UNIVERSITAT DE BARCELONA

# Methyl-CpG binding protein 2 deregulation: from Rett syndrome to MeCP2 duplication disorder

Paolo Petazzi

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# METHYL-CPG BINDING PROTEIN 2 DEREGULATION: FROM RETT SYNDROME TO MECP2 DUPLICATION DISORDER

**Memoria tesis doctoral**

**Paolo Petazzi**

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INSTITUT D'INVESTIGACIÓ BIOMÈDICA DE BELLVITGE

PEBC
Cancer Epigenetics and Biology Program
Programa d'Epigenètica i Biologia del Càncer
Programa de Epigenética y Biología del Cáncer

MARIE CURIE ACTIONS

# METHYL-CPG BINDING PROTEIN 2 DEREGULATION: FROM RETT SYNDROME TO MECP2 DUPLICATION DISORDER

Memoria presentada por Paolo Petazzi para optar al grado de Doctor por la Universidad de Barcelona

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That's all. Thanks again to everyone and remember that you are free to have a look at this thesis. You might even find something of your interest.

# INDEX

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# ABBREVIATIONS

| | |
|---|---|
| 5hmc | 5-hydroxymethylcytosine |
| 5mc | 5-methylcytosine |
| AcK | Acetylated Lysine |
| AMPA | α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| AP1 | Activator Protein 1 |
| APP | Amyloid Precursor Protein |
| Arc | Activity-related Cytoskeleton-associated Protein |
| Arhgef26 | Rho Guanine Nucleotide Exchange Factor 26 |
| ASD | Autism-Spectrum Disease |
| ATP | Adenosine Triphosphate |
| ATRX | Alpha thalassemia/mental retardation syndrome X-linked |
| CA1/3 | Cornus Ammoni 1/3 |
| cAMP | Cyclic Adenosine Monophosphate |
| CBP | CREB-Binding Protein |
| CDKL5 | Cyclin-dependent Kinase-like 5 |
| CGI | CpG Island |
| ChIP | Chromatin Immunoprecipitation |
| CMV | Cytomegalovirus (promoter) |
| CNS | Central Nervous System |
| CpG | Cytosine-phospho-Guanine |
| CpH | Cytosine-phospho-(Adenine/Guanine/Thymine) |
| CSF | Cerebrospinal fluid |
| DAPI | 4 ',6-diamino-2-fenilindol |
| DG | Dentate Gyrus |
| DIV | Days In Vitro |
| DLX 5/6 | Distal-less Homeobox 5/6 |
| DNA | Deoxiribonucleic acid |
| DNMT | DNA Methlytransferase |
| EEG | Electroencephalography |
| Egr1/2 | Early Growth Response 1/2 |

| | |
|---|---|
| Evf2 | Embryonic Ventral Forebrain 2 (lncRNA) |
| FDR | False Discovery Rate |
| FoxG1 | Forkhead Box Protein G1 |
| GABA | Gamma-aminobutyric Acid |
| Gabrr2 | GABA Receptor subunit Rho-2 |
| GEO | Gene Expression Omnibus |
| GFP | Green Fluorescent Protein |
| GO | Gene Ontology |
| HAT | Histone Acetyltransferase |
| HDAC | Histone Deacetylase |
| hESCs | human Embryonic Stem Cells |
| HH | Hamilton-Hamburger |
| HIP | Hippocampus |
| HOTAIR | Hox Antisense Transcript RNA |
| HP1 | Heterochromatin Protein 1 |
| ICF | Immunodeficiency, Centromere instability and Facial anomalies (syndrome) |
| IDP | Intrinsically Disordered Protein |
| IEGs | Immediate-Early Genes |
| IGF-1 | Insulin-like Growth Factor 1 |
| IgG | Immunoglobulin G |
| IRES | Internal Ribosomal Entry Site |
| KA | Kainic Acid |
| KBS | Kaiso-Binding Site |
| LINE-1 | Long Interspersed Element 1 |
| LPS | Lipopolysaccharide |
| LSD1 | Lysine-specific Demethylase 1 |
| LTP | Long-term Potentiation |
| MAP-2 | Microtubule-associated Protein 2 |
| MBD | Methyl-Binding Domain |

| | |
|---|---|
| MBPs | Methyl-Binding Proteins |
| MeCP2 | Methyl-CpG Binding Protein 2 |
| MEF2 | Myocite-Enhancer Factor 2 |
| MeK | Methylated Lysine |
| mEPSC | miniature Excitatory Postsynaptic Currents |
| MeR | Methylated Arginine |
| MZ | Mantle Zone |
| NMDA | *N*-Methyl-D-aspartic acid |
| Npas4 | Neuronal PAS domain protein 4 |
| NPCs | Neural Precursor Cells |
| Nr4a1/3 | Nuclear Receptor Subfamily 4 Group A member 1/3 |
| ORF | Open Reading Frame |
| PcG | Polycomb Group (proteins) |
| PFC | Prefrontal Cortex |
| PhS | Phosphorylated Serine |
| PKA | Protein Kinase A |
| PRC | Polycomb Repressor Complex |
| PSD-95 | Postsynaptic Density Protein 95 |
| PTM | Post-translational Modification |
| RNA | Ribonucleic acid |
| RNAPII | RNA Polymerase II |
| RT-qPCR | Reverse Transcription-quantitative Polymerase Chain Reaction |
| RTS | Rubinstein-Taybi Syndrome |
| SCN | Suprachiasmatic Nucleus |
| SRA | Set and Ring Associated Domains (Protein) |
| SRF | Serum-Response Factor |
| TET | Ten translocation (protein) |
| TRD | Transcriptional Repressor Domain |
| TrxG | Trithorax Group (porteins) |
| TSS | Transcription Start Site |

| | |
|---|---|
| TUJ1 | Class III beta-Tubulin (Protein) |
| UCR | Ultra Conserved Region |
| UTR | Untranslated region |
| VGLUT1 | Vesicular Glutamate Transporter 1 |
| VZ | Ventricular Zone |
| WT | Wild type |
| XCI | X-chromosome Inactivation |
| XIST | X-inactive Specific Transcript |

# GENERAL INTRODUCTION

# 1. EPIGENETICS

The knowledge of the mechanisms of genetics has been advanced enormously over the last 60 years starting from the discovery of the DNA double helix. Most certainly, the information contained within this static DNA code is only a starting point. Moreover, a number of seemingly straightforward questions cannot be answered without going beyond the highly conserved script. Undoubtedly, many ask: Why and how is an endothelial cell different from a brain cell when the static DNA genome is identical? To answer this and many other complex and fascinating phenomena, we must, instead, go over or above (epi-) genetics because the DNA blueprint is identical in each of the abovementioned somatic cells. A term coined in the 1957 by Conrad Waddington, epigenetics was first referred to all the developmental changes that take place from the fertilized egg to a mature organism (Waddington, 1953). Later on, the definition of epigenetics switched to a more biomolecular meaning, referring to the mitotically and/or meiotically heritable changes in gene expression that are not due to alterations in DNA sequence (Russo et al., 1996). Very recently Adrian Bird tried to merge the first two definitions in order to avoid the constraints imposed by stringently requiring heritability. He proposed the following description of epigenetics: the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states (Bird, 2007).

The chromosomal plasticity mentioned in the Adrian Bird definition could not occur if the DNA were to exist in a "naked state". **Figure 1** provides a visual representation of the layer of covalent modifications added to the eukaryotic DNA, as well as proteins and RNAs which themselves can be modified, all of which contribute to a layer of chemical complexity and significant implications that epigenetics strives to classify and comprehend.

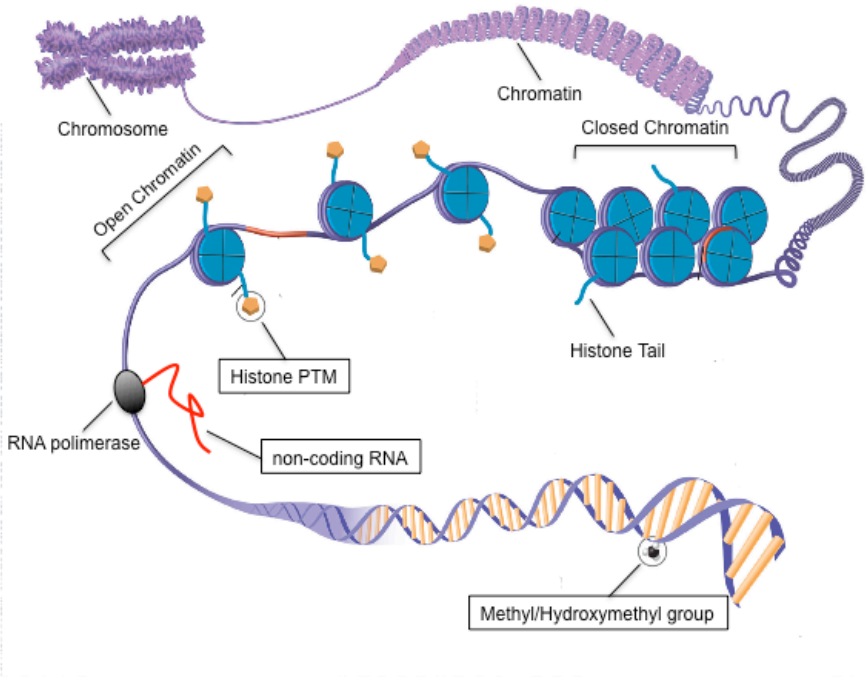


**Figure 1**: Graphical representation of the different layers of compaction of eukaryotic DNA, from the whole chromosome to base-resolution DNA. Text in boxes indicate an epigenetic modification

Division and differentiation are fundamental processes for sending cells of common origin to different destinations. Logically, the timing and regulation of these processes must be dependent on more than an identical code contained in all body cells. Thus, the epigenome comprises the immense repertoire of chemical alterations that regulate the expression of genes and, hence, dictate the function of cells and the role of proteins. A number of genetic determinants, as well as lineage-specific markings, and environmental responses are used to construct the epigenome (Bernstein et al, 2007).

