methytransferase MQ1-induced DNA methylation. To exclude the overall regulation effect of sh-TET1, we employed an inactive Cas9-mediated targeted DNA methylation system involving MQ1 and its mutant $MQ1^{147L}$ with better enzyme activity (Lei et al., 2017) to manipulate targeted methylation of *Th* promoter in DA neurons. Based on targeted de-methylation results above, we focused on sgRNA targeting NBRE-1. MQ1 and $MQ1^{147L}$ successfully caused more than 50% *Th* gene silencing in MeCP2-overexpressed DA neurons (Fig. 5E). MeDIP analysis of NBRE-1 region confirmed that 5mC was enriched by sg-NBRE-1 guided MQ1 and $MQ1^{147L}$ (Fig. 5F). As a consequence, ChIP analysis demonstrated an increased recruitment of MeCP2 onto NBRE-1 in DA neurons experiencing overexpressed MeCP2 and MQ1-mediated targeted methylation (Fig. 5G). This result supports the notion that MeCP2 functions as a repressor specifically recognizes methylated CpG sites in both DA progenitors and DA neurons. Conversely,

decreased recruitments of TET1 and NURR1 were detected at NBRE-1 region (Fig. 5H and 5I), suggesting a counteracting role of MeCP2 with transcription activators of DA phenotype genes.

Discussion

Here we performed a series of gain-of-function experiments to investigate the effects of abnormal timing and dose of MeCP2 expression on midbrain DA neuron development, particularly on the acquisition and maintenance of DA phenotype. Evidence exist for a long time in attempt to establish a direct link between MeCP2 and catecholamine system in CNS (Nomura et al., 1985). For instance, abnormal concentrations of biogenic amines have been observed in brains of MeCP2-null mouse and cerebrospinal fluid of Rett syndrome patients (Roux and Villard, 2010, 2). However, these alterations are supposed to be independent of DA neuron development, as MeCP2-knockout embryonic stem cells are capable of differentiating into TH-expressing DA neurons in vitro (Okabe et al., 2010), and rescue of MeCP2 expression in Rett syndrome mice in postnatal stage rectified neuronal and behavior defects (Robinson et al., 2012; Lang et al., 2013). Based on these loss-of-function studies, it seems reasonable to conclude that the major role of MeCP2 is to maintain DA neuron function after neuronal differentiation. Our finding provide evidence for a novel role of MeCP2 in DA neuron differentiation, proposing that restricting appropriate expression level of MeCP2 in DA progenitors is necessary for their phenotype specification. Converse with Rett syndrome, MeCP2 overexpression correlates with duplication or triplication of this X-linked gene, which causes neurodevelopmental disorder MeCP2 duplication syndrome. Patients with this disease manifest autism-like behaviors and multiple brain dysfunctions. It remains interesting to investigate the midbrain DA neuron development and catecholamine system in

MeCP2 duplication syndrome patients and MeCP2-overexpressing mice.

MeCP2 functions as transcription activator and repressor depending on genes and regulatory context (Chahrour et al., 2008). Our data support the notion that MeCP2 is a transcriptional repressor for DA phenotype genes. This is consistent with previous studies that identified *Th* and *Aadc* as direct target genes of MeCP2 (Yang et al., 2011; Urdinguio et al., 2008). Furthermore, MBD and TRD are both indispensible for MeCP2-mediated repression, as preventing DNA binding of MeCP2 or overexpressing TRD mutation R168X failed to repress *Th* gene expression. The function of MBD determines whether or not MeCP2 is recruited to DNA sequences, allowing TET1-mediated de-methylation to distinguish DNA binding of MeCP2 in DA progenitors and DA neurons. The function of TRD is to recruit other repressive complexes, so as to block the recruitment of other activators such as NURR1 and TET1. Interestingly, one study using MeCP2-overexpressing mice demonstrate that the duplication toxicity requires both domains to be functional (Heckman et al., 2014), raising the possibility that too much MeCP2 protein might function as a universal transcription repressor rather than activator.

The molecular mechanisms proposed here raise at least two neuropathological concerns (Fig. 6). First is premature MeCP2 expression in DA progenitors. We previously have shown that the promoter regions of other DA phenotype genes such as *Aadc* and *Dat* were also hypermethylated like Th in DA progenitors (He et al.,

2015), and their gene expressions are also under transactivation of master regulator NURR1(van Heesbeen et al., 2013). Therefore, the mode of action of premature MeCP2 expression on *Th* gene expression is very likely to be universal to other DA phenotype genes, leading to overall dysfunction of DA signaling pathway in functional DA neurons, including DA synthesis, transport and re-uptake. Second is failed de-methylation of DA phenotype genes in DA neurons. In Figure 5, MeCP2 overexpression in DA neurons enhanced the gene repression effect induced by targeted methylation of *Th* promoter, suggesting that the maintenance of DA phenotype genes in DA neurons is sensitive to DNA methylation. As MeCP2 is highly expressed in adult midbrain DA neurons, genetic deficiency such as TET1 mutation or environmental cues that induce DNA methylation might serve as potential risk factors for DA neuron dysfunction. However, although DNA methylation was induced in both MeCP2-overxpressed and normal DA neurons, the transcription of *Th* gene was only repressed in MeCP2-overexpressed DA neurons. Consistently, DNA bindings of repressors and activators were also not altered in normal DA neurons experiencing MQ1-mediated targeted DNA methylation. These results might suggest the existence of an unidentified regulation mode that protects DA neurons from DNA methylation-related gene silencing.

Materials and Methods

Cell culture

DA progenitors were extracted from ventral regions of mouse embryonic day 11 mesencephalon, mechanically dissociated into single cells and plated onto poly-L-ornithine and fibronectin double-coated glass coverslips and culture plates. Cells were allowed for proliferation by daily addition of basic fibroblast growth factor and epidermal growth factor in Neurobasal media supplemented with N2 and B27 supplement. DA neuron differentiation was conducted by withdrawal of mitogens from culture media for 3 to 6 days. For NURR1 overexpression experiments, NPCs were extracted from rat embryonic day 13 cortices and cultured by similar approach to mouse DA progenitors, except that only basic fibroblast growth factor was used for proliferation and B27 supplement was not included in the media. All culture media were changed every other day and cells were maintained at 37℃ in humidified 5% CO_2 incubators.