The first link between cellular processes and epigenetics was represented by X-chromosome inactivation, a gene dosage compensation mechanism by which one of the two X chromosomes in the female is transcriptionally silenced (Riggs et al., 1975; Holliday and Pugh, 1975). The awareness of the role of epigenetics in human disease

was first risen in oncology, but it was soon clear that many neurodevelopmental, neurodegenerative and immunological disorders arise as a consequence of epigenetic mechanisms disruption (Holliday, 1987; Nicholls, 1993).

## 1.1 Molecular epigenetics

The mechanisms by which epigenetics affects so deeply the cell physiology are mediated by a large number of actors, most of them represented by covalently modified nucleotides and amino acids, non-coding RNAs and proteins. These epigenetic players interact among themselves and the sum of all the contributions yields a stable effect on gene expression in a very concrete chromosomal region or even in an entire chromosome, as it occurs with X-chromosome inactivation. Here, to facilitate the comprehension, I will divide the epigenetic players in marks and readers.

### 1.1.1 Epigenetic marks

Epigenetic marks are defined as genomic features not directly governed by genetic code. In this chapter I will review the main epigenetic marks with a look on the state-of-the-art knowledge

#### 1.1.1.1 DNA methylation

In vertebrates, DNA methylation occurs almost exclusively in the context of CpG dinucleotides, and most CpGs in the genome are methylated (Bird et al., 2002). CpG methylation consists of the covalent addition of a methyl group at the 5-position of cytosines that are followed by guanines. CpGs tend to cluster in region, called CpG islands (CGI) (Fig 2), which are characterized by high G+C and CpG content (Bird, 2002). About 60% of human gene promoters are associated with CGIs. Since DNA methylation is strongly associated with transcriptional silencing, it has been suggested that most of CGIs are always unmethylated, but a small proportion are subjected to tissue-specific methylation during development (Strichman-Almashanu

et al., 2002). On the other hand, the CpG dinucleotides located outside CGIs are usually methylated in somatic tissues and display a lower than expected frequency. The current explanation of this unexpected frequency is related with the maintenance of chromosomal stability, translocation prevention and endoparasitic sequence silencing (Bird, 2002) (**Figure 2**).

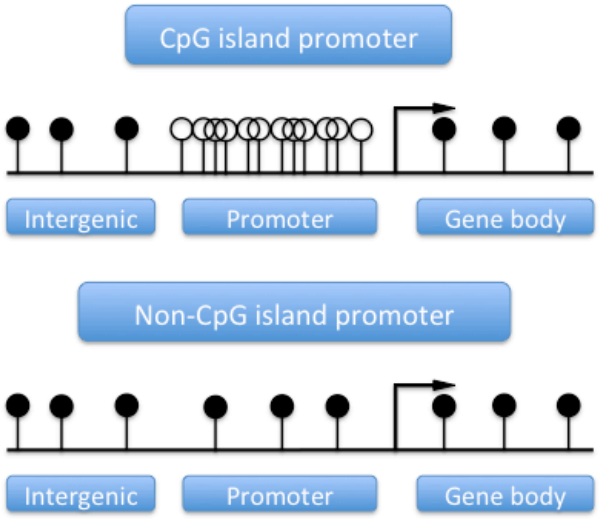


**Figure 2**: CGI versus non-CGI promoter. Black dots represent methylated CpG while white ones are un-methylated. *Adapted from Stirzaker et al., 2014*

There are evidences that non-methylated CGIs are organized in a characteristic chromatin structure that predisposes them toward promoter activity. This idea is supported by genome-wide chromatin immunoprecipitation (ChIP) and transcriptome analysis. RNA polymerase II (RNAPII) is bound at the CGI promoters of many inactive genes in embryonic stem (ES) cells (Guenther et al., 2007). Moreover, a study of chromatin at lipopolysaccharide (LPS)-inducible genes in macrophages found that these genes fall into two categories: those that requires SWI/SNF chromatin remodeling complexes for their activation and those that do not, and these groups correspond with non -CGI and CGI promoters, respectively (Ramirez-Carrozzi et al., 2009).

Methylation of other nucleotides has an established functional role in plants

(Chan et al., 2005) and might also act in mammals. In fact, non-CpG methylation has been described in human embryonic stem cells (hESC) and brain tissue (Lister et al., 2009). Recently, a study performed in mouse dentate gyrus (DG) described a potential compensatory role for CpH methylation in genome regions devoid of CpG (Guo et al., 2013).

Mammalian DNA methylation has been involved in a various range of cellular function and pathologies, including tissue-specific gene expression, cell differentiation, genomic imprinting, X chromosome inactivation, regulation of chromatin structure, carcinogenesis and aging (Bird, 2002). In addition, CpG methylation is crucial for normal development (Li et al., 1992; Okano et al., 1999). At the molecular level, a methylated cytosine can function to promote or preclude recruitment of regulatory proteins. In the first case, the methylation mark is read by a family of methyl-CpG binding proteins which in turn can recruit histone deacetylases (HDACs) and, therefore, mediate transcriptional repression (reviewed in Bird, 2002). Alternatively, the methylated CpG can exclude DNA binding proteins from their target sites, as has been shown for CTCF binding at the *H19* locus (Hark et al., 2000).

The enzymes that methylate the DNA are DNA methyltransferases (DNMTs). These proteins catalyse the transference of a methyl-group from an S-adenosyl-L-methionine to the cytosine. In mammals, the DNMT family consists of five proteins: DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L (Bestor, 2000). Once established during embryonic development by the *de novo* DNMT3A and B, DNA methylation is maintained through cell divisions by the maintenance DNA methyltransferase DNMT1. In humans, mutations in DNA methylation-related proteins or alterations of global DNA methylation profiles results in diseases, such as Immunodeficiency, Centromere instability and Facial anomalies syndrome (ICF) (Jiang et al., 2005) and cancer (Esteller, 2008).

### 1.1.1.2 5-hydroxymethylcytosine

In 2009 5-hydroxymethylcytosine (5hmC) was characterized as a relatively abundant and enzymatically-derived modification of 5mC in mammalian DNA (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009). Although 5hmC may merely represent an intermediate in an active, replication-independent DNA demethylation process, when present in DNA, 5hmC is a strong inhibitor of the DNA methyltransferase methylation reaction catalyzed by DNMT1 (Valinluck and Sowers, 2007), leading to passive DNA demethylation over subsequent replication cycles. In fact, research over recent years also revealed that this DNA modification appears to play its own role in epigenetic gene control. 5hmC is strongly associated with genes and regulatory elements in the genome, is abundant in brain, ES cells, primordial germ cells, and fertilized oocytes (Jin et al., 2011b). 5hmC is depleted in many types of human cancer (Jin et al., 2011a).

The protein Ten-Eleven-Translocation 1 (TET1), previously implicated in chromosomal translocations in leukemia (Lorsbach et al., 2003), was the first enzyme shown to catalyze the oxidation of 5mC to 5hmC (Tahiliani et al., 2009). One year later another study demonstrated that other 2 proteins, TET2 and TET3, possess the same enzymatic activity (Ito et al., 2010). Therefore, 5hmC can be established by three different TET enzymes, which are differentially expressed during development and target diverse genomic regions such as gene body, promoters or enhancers. This suggests that 5hmC may play a multifaceted role in genome biology.

### 1.1.1.3 Histones post-translational modifications

In the nucleus of eukaryotic cells, DNA is wrapped around an octamer of basic proteins. The octamer consists of two copies of each of the core histones, H2A, H2B, H3 and H4 (Kouzarides, 2007). The core histones, together with 146 base pairs of DNA, form the fundamental unit of chromatin structure, the nucleosome. Higher order organization of the chromatin presuppose DNA package into fibers by the binding of the non-core histone H1 to the linker DNA between adjacent nucleosomes

(Kimmins and Sassone-Corsi, 2005). Intriguingly, histone core proteins not only serve as structural proteins: N-terminal regions of histones protrude from the nucleosome and are recognized by other proteins. There are many types of post-translational modification (PTM) of the residue at histone tails, including methylation of lysines and arginines, acetylation, phosphorylation, ubiquitination, SUMOylation, and ADP-ribosylation (Kouzarides, 2007).

The vast majority of histone PTMs remains poorly understood, but in the last 20 years we have seen considerable progress in the understanding of lysine acetylation and methylation. Whereas lysine acetylation is almost always associated with chromatin accessibility and transcriptional activity due to the reduction of the positive charge, lysine methylation can have different effects depending on which residues is modified (**Table 1**).

| *Modification* | *Enzyme* | *Function* |
|---|---|---|
| **ACETYLATION** | | |
| H2AK5 | Tip60, p300/CBP | Transcriptional activation |
| H2BK5/K12/K15 | Atf2, P300/CBP | Transcriptional activation |
| H3K9 | Gcn5, Src-1 | Transcriptional activation |
| H3K14 | Gcn5, taf1, Tip60 | Transcriptional activation, DNA repair, RNA pol II and III transcription |
| H3K27 | Gcn5 | Transcriptional activation |
| H4K8 | Gcn5, Pcaf, Tip60, Atf2, p300/CBP | Transcriptional activation, DNA repair, Telomeric silencing |
| **METHYLATION** | | |
| H3K4 | Set7/9, MLL, ALL-1 | Transcriptional activation |
| H3K9 | SetDB1, Suv39h | Transcriptional silencing, genomic imprinting |
| H3K27 | EZH2, EZH1, G9a | Transcriptional silencing, X-inactivation |
| H3K36 | Set2 | Transcriptional elongation |
| H3K79 | Dot1 | Transcriptional elongation |
| H4K20 | Pr-Set7, Suv4-20h | Transcriptional silencing, heterochromatin |
| H3R8 | Prmt5 | Transcriptional silencing |
| H4R3 | Prmt1, 5 | Transcriptional activation |
| **PHOSPHORYLATION** | | |
| H2AS139 | ATR, ATM | DNA repair |
| H3S10 | MSK1, MSK2, Aurora-B kinase | Mitosis, Meiosis, Transcriptional activation |
| H3S28 | MSK1, MSK2, Aurora-B kinase | Mitosis |

**Table 1**: Most common histone post-translation modification. The enzymes responsible for the modification are also listed.

Methylation of histone H3 lysine 4 (H3K4) and H3 lysine 36 (H3K36) correlates with transcribed chromatin. In contrast, methylation of H3 lysine 9 (H3K9), H3 lysine 27 (H3K27) and H4 lysine 20 (H4K20) is generally linked to gene silencing (Bernstein et al., 2007). Most PTMs are distributed in distinct localized patterns within the upstream region, the core promoter, the 5' end or the 3' end of the open reading frame (ORF) **(Figure 3)**. Indeed, the location of a modification is tightly regulated and is crucial for its effect on transcription. For instance, SET2-mediated methylation of histone H3K36 normally occurs within the ORF of actively transcribed genes. However, if SET2 is mistargeted to the promoter region through artificial recruitment, it represses transcription (Landry et al., 2003; Strahl et al., 2002).

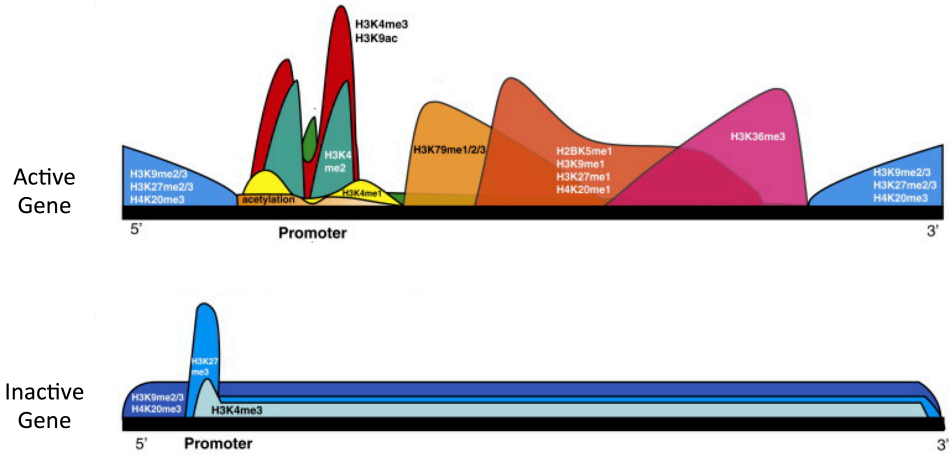


**Figure 3**: Histone marks distribution along active (upper panel) and inactive genes (lower panel). *Adapted from Barth and Imhof, 2010*

Starting from their identification decades ago, histone PTMs have been proposed to possess several functions. However, common features have begun to emerge in recent years. First, with the exception of methylation, histone modifications result in a change in the net charge of nucleosomes, which could loosen inter- or intra-nucleosomal DNA-histone interactions. This idea is supported by the observation that acetylated histones are easier to displace from DNA both in vivo (Reinke and Horz, 2003; Zhao et al., 2005) and in vitro (Chandy et al., 2006; Hassan

et al., 2006). Second, it is widely accepted that histone PTMs can be recognized by other proteins (Seet et al., 2006). Given the diversity of covalent modifications, it has been proposed that individual histone modifications or modification patterns might be read by other proteins, thus influencing chromatin dynamics and function (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 2000). Also, the outcome of a particular modification is dependent on the effector proteins that recognize it. Third, some modifications directly influence higher-order chromatin structure. For instance, acetylation of H4K16 inhibits the formation of compact 30 nm fibers (Shogren-Knaak et al., 2006). Finally, the mechanisms discussed above are not necessarily mutually exclusive. For example, acetylation of H4K16 also impairs the efficiency of ATP-dependent chromatin assembly and mononucleosome mobilization by the ACF histone chaperone (Shogren-Knaak et al., 2006), therefore suggesting that a single modification can elicit multiple effects on chromatin structure.

Since histone PTMs are very dynamic and they must quickly respond to cell signals, the enzymes for the establishment and removal of these modifications have a critical role for the correct spatio-temporal regulation of this epigenetic mark. In addition to the widely known Polycomb (PcG) and Trithorax (trxG) group proteins, numerous enzymes are responsible for the wide range of histone modifications, with often multiple enzymes capable of modifying a particular histone tail residue (Kouzarides T. 2007). For instance, during the early embryogenesis, when cells lack DNA methylation, the transcriptional repression is mainly due to PcG based mechanisms (Schuettengruber B. 2007). PcG mediates transcriptional repression of key developmental genes by two different complexes, Polycomb Repressor Complex (PRC) 1 and PRC2. Both PRC1 and PRC2 can interplay to repress transcription. Essentially, EZH2, a component of PRC2, trimethylates H3K27 (Cao et al., 2002).

Over the past decade numerous histone demethylases have been identified adding complexity to the histone code. These enzymes can be grouped in proteins containing the Jumonji (JMJMC) domain, and a second group consisting of amine oxidase family of proteins (Shi Y. 2004; Tsukada Y. 2006). The latter group includes the first histone demethylase identified, Lysine-specific Demethyase 1 (LSD1/KDM1)

(Shi Y. 2004).

Histone acetylation is regulated by the opposing action of histone acetyl transferases (HATs) and histone deacetylases (HDACs). The addition of acetyl groups is catalyzed by HATs, which are divided into the GNAT, MYST and p300/CBP subfamilies (Berndsen and Denu, 2008), whereas the removal of acetyl groups is mediated by HDACs, which, in mammals, are divided into four groups: the zinc-dependent class I, II and IV HDACs, and the NAD-dependent class III HDACs, which are also known as sirtuins (Haberland et al, 2009).

### 1.1.1.4 Non-coding RNAs

Our understanding of RNA biology has increased enormously in the last decade thanks to the recent advent of advanced sequence technology and findings from large-scale consortia focused on characterizing functional genomics elements, such as ENCODE (Thurman et al., 2012) or FANTOM (Carninci et al., 2005). We are now aware of the incredible large number of non-coding RNAs (ncRNAs) that the human genome is able to encode. Moreover, several species of ncRNAs exist with different size and origin of transcription, and the vast majority of them are supposed to carry out transcriptional regulatory tasks. ncRNAs are commonly classified based on their size in two major groups: small ncRNA (sncRNAs, 20-30nt) and long ncRNAs (lncRNAs, >200nt).

sncRNAs are involved in post-transcriptional regulation of target RNAs via RNAi, and/or modifying other RNAs, including microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs) and small nucleolar RNAs (snoRNAs). miRNAs are a class of small ncRNA with a role in post-transcriptional gene silencing through base-pairing with the 3'-UTR of the target transcript. This interaction leads to degradation of the target or to inhibition of translation (Kim et al., 2009). miRNAs classical biogenesis pathway involves processing by the DROSHA-containing complex, export into the cytoplasm through exportin 5, and cleavage by the DICER1 ribonuclease. snoRNAs and piRNAs are two types of small non-coding less characterized than

miRNAs. The firsts guide RNA-modifying enzyme complexes to other RNA molecules (such as rRNAs) in the nucleolus (Kiss, 2002). On the other hand, piRNAs are DICER-independent and can originate from transposon elements or from piRNA clusters. Although piRNAs were initially found in germ cells, recent studies have established that they are expressed in somatic cells, including neurons (Yan et al., 2011).

By definition lncRNAs are transcript of at least 200 nucleotides in length. Many lncRNAs are post-transcriptionally processed like mRNA and therefore undergo 5' capping, polyadenylation, alternative splicing, RNA editing and trafficking (Kapranov et al., 2007). lncRNAs can be transcribed from different sites within the genome:

- from intergenic regions (Guttman et al., 2009; Khalil et al., 2009);
- in antisense, overlapping, intronic and bidirectional orientations relative to protein-coding genes (reviewed in Qureshi and Mehler, 2012);
- from gene regulatory regions: UTRs (Mercer et al., 2011), promoters (Hung et al., 2011) and enhancers (Orom et al., 2010);
- from telomeres (Azzalin et al., 2007).

Several roles (**Figure 4**) have been described for lncRNAs in transcriptional and epigenetic regulation. lncRNAs can recruit transcription factors and chromatin-modifying complexes to specific nuclear and genomic sites (Khalil et al., 2009). They are also involved in alternative splicing and other post-transcriptional RNA modifications through the assembly of nuclear domains containing RNA-processing factors (Wang and Chang, 2011; Caudron-Herger and Rippe, 2012). Notably, lncRNAs can exert their function on the genomic loci they are derived from (*in cis*) or far away (*in trans*). The structural and functional activities of lncRNAs have recently been categorized within a well-designed framework as molecules of signals, decoys, guides, and scaffolds (Wang and Chang, 2011).