Plasmids and gene delivery

cDNAs encoding full length mouse MeCP2 and NURR1 was generated by reverse transcriptase-PCR from RNA extracted from adult mouse brain. Plasmid expressing Dx-inducible MeCP2 was made by cloning the cDNA into FUW-TetO-MCS vector (Addgene plasmid #84008). Plasmid expressing MeCP2 and GFP tagging was made by cloning the cDNA into pLVX-EF1α-IRES-ZsGreen1 vector (TaKaRa plasmid #631982). R168X mutation was introduced by site-directed mutagenesis. Plasmid

expressing NURR1 was made by cloning the cDNA into pLVX-based vector. Plasmid expressing rat-specific shTET1 was purchased from Origene (#309902). For targeted DNA de-methylation, sgRNAs were cloned into pPlatTET-gRNA2 vector (Addgene plasmid #82559). Plasmids expressing sgRNAs were made by cloning the annealed oligo sgRNA sequences into PX459-based vector (Addgene plasmid #62988). The oligo sequences for sg-NBRE-1, -2, -3 are 5'-GAGGGGCTTTGACGTCAGCC-3', 5'-AATTAGATCTAATGGGACGG-3', 5'-ACTTTGTTACATGGGCTGGG-3', respectively.

For the production of viruses, the virus vectors and 2nd generation packaging vectors were introduced into HEK293T packaging cell line by transient transfection with Lipofectamine 2000 (Invitrogen). One day later, supernatants were harvested, concentrated with PEG8000 (Sigma), and stored until use. For viral transduction, cells were incubated with the viral supernatant containing polybrene (4 μg/ml, Sigma) for 6 hours.

For targeted DNA methylation, MQ1 and $MQ1^{Q147L}$ plasmids (addgene plasmid #89634, 89637) and sgRNAs were co-transfected into DA neurons with Lipofectamine Stem (Invitrogen) for 6 hours.

Immunofluorescent staining and imaging

Cells were fixed with 4% paraformaldehyde for 20 minutes, permeabilized and blocked with phosphate buffered saline with 0.3% Triton-X100 and 1% bovine serum albumin for 40 minutes, then incubated with first antibodies diluted with blocking

solution at 4□ overnight. Alexa Fluo series of second antibodies (Thermo Scientific) were applied accordingly for two hours at room temperature. Cells were finally mounted in 4',6-diamidino-2-phenylindole (DAPI) and examined using fluorescence microscope (Leica DMi8). Embryonic and postnatal ventral midbrain tissues were fixed with 4% paraformaldehyde and dehydrated with 30% sucrose overnight, and cryosectioned at 14 μm thickness. The first antibodies used include rabbit anti-MeCP2 (Cell Signaling Technology), mouse anti-NURR1 (R&D Systems), mouse anti-TH (Sigma), rabbit anti-NURR1 (Santa Cruz Biotechnology).

Real-time PCR analysis

RNA was extracted, reverse-transcribed (TaKaRa), amplified, and applied to real-time PCR analyses (Roche). The comparative cycle threshold method was used for quantification. Each experiment was repeated at least once to guarantee same trend of gene expression. Data from one experiment was used to generate representative histogram. The primer sequences are listed in Supplemental Table 1.

ChIP, MeDIP and hMeDIP

For ChIP experiments, cells were cross-linked with 1% paraformaldehyde for 15 minutes and Chromatins were sheared into an average 200–400 bp in length by sonication (Diagenode) and immunoprecipitated with following antibodies: rabbit anti-MeCP2 (Cell Signaling Technology), rabbit anti-NURR1 (Santa Cruz Biotechnology) and rabbit anti-TET1 (Abcam). Immunoprecipitated DNA fragments

were collected by magnetic beads (Active Motif), purified, and subjected to real-time PCR using primers specific to regions spanning three NBREs on *Th* promoter. Data were normalized to values of the input DNA. For MeDIP and hMeDIP experiments, genomic DNA were extracted from cells, sheared, immunoprecipitated, collected and subjected to real-time PCR in a similar way as ChIP with following antibodies: mouse anti-5mC (Abcam) and rabbit anti-5hmC (Active Motif). The primers are listed in Supplemental Table 1.

Cell counting and statistics

Immunoreactive or DAPI-stained cells were counted in at least 10 random regions of each culture coverslip using an eyepiece grid at a magnification of 50 to 400X. Data are expressed as mean ± S.E.M. of three to five independent cultures. Statistical comparisons were made using Student's t-test or one-way ANOVA with Tukey's post hoc analysis (Graphpad Prism).

Competing Interest Statement

The authors declare no competing interests.

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Author Contributions

X-B.H. conceived the study and wrote the manuscript. X-B.H. performed cell culture, immunostaining and epigenetic experiments. F.G. performed molecular cloning, virus production, PCR experiments and helped with data collection and data analysis. Both authors have read and approved the final version of the manuscript.

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Figure legends

Figure 1. Overexpressing MeCP2 in different periods of dopamine (DA) neuron differentiation. (A) Schematic overview depicting time- and dose-dependent overexpression of MeCP2 by FUW-TetO-mMeCP2 lentivirus within 6 days of DA neuronal differentiation in vitro (DIV). Two concentrations of Doxycycline (Dx) were treated from early (DIV 1-3) or late (DIV 4-6) period of differentiation, in which DA phenotype was specified and maintained, respectively. (B) Representative images and quantification of percentage of cells expressing DA phenotype marker tyrosine hydroxylase (TH) after 6 days of differentiation. (C) Transcriptional changes of DA phenotype genes *Th* and *Aadc* in response to MeCP2 overexpression. 2 μM Dx was added within DIV 1-3 or DIV 4-6. Analysis was performed DIV 6. Independent experiments with similar trend of results were performed at least twice, and representative data from one experiment was shown. (D) Representative images and quantification of percentage of cells expressing TH showing that overexpressions of MeCP2 and its transcription repression domain mutation R168X in cultured DA progenitors have different impact on DA phenotype specification. Ectopic genes were delivered by lentiviruses carrying ZsGreen1 fluorescent protein (GFP). (E) Quantification of cells double positive for TH and GFP. Note that no double positive cells in MeCP2-overxpressed group, indicating no cells overexpressing MeCP2 acquired DA phenotype after differentiation. Scale bar represents 50 μm. Cell numbers were counted in 10 random areas of each culture coverslip. Data represent

mean ± S.E.M. n=3 independent culture. * $P < 0.05$, ns, not significant; one-way ANOVA with Tukey's post hoc analysis.