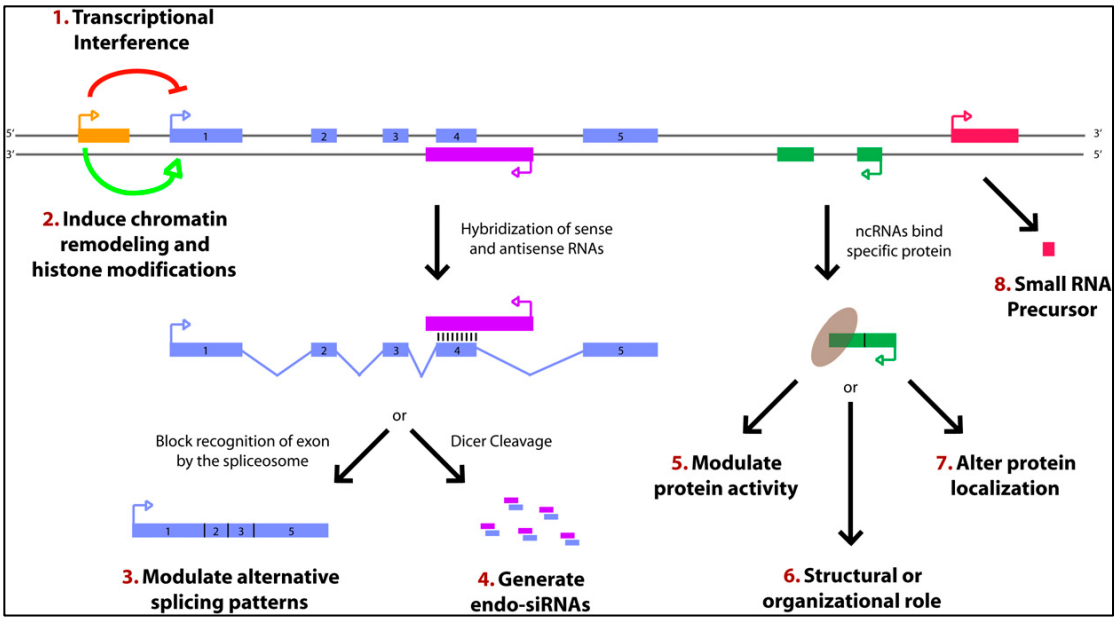


**Figure 4**: Proposed roles for long non-coding RNAs. *Taken from Wilusz et al., 2009*

## 1.1.2 Epigenetic readers

Epigenetic readers are effector proteins that recognize and are recruited to histone or nucleotide specific marks. Enzymes that "write" or "erase" epigenetic marks may also contain such reader domains, leading to the formation of multifunctional complexes.

### 1.1.2.1 Methyl-CpG binding proteins

Methylated DNA can be specifically recognized by a set of proteins called methyl-CpG-binding proteins (MBPs), which belong to three different structural families in mammals: the MBD family, the KAISO and KAISO-like proteins and the SRA domain proteins. The simplified mechanistic view is that, once bound to methylated DNA, MBPs translate the DNA methylation signal into appropriate functional states, through interactions with diverse partners.

The most studied group of MBP proteins is the MBD family, which consists of four MBD proteins that bind methylated DNA: MeCP2, MBD1, MBD2 and MBD4, and three other proteins, MBD3, MBD5 and MBD6, that are members of this family but do

not bind methylated DNA (Hendrich and Tweedie, 2003; Laget et al., 2010). Although the MBD proteins share a common capacity for binding symmetrically methylated CpGs via the Methyl-binding domain (MBD), each protein shows distinct domain structure and interacts with different proteins. This suggests that the MBD proteins have primarily non-overlapping functions and play roles in diverse biological processes. Several MBD proteins have been shown to repress methylated promoters via HDAC2 and chromatin remodelers recruitment and subsequent chromatin compaction (Parry and Clarke, 2011). The MBD family has been extensively studied, though several unanswered question still remain to uncover, especially about biological significance, because mice lacking any of these protein (with the exception of MeCP2) don not develop clear phenotypes. Recently, a new role for one of the member of the MBD family, MeCP2, has been discovered. A study showed that, in brain, MeCP2 binds 5hmc and 5mc with similar affinities (Mellén et al., 2012). This finding expands the role of MeCP2 from a mere transcriptional repressor to a chromatin regulator able to either promote or repress gene expression depending on the DNA modification is bound to. In addition, MBD3 has also been shown to bind to 5hmc-containing DNA in ES cells (Yilidrim et al., 2011)

Contrary to MBD proteins, KAISO family protein could discriminate methylated from unmethylated DNA (Prokhortchouk et al., 2001). KAISO protein was independently shown to also bind a non-methylated consensus site, CTGCNA, called the KAISO binding site (KBS) (Daniel et al., 2002). Kaiso has two close paralogs in mammalian genomes: Zbtb4 and Zbtb38 (Sasai and Defossez, 2009). These proteins, like KAISO, bind methylated DNA but can also bind a non-methylated consensus (Filion et al., 2006). The KAISO group of proteins contains a BTB/POZ domain at the N terminus and three tandem Kruppel-like C2H2 zinc fingers at the C terminus. The BTB/POZ domain permits protein–protein interactions (Perez-Torrado et al., 2006), and zinc fingers allow binding of a sequence-specific DNA (KBS) (Daniel et al., 2002; Ruzov et al., 2009). Depletion of Kaiso in Xenopus embryos results in de-repression of methylated genes before the midblastula transition (Ruzov et al., 2004; Ruzov et al., 2009). Recently, KAISO was showed to localize at centrosomes and spindle

microtubules during mitosis, revealing a new functional role for KAISO in centrosome separation during mitosis, microtubule nucleation and/or the G2-M checkpoint (Soubry et al., 2010).

The third and last family of MBPs was discovered through the efforts of Yusuke Nakamura and his team, who established that UHRF1 and UHRF2, two related proteins, could bind methylated DNA via their SRA domains (Unoki et al., 2004). It was soon discovered that UHRF1 is an essential protein that binds hemi-methylated DNA and recruits DNMT1 to facilitate maintenance DNA methylation; in the absence of UHRF1, there is a precipitous loss of DNA methylation (Bostick et al., 2007). In fact, its deletion leads to rapid developmental arrest (Sharif et al., 2007). Interestingly, base specific interactions from UHRF1 are limited to the 5-mCpG/CpG pair, suggesting that in contrast to the MBD family, sequence context outside of the mCpG site is not critical for recognition. UHRF1 contains at least five functional domains (Hashimoto et al., 2009): an ubiquitin-like domain (UBL) at the N-terminus; a tandem Tudor domain (TTD) that binds histone H3 tails trimethylated on residue K9 (Karagianni et al., 2008); a plant homeodomain (PHD), which can bind modified histones; a SET and RING Associated domain (SRA) that binds methylated DNA; and finally a Really Interesting New Gene (RING) domain at its C-terminus, which is endowed with catalytic activity as an E3 ubiquitin ligase.

### 1.1.2.2 Readout of histone PTMs

The high density of modificated sites and various types of histone PTMs plus the additional DNA methylation nearby PTMs might indicate that many chromatin marks are not acting independently, but instead they influence each other function. The cross talk between different epigenetic players is crucial to the final effect on chromatin organization and gene expression. Here I review some of the most-known histone readers and their respective mechanisms of action.

Acetylated lysines (AcKs) can be recognized by bromo domains (Dhalluin et al., 1999) and by the tandem PHD domain (Lange et al., 2008; Zeng et al., 2010). Many

bromo domains bind to multiple acetylated histones and the tandem PHD domain of human DPF3B also prefers acetylated H3 and H4 (Lange et al., 2008), indicating the lack of unique sequence recognition by these readers. Since the interaction between AcK and its readers is relatively weak, multiple domains working in tandem are common. For instance, the chromatin-remodeling complex, RSC, has three bromo domain-containing subunits (Rsc1, Rsc2 and Rsc4) (Cairns et al., 1999), the SAGA-HAT complex contains two-bromo domain proteins (Gcn5 and Spt7) and the polybromo (PB) protein alone consists of six-bromo domains (Charlop-powers et al., 2010).

One of the most stable histone marks is lysine methylation, and it presents four types of states: unmethylated (me0), mono- (me1), di- (me2) and tri- (me3) methylation. Domains that recognize histone MeK include PHD, chromo, WD40, Tudor, double/tandem Tudor, MBT, Ankyrin Repeats, zf-CW and PWWP domains, a long list that may continue to grow in coming years. Unlike acetylation, methylation is highly site-specific and is maintained by histone methyltransferases and demethylases that possess stronger site-specificity than HAT and HDAC (Tsukada et al., 2006). The recognition of methyl states occurs in two ways. At some lysines, different methyl-states recruit different sets of effectors. For instance, Pdp1 binds to H4K20me1 for cell-cycle regulation, whereas Crb2 recognizes H4K20me2 to control a DNA damage checkpoint (Wang and Jia, 2009). However, at other sites, methyl states only control the binding strength of the same chromatin regulators. For example, Rpd3S binds to K36me1 nucleosomes at a similar affinity to the unmodified ones, K36me2 shows stronger binding and K36me3 displays the highest affinity (Li et al., 2009).

Several proteins have been shown to specifically interact with H3K9me. HP1 binds via its chromodomain to histone H3 peptides in vitro with a slight preference for K9me2/3 over K9me1. Consistent with this, HP1 cellular distribution largely overlaps that of H3K9me3. In addition, Ankyrin repeats of G9A were shown to bind H3K9me1/2 in vitro (Collins et al. 2008). Also, a PHD region in UHRF1 displays affinity for H3K9me (Papait et al. 2008). However, the downstream working

mechanisms of the different H3K9me interacting factors are still unknown. An important aspect of H3K9me biology seems to be cross talk with DNA methylation systems.