Figure 2. Expression patterns of NURR1, MeCP2 and TH in developing and adult mouse midbrains and cell model of embryonic mouse midbrain dopamine (DA) neuron differentiation. (A) Sagittal (Embryonic day (E) 10) and coronal (E13 and Postnatal day (P) 28) views of MeCP2-positive cells co-express NURR1 or TH in mouse ventral midbrains. TH is not detected at E13. Both ventral tegmental area (VTA) and substantia nigra (SN) of midbrain is shown at P28. Scale bar represents 100 μm. (B) Cultured DA progenitors were allowed for 6 days of DA neuronal differentiation in vitro (DIV). DIV 0, 3 and 6 were selected for immunofluorescent labeling of cells expressing NURR1/MeCP2 and TH/MeCP2. Scale bar represents 20 μm.

Figure 3. Roles of MeCP2 in NURR1-mediated transactivation of dopamine (DA) phenotype genes. (A, B) Rat cortical neural progenitor cells were transduced with NURR1, NURR1+MeCP2 and NURR1+R168X and allowed for spontaneous differentiation for 3 days. R168X is a truncated mutation of MeCP2 which does not contain transcription repression domain. ZsGreen1 fluorescent protein (GFP) marks cells transduced with MeCP2, R168X or control vector. Representative images and quantification of cells expressing TH are shown as (A) and (B), respectively. (C) Quantification of MeCP2 or R168X-transduced cells expressing TH. (D) Transcriptional repression of NURR1-mediated expression of DA phenotype genes by

MeCP2 but not R168X. (E) Schematic of 1kilo bp length of rat *Th* promoter and locations of three Nurr1 binding sites (NBREs). (F-I) Chromatin immunoprecipitation (ChIP) analyses were performed to measure the DNA binding of MeCP2 (F and H) and of NURR1 (G and I) onto NBREs in DA progenitors and DA neurons transduced with MeCP2 or R168X. For ChIP assays, independent experiments with similar results were performed at least twice, and representative data from one experiment was shown. Scale bar represents 20 μm. Cell numbers were counted in 10 random areas of each culture coverslip. Data represent mean ± S.E.M. n=3 independent culture. * $P < 0.05$, ns, not significant; one-way ANOVA with Tukey's post hoc analysis.

Figure 4. DNA de-methylation in dopamine (DA) progenitors prevents MeCP2 overexpression-induced transcriptional repression of DA phenotype genes. (A) DNA methylation (5mC) and hydroxymethylation (5hmC) states within 1kilo bp promoter region of DA phenotype gene *Th* at differentiation day (DIV) 0 and DIV 6. (B) Chromatin immunoprecipitation (ChIP) experiment showing increased TET1 recruitment onto three NURR1 binding sites (NBREs) within *Th* promoter along with differentiation. (C) Doxycycline (Dx)-induced overexpression of MeCP2 in DA progenitors caused DNA methylation of *Th* promoter after DA neuronal differentiation. (D) TET1 recruitment onto NBREs within *Th* promoter at DIV 6 was reduced by overexpression of MeCP2 in DA progenitors. (E) Quantification of TH+ cells at DIV 6. Vitamin C (VC) was added in DA progenitors overexpressing MeCP2

or truncated mutation R168X from DIV 0 to DIV 6. Data represent mean ± S.E.M. n=3 independent culture. * $P < 0.05$, ns, not significant; one-way ANOVA with Tukey's post hoc analysis. (F) Enrichment of MeCP2 onto NBREs is reduced by VC in MeCP2-overexpressed DA progenitors. (G) Schematic showing strategy of targeted recruitment of inactive Cas9-TET1 catalytic domain (dCas9-TET1CD) guided by small guiding RNAs (sgRNAs) onto three NBREs within *Th* promoter. (H) Targeted de-methylation guided sgRNA targeting the first NBRE on *Th* promoter (sg-NBRE-1) in DA progenitors caused decreased DNA methylation at and near local DNA regions after differentiation. (I) Targeted de-methylation guided by sg-NBRE-1 re-activates *Th* gene expression in MeCP2-overexpressed DA progenitors Effect of targeted de-methylation of NBRE-1 in DA progenitors with or without MeCP2 overexpression on gene expression of *Th* after differentiation. (J) ChIP experiments showing the effect of targeted de-methylation of NBRE-1 in DA progenitors with or without MeCP2 overexpression on recruitments of MeCP2 and NURR1 onto NBRE-1 after differentiation. independent experiments with similar results were performed at least twice, and representative data from one experiment was shown.

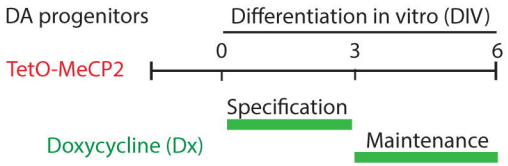
Fig 5. Targeted DNA methylation in DA neurons permits overexpressed MeCP2 counteracting TET1-dependent DA phenotype maintenance. (A) Inhibition of TET1 by short hairpin RNA in DA neurons causes DNA methylation of *Th* promoter. (B and C) Effect of TET1 inhibition on recruitment of MeCP2 (B) and NURR1 (C) onto the first NURR1 binding site (NBRE-1) in DA neurons with or without MeCP2

overexpression. (D) Effect of TET1 inhibition on *Th* gene expression in DA neurons with or without MeCP2 overexpression. (E and F) Targeted recruitments of dCas9-MQ1 and its activity-enhancing mutation $MQ1^{147L}$ onto NBRE-1 within *Th* promoter in normal or MeCP2-overexpressed DA neurons showed different impact on *Th* gene expression (E) and DNA methylation (F). (G-I) Effects of targeted DNA methylation of NBRE-1 in normal or MeCP2-ovexpressed DA neurons on recruitments of MeCP2 (G), TET1 (H) and NURR1 (I) onto NBRE-1. independent experiments with similar results were performed at least twice, and representative data from one experiment was shown.

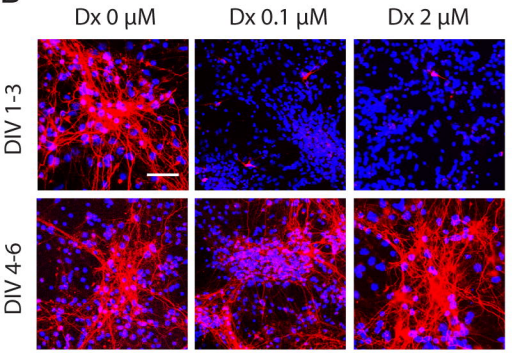
Fig 6. Proposed model illustrating roles of MeCP2 in midbrain dopamine (DA) neuron development. *Th* serves as a representative for DA phenotype genes. (A) Under normal development, DA phenotype is not yet specified in DA progenitors due to hypermethylation of the promoters of DA phenotype genes and lack of NURR1. Along with differentiation, TET1 induces gene-specific DNA de-methylation, in particular of NURR1 binding sites (NBREs), which allows recruitment of NURR1 for gene transactivation. The expression of DA phenotype genes is maintained after differentiation in DA neurons. (B) MeCP2 is normally not expressed in DA progenitors. When MeCP2 is prematurely expressed, for instance, in MeCP2 duplication syndrome, the hypermethylated promoters of DA phenotype genes become targets for MeCP2 recruitment and its mediated gene repression. This prevents TET1-mediated DNA de-methylation of DA phenotype genes along with DA

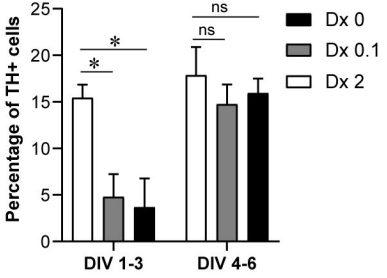
neuronal differentiation. As a consequence, DA progenitors and neurons after differentiation are not able to acquire DA phenotype. (C) In DA neurons, TET1 continues de-methylating DA phenotype genes, allowing constitutive NURR1 binding and maintenance of DA phenotype. TET1 dysfunction leads to hypermethylation of DA phenotype genes, allowing binding of excess MeCP2, failed NURR1 binding and subsequent loss of DA phenotype.

A

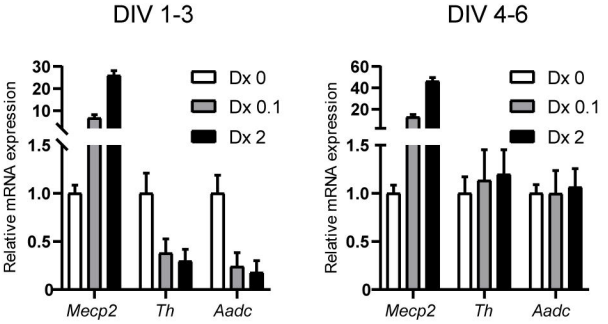

DA progenitors
Differentiation in vitro (DIV)
0
3
6
TetO-MeCP2
Specification
Doxycycline (Dx)
Maintenance

B

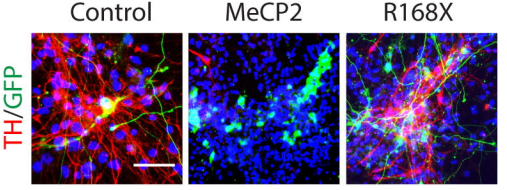

Dx 0 μM
Dx 0.1 μM
Dx 2 μM
DIV 1-3
DIV 4-6

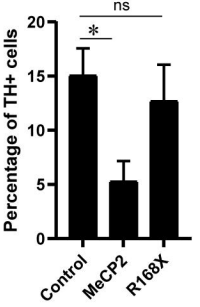

Percentage of TH+ cells
25
20
15
10
5
0
*
*
ns
ns
Dx 0
Dx 0.1
Dx 2
DIV 1-3
DIV 4-6

C

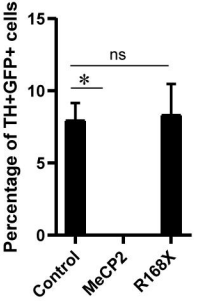

DIV 1-3
DIV 4-6
Relative mRNA expression
30
20
10
1.5
1.0
0.5
0
60
40
20
Dx 0
Dx 0.1
Dx 2
Mecp2
Th
Aadc