In contrast with lysine methylation, methyl-arginine (MeR) can be found in three states depending on the position of the methyl group: monomethylation (me1), symmetrical dimethylation (me2s) and asymmetrical dimethylation (me2a). The ADD domain (containing a PHD motif) of the DNA methyltransferase DNMT3A recognizes H4R3me2s but not H4R3me2a, thereby linking histone MeR to DNA methylation and gene repression (Zhao et al., 2009). The Tudor domain of TDRD3 is a reader for H3R17me2a and H4R3me2a (Yang et al., 2010). TDRD3 acts as a transcription coactivator and is enriched at transcription start sites (Yang et al., 2010), which link MeR to a role in active transcription.

Protein domains that recognize phosphorylated amino acids in a non-histone context are well characterized and include SH2, BRCT, WW, FHA, WD40, 14-3-3 and LRR domains. However, only two readers have been identified for phosphorylated serine (PhS) in histones. The BRCT domain of MDC1 binds to PhS near the C-terminus of histone H2AX (Stucki et al., 2005). Instead, PhosphoS10 histone H3 is read by the 14-3-3 family. Mammalian 14-3-3ζ recognizes H3S10ph peptide using a deep scaffold (Macdonald et al., 2005). Interestingly, the binding of the yeast 14-3-3 proteins Bmh1 and Bmh2 to H3S10ph peptides is stimulated by H3K14ac, and H3K14ac is important for the recruitment of Bmh1 *in vivo* (Walter et al., 2008). Future structural analysis would provide more insight into how the same family of readers responds differently to the PTM near its primary target.

To add some more complexity to the topic, other epigenetic modifications can regulate histone readers. For example, ncRNA plays important roles in targeting chromatin regulators to their cognate sites. The CBX7 subunit of the PRC1 complex not only contains a chromo domain that reads K27me2, but also recognizes an antisense ncRNA transcribed from the *INK4b/ARF/INK4a* locus using a different binding surface. Therefore, ncRNA association is important for PRC1 targeting and repression functions (Yap et al., 2010). Similarly, short ncRNA generated from PRC2-

repressed promoters forms stem-loop structures that interact with PRC2 and control its localization (Kanhere et al., 2010). Finally, *HOTAIR* ncRNA and *XIST* RNA also help targeting PRC2 *in cis* (Tsai et al., 2010; Zhao et al., 2008). However, in these cases, it is not clear how RNA binding coordinates with PTM recognition.

## 1.2 Neuroepigenetics

In the last 25 years it has become clear that experience, in the form of environmental factors, maternal behavior, psychological or physical stress, learning or drug exposure, leads to active regulation of the chemical and three-dimensional structure of DNA in the nervous system, i.e., that experience is transduced into epigenetic mechanisms in the central nervous system (CNS) (Borrelli et al., 2008; Champagne and Curley, 2009; Day and Sweatt, 2010; Renthal and Nestler, 2008).

The definition of epigenetics does not fit properly when it comes to neurons. In fact, mature neurons are non-dividing cells and, because of this, no genomic modification can be inherited by a descendent cell. The other epigenetic features are conserved in neurons, like the long-lasting, permanent and self-regenerating properties of epigenetic marks. For all these reasons, Day and Sweatt (2010) proposed to adopt the term neuroepigenetic to highlight the difference with classical, heritable epigenetics.

One of the most interesting topics in neuroepigenetic is memory formation. In recent years, epigenetics modification of DNA and chromatin have been identified as essential mediators of memory formation through the regulation of gene expression (Sultan and Day, 2011), with CpG cytosine methylation playing a crucial role in memory consolidation and stabilization over time. Indeed, several studies reported rapid and reversible changes in DNA methylation at memory-associated genes, suggesting the presence of both active DNA methylation and demethylation processes in response to neuronal activity (Lubin et al., 2008; Miller et al., 2010).

Another quite important epigenetic feature in the brain is the presence of 5'-hydroxymethylcytosine. In the CNS, 5-hmc is substantially enriched relative to many

other tissues and cell types, for instance, 10 times more in brain than in some peripheral tissue or embryonic stem (ES) cells (Globisch et al., 2010). Moreover, the genomic location of 5-hmc in the brain seems to be different in comparison with ES cells. Whereas 5-hmc is preferentially found in enhancers and promoters of pluripotency-related genes in ES cells (Pastor et al., 2011; Yu et al., 2012), in the brain it is enriched in gene bodies and depleted from transcription start sites (TSS) (Szulwach et al., 2011). Finally, the striking finding that MeCP2 binds to 5hmc within active transcription units (Mellén et al., 2012) expands our understanding of neuroepigenetics and leaves several unanswered question.

Acetylation of histones has a prominent role in both development and adult function of the brain. For instance, the protein p300 and its close homolog CBP are transcriptional coactivators with HAT activity that participate in the activities of numerous transcription factors, including CREB, SRF, MEF2 and C-JUN, among others (Goodman and Smolik, 2000). Although CBP/p300 is widely distributed throughout the developing neural tube, in the adult CNS it is mostly confined to subpopulations of cortical cells and motor neurons (Partanen et al., 1999). A peculiar protein with HAT activity is CLOCK, which is fundamental for the proper expression of core circadian clock genes in the suprachiasmatic nucleus (SCN) of the hypothalamus, and for the maintenance of circadian rhytmicity (Doi et al., 2006). The counterparts of HATs, HDACs, are expressed in the nervous system, often in a developmentally regulated manner (Broide et al., 2007). HDAC1 is confined to neural stem cell and glia, whereas HDAC2 is mostly found in post-mitotic neuroblasts and differentiated neurons (MacDonald and Roskams, 2008).

The importance of histone modification in neuronal cells becomes paramount when it comes to activity-dependent regulation. After depolarizing stimuli, the $Ca^{2+}$-dependent signal cascade provokes phosphorylation of CBP, which in turn acetylate the promoter (**Figure 5**). At the same time the phosphorylation of histone H3 on serine 10 cooperate with histone acetylation to induce chromatin unfolding and subsequent gene expression (Impey et al., 2002). Moreover, certain neuronal genes as Bdnf are maintained in a repressed state through a mechanism that includes the

recruitment at the promoter of LSD1, which is a lysine demethylase, HDAC2 and MeCP2. After synaptic stimulation, the co-repressor complex is dissociated from gene regulatory regions and co-activators are recruited at the TSS (Riccio et al., 2010).

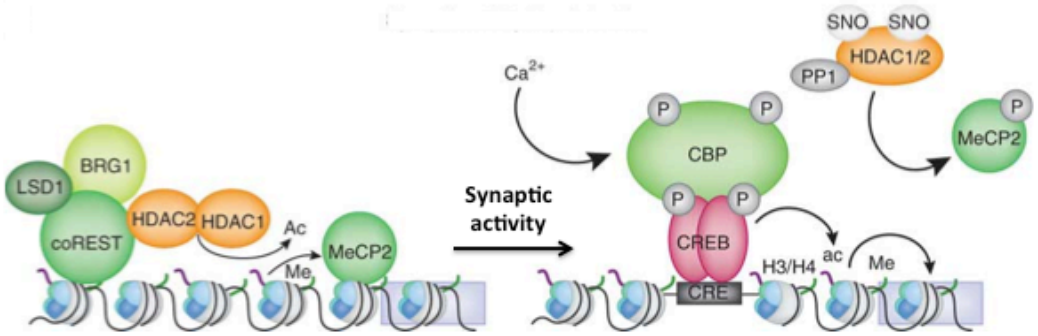


**Figure 5**: In neurons, certain activity-dependent genes such as Bdnf are maintained in a repressed state through a mechanism that includes the recruitment of coREST, HDAC1, HDAC2 and MeCP2. Following synaptic stimulation, HDAC2, and possibly HDAC1, are S-nitrosylated (SNO), whereas MeCP2 is phosphorylated, resulting in the dissociation of the co-repressor complex from gene regulatory regions and the recruitment of co-activators such as CREB and the histone acetyltransferase CBP. *Adapted from Riccio, 2010.*

Several ncRNAs have also been associated with neural development, maintenance and plasticity. For example, Evf2 is a lncRNA transcribed from an enhancer that affects forebrain development by modulating the expression of DLX5/6 transcription factors. It has been shown that Evf2-deficient mice present a decrease in the population of GABAergic interneurons within the early postnatal hippocampus, as well as defects in synaptic inhibition (Bond et al., 2009). Furthermore, a number of miRNAs have very important roles in synaptic functions by targeting CREB, which is vital for long-term potentiation (LTP) and memory (Wu et al., 2006). One intriguing report identified thousands of ncRNAs in neuronal cells derived from enhancer elements, called enhancer RNAs (eRNAs). The transcription of eRNAs is activity-dependent and correlates with the expression of proximally located protein-coding genes, suggesting that the process of eRNA transcription itself is responsible for promoting activity-dependent gene transcription (Kim et al., 2010).

## 1.3 Epigenetics in neurological diseases

There is a considerable body of evidence implicating disruption of epigenetic mechanisms as a causal basis for human cognitive dysfunction. When considering these cases it is important to distinguish between a developmental need for epigenetic mechanisms, to allow the formation of a normal nervous system, versus an ongoing need for these mechanisms as part of cognitive processing per se in the adult. The majority of the attention to date has focused on developmental roles for epigenetics in establishing the capacity for cognitive function in the adult. However, many experimental results of recent years implicate an ongoing and active role for epigenetic mechanisms in cognition and behavior in the adult.