D

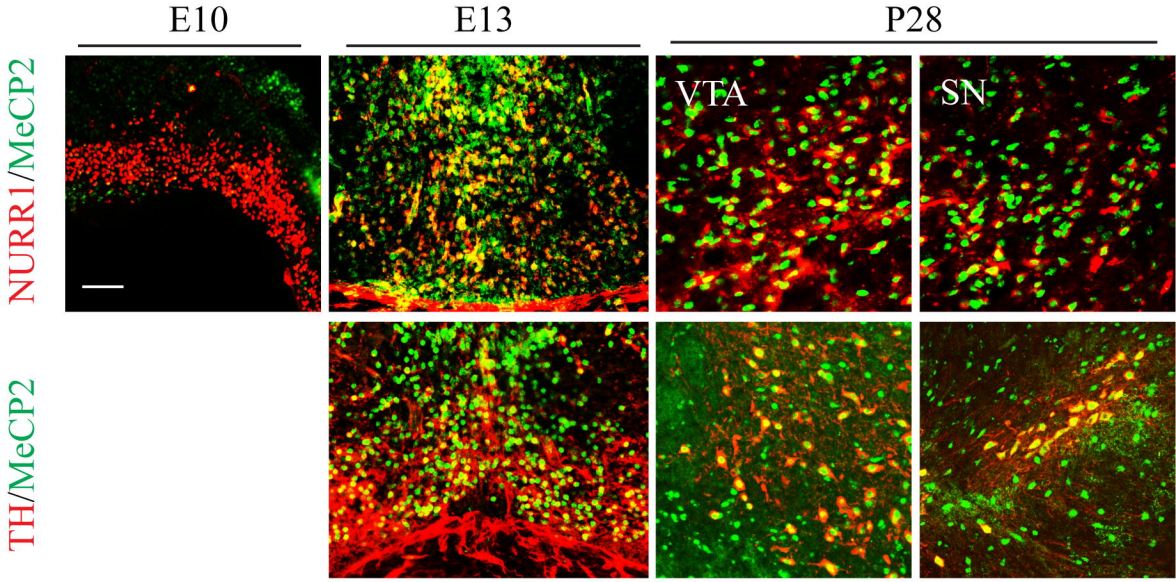

Control
MeCP2
R168X
TH/GFP


Percentage of TH+ cells
20
15
10
5
0
*
ns
Control
MeCP2
R168X

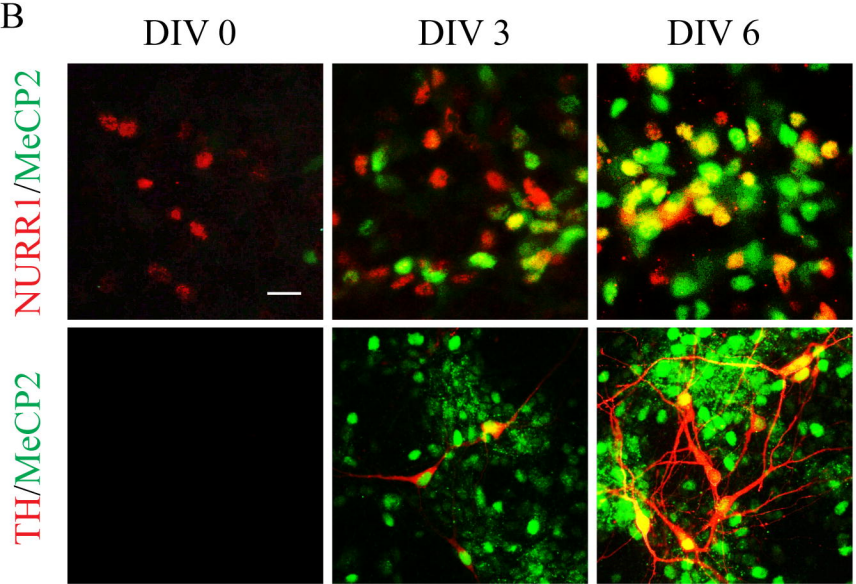
E


Percentage of TH+GFP+ cells
15
10
5
0
*
ns
Control
MeCP2
R168X

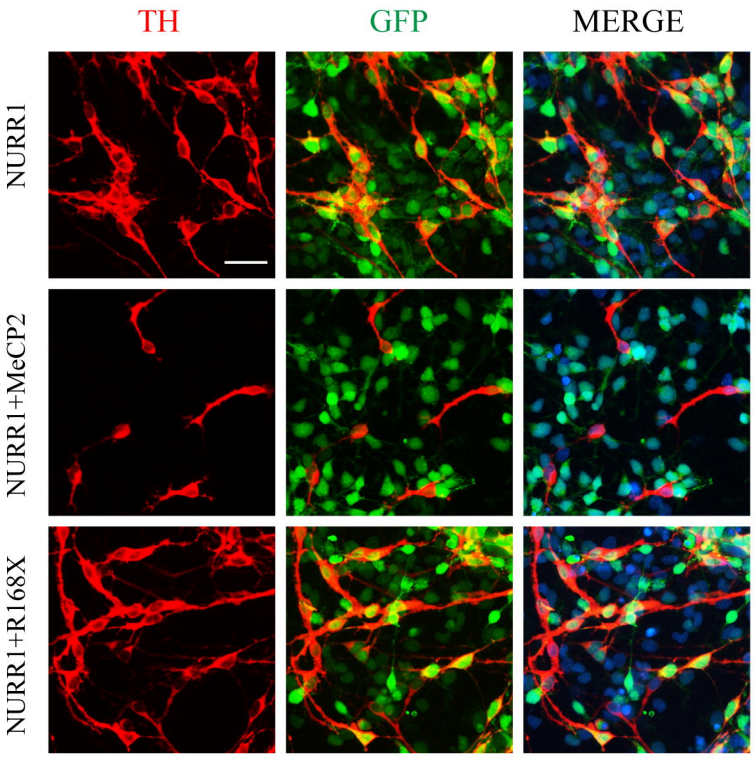
A


E10
E13
P28
VTA
SN
NURR1/MeCP2
TH/MeCP2

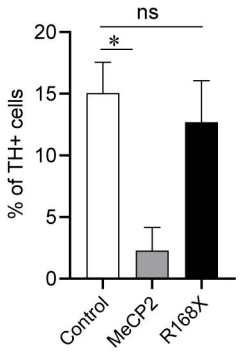
B


DIV 0
DIV 3
DIV 6
NURR1/MeCP2
TH/MeCP2

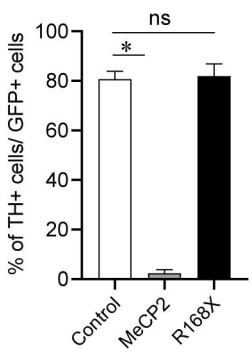
A


TH
GFP
MERGE
NURR1
NURR1+MeCP2
NURR1+R168X

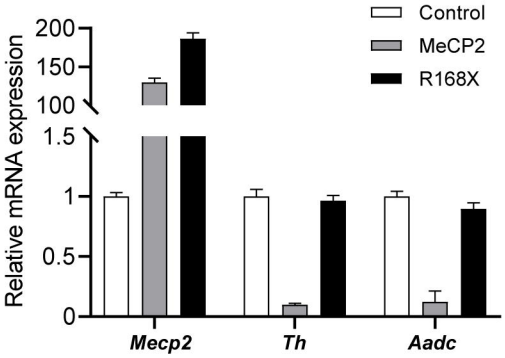
B


ns
*
% of TH+ cells
20
15
10
5
0
Control
MeCP2
R168X

C


ns
*
% of TH+ cells/ GFP+ cells
100
80
60
40
20
0
Control
MeCP2
R168X

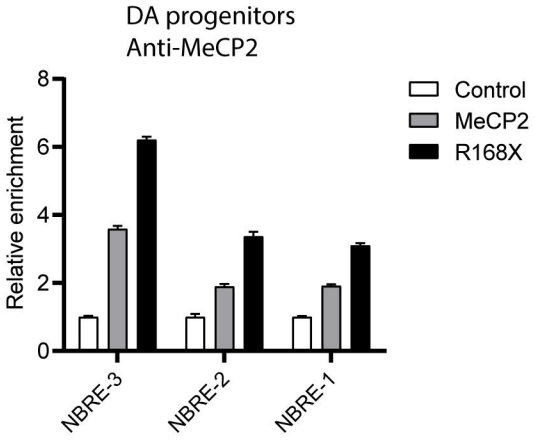
D


Control
MeCP2
R168X
Relative mRNA expression
200
150
100
1.5
1
0.5
0
Mecp2
Th
Aadc

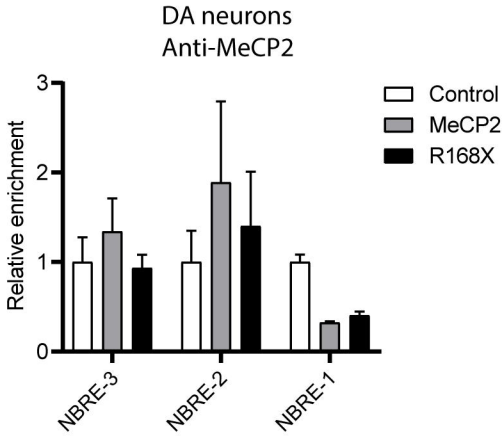
E

Th
NBRE-3
NBRE-2
NBRE-1
- 915
- 795
- 430
- 327
- 105
+ 5

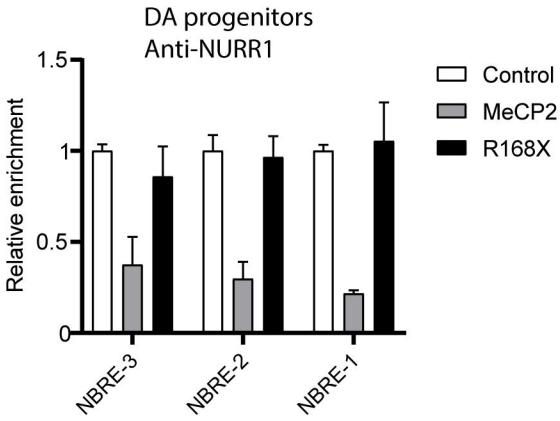
F


DA progenitors
Anti-MeCP2
Control
MeCP2
R168X
Relative enrichment
8
6
4
2
0
NBRE-3
NBRE-2
NBRE-1

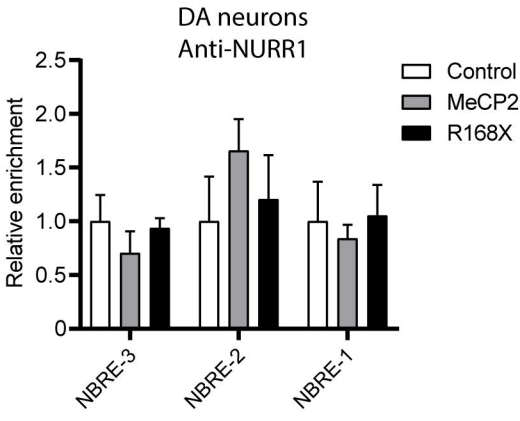
H

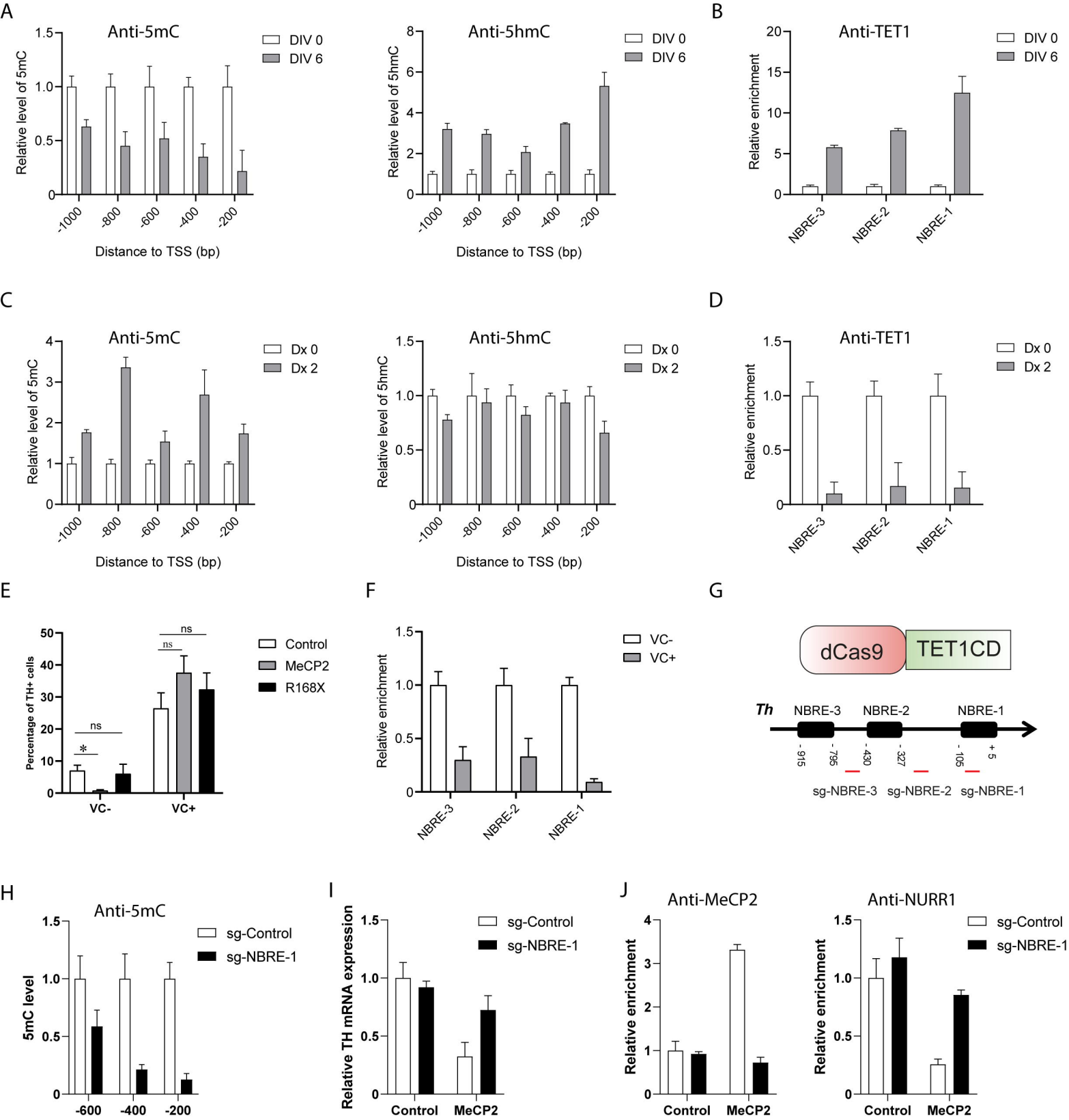

DA neurons
Anti-MeCP2
Control
MeCP2
R168X
Relative enrichment
3
2
1
0
NBRE-3
NBRE-2
NBRE-1