Listed below are the most common neurodevelopmental diseases caused by epigenetic defects (**Table 2**):

- Rubinstein–Taybi syndrome (RTS), an inherited autosomal dominant disease, is caused by a mutation of the gene encoding CBP, the transcriptional co-activator and HAT discussed in previous sections. Several studies using animal models to investigate the molecular basis of RTS indicate that deficiency in CBP has severe consequences for long-term memory formation (Petrij et al., 1995).
- ATRX syndrome is an X-linked disorder caused by mutations in a chromatin-remodeling protein (McDowell et al., 1999; Berube et al., 2000). The ATRX protein main function is to interact with constitutive heterochromatin domains and with HP1, a protein known to affect chromatin structure during the cell cycle. ATRX also interacts with the polycomb group protein EZH2 (Cardoso et al., 1998) and MeCP2 (Nan et al., 2007).
- Rett syndrome (RTT) is an inherited X-linked disease that is due, in 90% of the cases, to loss-of-function mutations in the gene encoding MeCP2, the methyl-DNA binding protein (Amir et al., 1999). Using genetic animal models, it was discovered that overexpression of MeCP2 enhanced long-term memory formation and the induction of hippocampal long- term potentiation (LTP), indicating that MeCP2 modulates memory formation and induction of synaptic

plasticity (Collins et al., 2004).

- Fragile X syndrome, the most commonly inherited form of mental retardation, is provoked by an abnormal expansion of repeated trinucleotide sequences within one of two different Fragile X genes: FMR1 and FMR2 (Ashley et al., 1993; Turner et al., 1996). Both FMR1 and FMR2 contain a polymorphic trinucleotide repeat, CGG and CCG respectively, in their 5' untranslated regions responsible for the loss of gene expression (Gecz et al., 1996; Gu et al., 1996). Expansion of these repeats results in hypermethylation of these regions and flanking CpG islands, leading to transcriptional silencing of the FMR and surrounding genes.
- The most widespread of senile dementias, Alzheimer's disease, is the consequence of an increase in soluble β-amyloid peptides in the brain (Kuo et al., 1996). These peptides are created by endoproteolytic cleavage of the transmembrane amyloid precursor protein (APP) by β- and γ-secretases (Selkoe, 1998). Interestingly, cleavage of APP results not only in production of an extracellular β-amyloid fragment, but also an intracellular fragment, the APP intracellular domain (AICD). This fragment regulates transcription through recruitment of the adapter protein Fe65 and the HAT Tip60, suggesting that some of the pathology of Alzheimer's disease might be due to dysregulation of histone acetylation (Cao et al., 2001).
- Finally, schizophrenia is a serious disorder of cognition, causing patients to be unable to function normally in social situations and in performing everyday cognitive tasks. An emerging body of evidence suggests that deficiencies in the extracellular protein reelin may contribute to the pathophysiology of schizophrenia, at least in a subset of patients (Costa et al., 2002). The promoter of reelin contains several sites for DNA methylation. Inhibitors of HDAC and DNMT activity increase expression of reelin, indicating that epigenetic mechanisms govern reelin expression (Chen et al., 2002).

| *Disease* | *Gene or sequence involved* | *Function* | *Epigenetic consequence* |
|---|---|---|---|
| Rubinstein-Taybi syndrome | CBP | Histone acetyltransferase | ↑ Histone acetylation |
| Rett Syndrome | MeCP2 | Binds to CpG and recruits HDACs | ↓ Histone acetylation |
| Fragile X mental retardation | Trinucleotide expansion in FMR1 and FMR2 genes | Expansions results in aberrant DNA methylation of FMR1 and FMR2 genes | ↑ DNA methylation ↓ Histone acetylation |
| Alzheimer's disease | Amyloid precursor protein | APP intracellular domain associates with the HAT Tip60 | ↑ Histone acetylation |
| Schizophrenia | Reelin | extracellular matrix protein involved in synapse development | ↑ DNA methylation around reelin gene |
| ATRX syndrome | ATRX | chromatin remodelling complex | ↓ DNA methylation |
| ICF syndrome | unknown | unknown | ↓ DNA methylation |
| Coffin-Lowry disease | RSK2 | Direct phosphorylation of histones and interaction with CBP | ↓ Histone phosphorylation |
| Epilepsy | several voltage- or ligand-gated channels | membrane excitability | ↑ Histone acetylation and phosphorylation |
| Huntington's disease | HTT | Mutant HTT inhibits CBP function | ↓ Histone acetylation ↑ H3K9me3 |

**Table 2**: Epigenetic deregulations occurring in most common neurodevelopmental and neurodegenerative diseases.

# 2. METHYL-CPG BINDING PROTEIN 2 (MECP2)

The story of MeCP2 is constantly evolving since its discovery already 22 years ago. This protein was identified and characterized by its virtue of binding methylated CpGs (Lewis et al., 1992). It was later on described its ability to repress transcriptional activity *in vitro* (Nan et al., 1997). Few years passed and Amir et al (1999) published a breakthrough paper showing that mutations in the *MECP2* gene are at the root of Rett syndrome (RTT), a neurodevelopmental disease that manifests in girls during early childhood. During the first decade of this century, many efforts were put to gain more insight on MeCP2 functions in normal physiology and in relation with RTT manifestations. One of the questions that have been raised lately is about MeCP2 partners, which is deeply bound to another aspect of MeCP2

mechanism of function: the diversification of biological roles of this extremely disordered protein. MeCP2 has been shown to interact with several proteins, SIN3A, HP1, ATRX, DNMT1, NCOR and CREB, among others (reviewed in Guy et al., 2011). Finally, in the last 3 years, other striking functions of MeCP2 have been discovered, as the ability to bind 5hmc in the brain (Mellén et al., 2012) and the mediation of the newly described AT-hook in the binding to DNA (Baker et al., 2013). In the rest of this chapter I will explain the MeCP2 state of the art.

## 2.1 MeCP2 gene structure

*MECP2* gene is composed of four exons that encode for two different isoforms of the protein, due to alternative splicing of exon 2 (**Figure 6**). The core promoter of *MECP2* includes a CpG island (Reichwald et al., 2000) and a region enriched in regulatory factor binding sites. Instead of the classical "TATA-dependent" promoter, the 5'-region of the *MECP2* show a high content of GC and a stretch of sequence containing a cluster of alternative transcription start sites (TSSs) (Carninci et al., 2006).

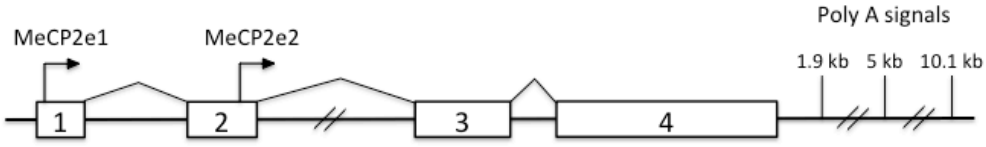


**Figure 6**: human *MECP2* genomic structure. The two arrows indicate the alternative ATGs. Rectangular boxes represent exons.

A recent study examined the conserved elements throughout the *MECP2* gene and its neighboring regions for regulatory activity, and identified four new enhancers and two silencer elements, as well as a conserved fragment spanning 1080 nucleotides immediately upstream of exon 1, which includes the *MECP2* core promoter and at least one positive and two negative regulatory elements. The identified regulatory elements show cell-specific activity differences. All four

enhancers and two silencers were able to interact with nuclear proteins in gel-shift assays, and the enhancer elements contain predicted binding sites for brain-specific transcription factors. Furthermore, three of the enhancers and both of the silencers were shown to interact in *cis* with the core promoter, providing further support for their potential role in transcriptional regulation (Liu and Francke, 2006).

*MECP2* possesses a large (8,5 kb), highly conserved 3'-untranslated region (3'-UTR) that contains multiple polyadenylation sites, which can be alternatively used to generate up to four different transcripts. These alternative *MECP2* transcripts display quantitative differences among different tissues (Coy et al., 1999; Pelka et al., 2005; Shahbazian et al., 2002) as well as in different stages of embryonic mouse development (Coy et al., 1999) and human post-natal brain development (Balmer et al., 2003) suggesting that 3'-UTR may play a critical role in transcript stability and therefore post-transcriptional regulation of RNA. According to the TargetScanS tool at the UCSC genome browser, the 3'-UTR of *MECP2* contains 52 predicted miRNA-binding sites. In addition, it has been experimentally demonstrated that miRNA132, a critical regulator of Creb and Bdnf, interacts with the 3'-UTR of the longest Mecp2 transcript (Klein et al., 2007).

## 2.2 MeCP2 protein structure

As previously stated, alternative splicing between exons 1 and 2 leads to the generation of two different MeCP2 isoforms (Mnatzakanian et al., 2004; Kriaucionis and Bird, 2004). MeCP2E1 is the longer isoform and contains 21 unique N-terminal residues highly enriched in acidic and hydrophobic residues and MeCP2E2 that differ from the previous by 9 unique residues. Although the two isoforms have distinct expression patterns, with MeCP2E2 being 10 times less abundant than the E1 isoform in the postnatal brain, the lack of any pathogenic mutations in the N-terminal residues distinguishing the two isoforms has led to consider them as mainly functionally equivalent (Itoh et al., 2012). Besides this difference, all the remaining amino acids are identical between the two isoforms and, in general, the primary

structure is highly conserved among vertebrates. At the secondary and tertiary structural levels, MeCP2 seems to be organized into five main distinct domains (Hansen et al., 2010): the N-terminal domain (NTD; residues 1–78), the MBD (residues 79–162), the intervening domain (ID; residues 163–206), the TRD (residues 207–310) and the C-terminal domain (CTD; residues 311–486) (**Figure 7**). In addition to these discrete domains, a recent publication revealed the existence of two AT-hook sub-domains located in the TRD of MeCP2 (Baker et al., 2013). The importance of these domains is supported by the fact that two very close mutations, R270X and G273X, differently affect the onset and the severity of RTT symptoms. In fact, such a divergence is due to an AT-hook domain that ends at G273. Finally, it is important to mention other less understood structural features of MeCP2: a nuclear localization signal (NLS) located within the TRD, a WW domain, linking MeCP2 to splicing (Buschdorf and Strätling, 2004) and two PEST sequences (73–94 and 389–426, respectively) (Thambirajah et al., 2009) usually correlated with rapid proteolytic degradation by the 26S ubiquitin proteasome system.