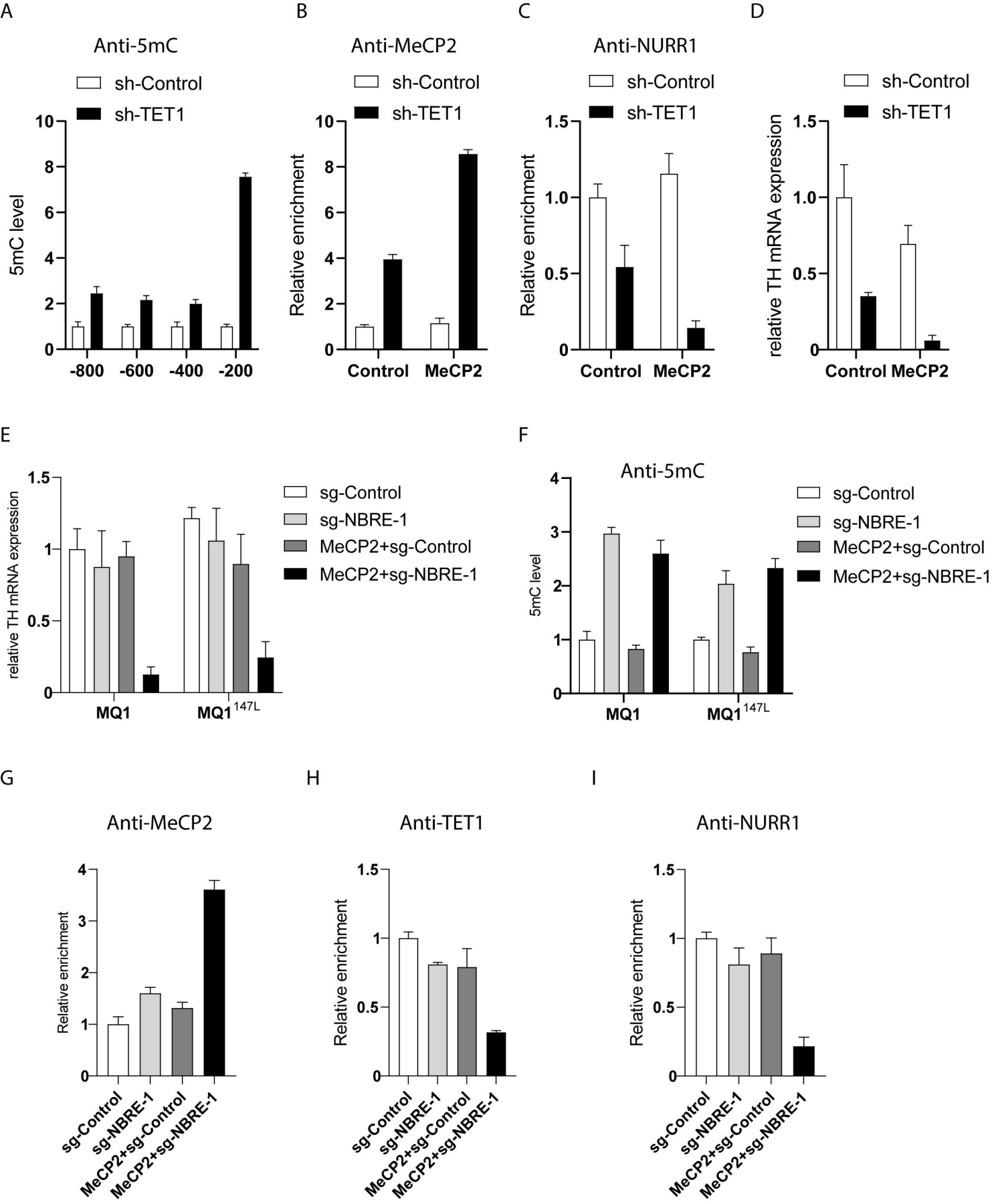
G

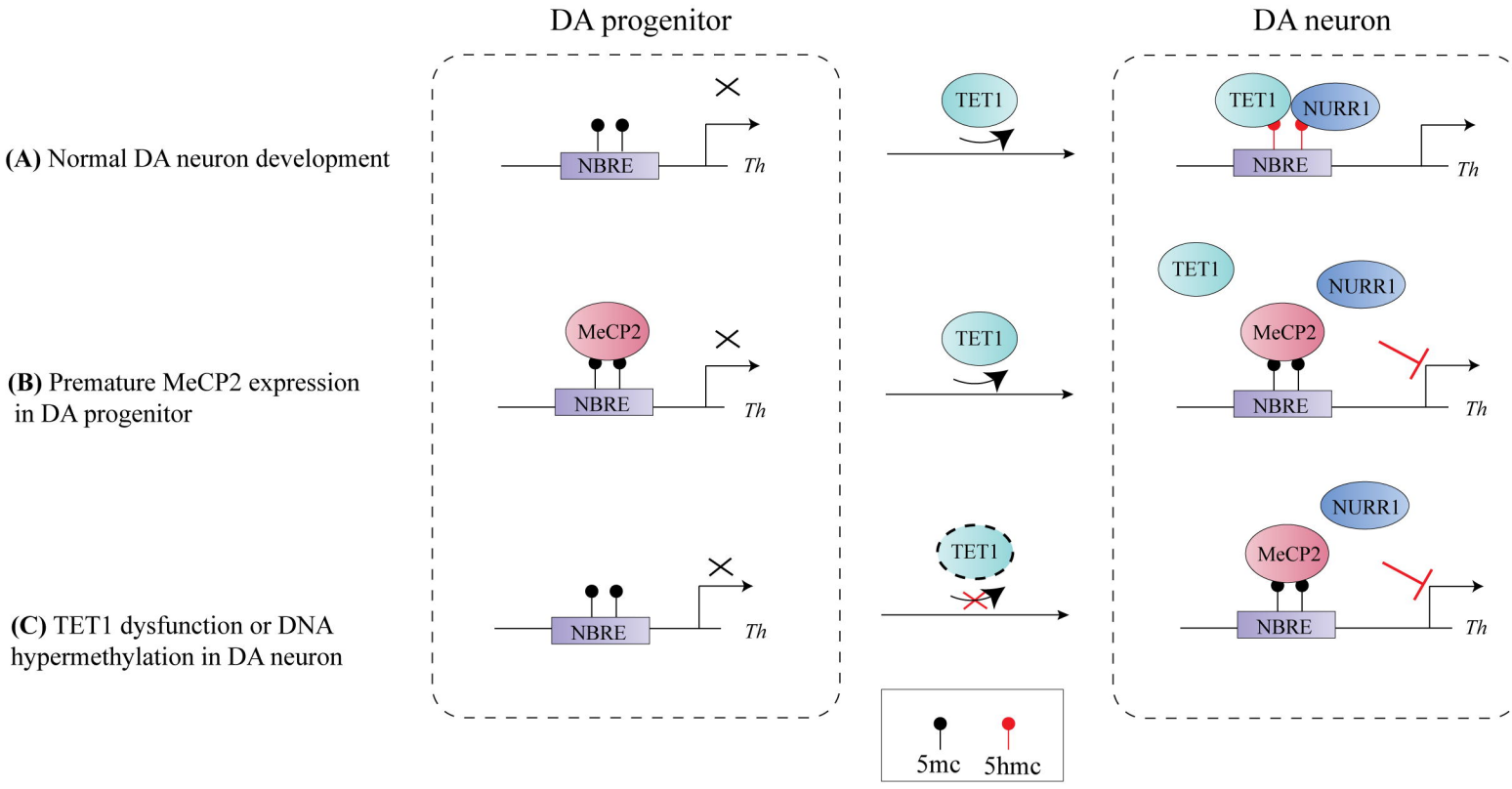

DA progenitors
Anti-NURR1
Control
MeCP2
R168X
Relative enrichment
1.5
1
0.5
0
NBRE-3
NBRE-2
NBRE-1

I


DA neurons
Anti-NURR1
Control
MeCP2
R168X
Relative enrichment
2.5
2.0
1.5
1.0
0.5
0
NBRE-3
NBRE-2
NBRE-1


A
Anti-5mC
DIV 0
DIV 6
Relative level of 5mC
Distance to TSS (bp)
Anti-5hmC
Relative level of 5hmC
B
Anti-TET1
Relative enrichment
NBRE-3
NBRE-2
NBRE-1
C
Dx 0
Dx 2
D
E
Percentage of TH+ cells
Control
MeCP2
R168X
ns
*
VC-
VC+
F
G
dCas9
TET1CD
Th
sg-NBRE-3
sg-NBRE-2
sg-NBRE-1
H
5mC level
I
Relative TH mRNA expression
J
Anti-MeCP2
Anti-NURR1


A
Anti-5mC
sh-Control
sh-TET1
5mC level
10
8
6
4
2
0
-800
-600
-400
-200
B
Anti-MeCP2
sh-Control
sh-TET1
Relative enrichment
10
8
6
4
2
0
Control
MeCP2
C
Anti-NURR1
sh-Control
sh-TET1
Relative enrichment
1.5
1.0
0.5
0
Control
MeCP2
D
sh-Control
sh-TET1
relative TH mRNA expression
1.5
1.0
0.5
0
Control
MeCP2
E
relative TH mRNA expression
1.5
1
0.5
0
MQ1
MQ1 147L
sg-Control
sg-NBRE-1
MeCP2+sg-Control
MeCP2+sg-NBRE-1
F
Anti-5mC
5mC level
4
3
2
1
0
MQ1
MQ1 147L
sg-Control
sg-NBRE-1
MeCP2+sg-Control
MeCP2+sg-NBRE-1
G
Anti-MeCP2
Relative enrichment
4
3
2
1
0
sg-Control
sg-NBRE-1
MeCP2+sg-Control
MeCP2+sg-NBRE-1
H
Anti-TET1
Relative enrichment
1.5
1
0.5
0
sg-Control
sg-NBRE-1
MeCP2+sg-Control
MeCP2+sg-NBRE-1
I
Anti-NURR1
Relative enrichment
1.5
1
0.5
0
sg-Control
sg-NBRE-1
MeCP2+sg-Control
MeCP2+sg-NBRE-1


DA progenitor
DA neuron
(A) Normal DA neuron development
(B) Premature MeCP2 expression in DA progenitor
(C) TET1 dysfunction or DNA hypermethylation in DA neuron
NBRE
Th
TET1
NURR1
MeCP2
5mc
5hmc