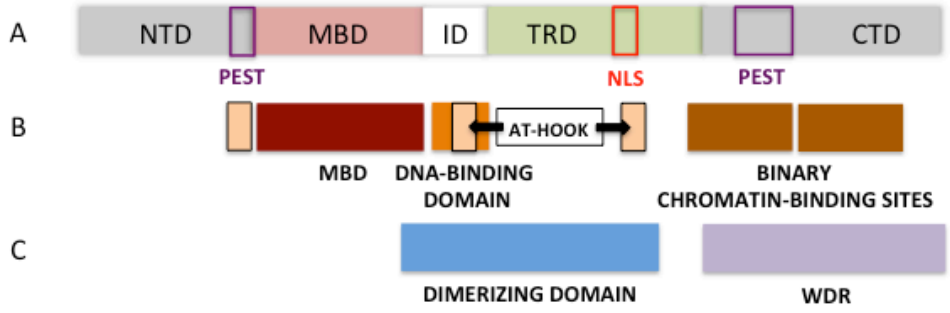


**Figure 7**: structure of hMeCP2E1. (A) Structural domains. (B) DNA- and chromatin-interacting domains. (C) protein-protein-interacting domains.

In the recent years researchers have started to think about MeCP2 as an intrinsically disordered protein (IDP) due to its unique structure and the high number of interacting partners. It is important to notice here that despite the MeCP2 domains have already been identified, the only three-dimensional structural information available is provided by the MBD. Theoretical predictions and

experimental evidences have showed that about 60% of MeCP2 protein is unstructured (Adams et al., 2007). IDPs such as MeCP2 usually acquire a concrete conformation upon binding to other proteins or nucleic acids.

Another source of functional plasticity is given by MeCP2 PTMs. MeCP2 has been demonstrated to undergo many PTMs, including acetylation (Gonzales et al, 2012; Zocchi and Sassone-Corsi, 2012), phosphorylation (Gonzales et al, 2012; Tao et al., 2009; Zhou et al., 2006; Cohen et al., 2011), ubiquitination (Gonzales et al, 2012), and sumoylation (Cheng et al., 2014). Even though the functional roles of the majority of MeCP2 PTMs remain largely unclear, some modifications have been successfully characterized. The best-known modifications of MeCP2 are, so far, phosphorylation of serine 421 and serine 80. Phosphorylation of S421 was the first PTM of MeCP2 to be described and was found to be induced during increased neuronal activity associated with influx of calcium ions. Though many tissues have been tested for this modification, it was found only on MeCP2 protein from brain tissue, suggesting that phosphorylation of S421 is solely a neuronal event (Zhou et al. 2006). On the other hand, MeCP2 S80 phosphorylation was found to be dependent on the neuronal calcium influx, similar to what was demonstrated for S421. However, in contrast to the previous S421 characterization, the S80 phosphorylation was inversely correlated with neuronal activity, suggesting alternative signaling between these residues in resting or depolarized neurons. Lately, a new study reported the phosphorylation of T308 as critical for the capability of MeCP2 of interacting with the NCoR transcriptional repressor (Ebert et al., 2013).

## 2.3 MeCP2 mechanisms of action

### 2.3.1 Transcriptional repression and chromatin compaction

The above-stated unique features of MeCP2 make this protein capable of a wide range of cellular functions. As already mentioned, MeCP2 was originally isolated as a nuclear factor able to bind *in vitro* a DNA probe containing at least one symmetrically methylated CpG dinucleotide. It was then showed that *in vivo,* the protein

accumulates in mouse cells at pericentromeric heterochromatin, which contains highly methylated satellite DNA. After the first experimental evidences on MeCP2 function, it was demonstrated that MeCP2 was able to repress transcription mainly, but not exclusively, through its TRD domain (Nan et al., 1997). Given the capability of the TRD to bind co-repressor complexes (SIN3A and NCOR) containing HDAC activity, the repressive activity of MeCP2 has subsequently been linked to chromatin compaction (Jones et al., 1998, Ng et al., 1999 and Kokura et al., 2001). Later on, the identification of other interacting partners, such as the chromatin remodeling complexes BRAHMA and ATRX, the co-repressors C-SKI, CoREST and LANA and a H3K9 histone methyltransferase (Chahrour and Zoghbi, 2007 and Guy et al., 2011) further reinforced the interplay between MeCP2 and chromatin. The capability of MeCP2 to work as an architectural chromatin protein was additionally supported showing that at high molar ratio to nucleosomes, MeCP2 mediates the formation of a highly compacted chromatin structure (Georgel et al., 2003). Noteworthy, this MeCP2 feature does not require additional factors apart from core histones, and appears independent of DNA methylation, thus raising the possibility that MeCP2 might affect the expression/structure of genomic unmethylated regions. This study is of particular relevance since it suggests, for the first time, that MeCP2 action might depend on its abundance and ability to bind DNA. Concordant with this last evidence, a major breakthrough of the field showed that MeCP2 in mature neurons might serve as an alternative linker histone and organizes a specialized chromatin structure able to reduce transcriptional noise (Skene et al., 2010). Again, this study proved this MeCP2 property to be exclusive of neuronal cells, since it is not present in glia, where the protein is 10 times less abundant. Moreover, the authors have reported that in mature neurons, characterized by $1.6 \times 10^7$ molecules of MeCP2 per nucleus, corresponding roughly to one molecule every second nucleosome, MeCP2 is genome-wide bound, tracks methylated DNA and affects chromatin structure. The role played by MeCP2 on global genomic architecture is further demonstrated by an increase in histone acetylation and H1 protein level selectively in Mecp2-null neurons. Strinkingly, these consequence were not observed in Mecp2-null glia (Skene et al.,

2010)

In line with the observation that molecular genetics seem to reflect the relevance of MeCP2 activity on chromatin structure, a very recent study revealed that two mutations, MeCP2-270X and MeCP2-273X, correlate with the impaired functionality of the methyl-binding protein in the ability to form higher-order structures with nucleosomal DNA in vitro and in the disruption of proper ATRX positioning at pericentric heterochromatin in vivo (Baker et al., 2013). In the same paper, the peculiar difference between the two mutations in phenotypic penetrance is explained with an AT-hook domain disrupted completely by the MeCP2-270X mutations but only slightly affected by MeCP2-273X. Mechanistically, in mature neurons MeCP2 uses its MBD to bind methylated CpG dinucleotides with high affinity. Once bound, MeCP2 may affect the nearby chromatin structure using one or more AT-hook domains, which interact with AT-rich sequences of DNA.

Neurons lacking MeCP2 have also been reported to increase mobilization of LINE-1 retrotransposons (Muotri et al., 2010). Indeed, previous data already showed that LINE-1 promoters are targets of MeCP2 that may be subject to methylation-dependent repression (Yu et al, 2001). Intriguingly, in mammals, there is evidence of an active role of LINE-1 transposition in neural progenitor development (Muotri et al., 2005; Coufal et al., 2009). Insertions of LINE-1 can impact gene expression and neuronal function, thereby increasing brain-specific genetic mosaicism (Muotri and Gage, 2006; Singer et al., 2010). Since MeCP2 is relatively low expressed in neural progenitor cells and the subsequent chromatin state is much more permissive toward mobile elements than that of mature neurons, a possible hypothesis is that mobilization of retrotransposons would represent a physiological process to expand the phenotypes of the developing neurons.

### 2.3.2 Transcriptional activation, RNA splicing and protein synthesis

Despite all the evidences proposing a chromatin-repression role for MeCP2, many other functions have been demonstrated, including transcriptional activation,

mRNA splicing regulation and protein synthesis modulation. The link between MeCP2 and splicing came from the finding that the methyl-binding protein interacts with YB1, a regulator of alternative splicing (Young et al., 2005). In agree with this, MeCP2 is capable of a direct binding to RNA (Jeffery and Nakienly, 2004) and, in the brain, it is part of a multiprotein complex containing PRPF3, a major component of the spliceosome, and SDCCAG1, a mediator of nuclear export (Long et al., 2011). Although a RTT transgenic mouse line characterized by a truncated form of MeCP2 displays an abnormal splicing of multiple genes in the brain (Young et al., 2005), more studies are required to understand the significance of MeCP2 involvement in mRNA splicing both in mouse models and human patients.

The ability to function as a transcriptional activator has been inferred from transcriptional profiling studies of RNA purified from hypothalamic and cerebella of RTT mice. The data were supported by the identification of an interaction between MeCP2 and the transcriptional activator CREB (Chahrour et al., 2008). Additionally arguments for the MeCP2 protein as a positive regulator of gene expression hypothesis comes from a study in which human embryonic stem cells were deprived of MeCP2 and let differentiated to neurons. Subsequent transcriptional analyses highlighted a global activator role for MeCP2. In fact, in addition to the majority of the genes being down-regulated in MECP2-null neurons, these have smaller nuclei and soma compared with control cells (Li et al., 2013). Further support to this theory is given by a preceding report showing *in vitro* decreased RNA synthesis in MeCP2 mutant mouse neurons (Yazdani et al., 2012). Strikingly, the finding that the genes with higher 5hmc/5mc ratio were more likely to be down-regulated in Mecp2-deficient neurons (Li et al., 2013), raised new interrogatives about MeCP2 mechanisms of action. In fact, it has been described how the bodies of genes are depleted of 5mc in the CNS and the same gene bodies became enriched in 5hmc (Kriaucionis and Heintz, 2009). But it was very recently that the connection between this epigenetic mark indicating permissive transcription state and the MeCP2 protein became clear. Mellén et al (2012) proposed a model, supported by experimental data, where MeCP2 binds to 5hmc in expressed genes in order to facilitate the

transcription, through the organization of dynamic chromatin domains.

Finally, it has been shown that the AKT/mTOR signaling pathway is impaired in both Mecp2-null mice and heterozygous females and protein synthesis was significantly decreased in these mice. These results have been independently confirmed by other authors, who also showed that the AKT/mTOR defect is rescued by BDNF and IGF-1 treatments (Li et al., 2013) Intriguingly, the AKT/mTOR pathway is disrupted also in a knockout mouse model of Cdkl5, whose protein product is a kinase functionally linked to MeCP2 and involved in several forms of neurodevelopmental disorders including a severe form of atypical Rett syndrome (Wang et al., 2012).

## 2.4 MeCP2 target genes

Since MeCP2 is now recognized as a transcriptional modulator, we would expect that the loss- or gain-of-function of MeCP2 would result in a deregulation, both positive and negative, of the expression of MeCP2 target genes. Many attempts to identify MeCP2 targets were made in the field of transcriptomic, mostly based on the paradigm microarray profiling and qRT-PCR validation. The systems were heterogeneous including human samples (post mortem brain tissues or immortalized cell lines derived from RTT patients) and mouse models (knock-out or mutant Mecp2 mice) (Ben-Shachar et al., 2009; Chahrour et al., 2008; Urdinguio et al., 2008; Jordan et al., 2007; Nuber et al., 2005; Tudor et al., 2002). Because of the high variability and the low interlaboratory reproducibilty of the aforementioned studies, only few validated target genes and no molecular pathways have been identified. Several reasons could possibly explain such variability, from the high heterogeneity of the samples analyzed (from cerebral areas of animal models to reprogrammed neurons obtained from patient's skin biopsies or human tissues), to the different timing of the analysis (from pre-symptomatic mouse studies to the fully-penetrant RTT analysis), or the statistical approach that could yield different results according to the stringency of the used method.

The very first studies of gene expression in the brains of Mecp2-null mice and RTT patients did not uncover any obvious changes in gene expression (Colantuoni et al., 2001; Tudor et al., 2002). Since these early studies, however, it has become clear that MeCP2 may control the expression of different sets of genes in separate areas of the brain, both negatively and positively, and in an activity-dependent manner (Chen et al., 2003; Cohen et al., 2008). Therefore, researcher started to refine the investigation by looking at concrete part of brain or simply by using more homogeneous models.

Given the great influence of MeCP2 deregulation on various neurological processes, one of the first genes to be associated with MeCP2 was the brain-derived neurotrophic factor (BDNF). Since then a great effort has been made in order to uncover the link between such an important protein and MeCP2. BDNF is a growth factor involved in neurogenesis, neuronal maturation and survival, Ca2+ homeostasis, and synaptic plasticity, as well as plays roles in learning and memory and a number of neurological diseases (Binder and Scharfman, 2004). In neuronal cultures, MeCP2 binds to the *BDNF* promoter III and represses transcription of BDNF (Chen et al., 2003; Martinowich et al., 2003). BDNF protein expression and mRNA is decreased by 70% in Mecp2-mutant brains compared to WT mice (Chang et al., 2006). This reduction in BDNF expression is thought to contribute to the pathogenesis of RTT. Importantly, these findings helped to gain a hint on the involvement of MeCP2 in activity-dependent regulation of gene expression. In fact, MeCP2 is phosphorylated in response to membrane depolarization, which causes it to be released from the promoters of target genes, included BDNF, allowing for transcriptional activation (Chen et al., 2003).

Other genes whose expression appears to be repressed by MeCP2 have been identified, including Dlx5, Dlx6 (Horike et al., 2005), Fxyd1, Reln, and Gtl2 (Jordan et al., 2007). Transcription of Dlx5 and Dlx6 was shown to be significantly enhanced in the frontal cortex region of Mecp2-null mice compared to controls (Horike et al., 2005). However, this work has more recently been refuted, showing that *DLX5/6* is not imprinted in human and there was no significant difference in expression

between wild type and null mice (Schule et al., 2007). Fxyd1 is also elevated in the frontal cortex of Mecp2-null mice as well as in RTT patients (Deng et al., 2007). Like Bdnf, Gabrb3 and Ube3a are significantly reduced in Mecp2-null mice, as well as in the brains of RTT patients (Samaco et al., 2005). Importantly, most of these genes are critical for normal development of dendritic morphology and the regulation of GABAergic function.

The first study that provided evidences of a transcriptional activation role for MeCP2 was shown by Chahrour and colleagues (2008). They analyzed the gene expression profiles in the hypothalamus of mice without MeCP2 and with double the amount with the aim to uncover more information into the molecular mechanism of MeCP2. Through the use of microarray analysis, a variety of genes were found to be altered in both cases. Surprisingly around 85% of these genes appeared to be activated and not repressed. Chromatin immunoprecipitation then confirmed that MeCP2 binds to the promoter region of six of the activated genes (Sst, Oprk1, Mef2c, Gamt, Gprin1 and A2bp1). The same article also reported an interaction between MeCP2 and the transcriptional activator CREB1 at the promoter regions of activated target genes (Chahrour et al., 2008).

When it became clear that ncRNAs are abundant in the CNS and they are also critical for neuronal function and brain development, researchers began to investigate hypothetical ncRNA targets of MeCP2. In the first work, Wu et al (2000) identified several up- and down-regulated miRNAs in cerebella of Mecp2-deficient mice and, for many of them a direct promoter binding of MeCP2 was demonstrated. In addition, it was shown that MeCP2 regulates the expression of a large cluster of miRNAs embedded within the Dlk1-Gtl2 imprinting domain, showing different levels of deregulation and suggesting the existence of miRNA-specific post-transcriptional regulation. Remarkably, MeCP2 silences miR-30a/d, miR-381 and miR-495, which in turn repress BDNF. This suggests a multilayered MeCP2-mediated transcriptional regulation of BDNF (Wu et al., 2010).

Similarly, in another study concerning miRNAs, microarray expression analysis of Mecp2-null brains revealed alterations of several miRNAs. Noteworthy,

deregulated miR-29 and miR-146 are known to have roles in neural and glial cells and its association with neurological disorders is reported (Urdinguio et al., 2010).

# 3. MECP2 IN NEURODEVELOPMENTAL DISORDERS

It is not really surprising that a functionally versatile protein such as MeCP2 may be crucial for the correct development and maturation of the CNS. Tightly regulated MeCP2 dosing is critical for neuronal morphology and dendritic spine density in many human diseases and animal models. In fact, MeCP2 impairments are the primary responsible for RTT syndrome (Chahrour and Zoghbi, 2007) and have also been shown to be involved in several other disorders, albeit in very few patients, as Prader-Willi syndrome (Samaco et al., 2004), Angelman syndrome (Watson et al., 2001), nonsyndromic mental retardation (Miltenberger-Miltenyi and Laccone, 2003), and autism (Zoghbi, 2005).

## 3.1 Rett Syndrome

RTT is a postnatal progressive neurodevelopmental disorder that affects predominantly girls and whose symptoms first manifests during early childhood. Indeed, RTT is the second major cause of mental retardation in girls after Down's syndrome and has a rough incidence in women of 1 in 10,000. It bears the name of the Austrian pediatrician who first described the RTT clinical profile of 22 patients (Rett, 1968), even though the disease have not been recognized by the international medical community until 1983, when Dr. Hagberg and his colleagues reported 35 cases of RTT in English literature (Hagberg et al., 1983).

### 3.1.1 Clinical features of RTT

Patients with RTT undergo an appareantly normal post-natal developmental stage until 6-18 months of age and then begin losing achieved milestones, such as the ability to walk and speak. Even at the very beginning of RTT diagnosis, the variability of the symptoms among patients of the same age is noteworthy. The shared early

indicators of RTT include: microcephaly, developmental stagnation and muscular hypotonia. Along with the syndrome progression, patients lose purposeful use of their hands and instead develop stereotypic hand wringing or washing movements, representing the most typical feature of girls affected by RTT (Chahrour and Zoghbi, 2007). By the second-third year of age, complete loss of language and social withdrawal become apparent in RTT patient, together with other autistic features, such as lack of eye-to-eye contact, expressionless face, indifference to the surrounding environment and self-abusing behavior (Nomura et al., 2005). The first autonomic dysfunction to manifest is hyperventilation during wakefulness, followed by breathing abnormalities including apnea and breath holding. One of the most devastating manifestations of RTT is the presence of epileptic features, which vary in severity with the most common type being tonic-clonic seizures (Jian et al., 2006). However, the seizure episodes tend to decrease in intensity after the teenage years. Another feature that seems to ameliorate is the social part of the autistic behavior, since some patients have been reported to respond to some social input between 5 to 10 years of age. As the patients get adult, they suffer severe motor deterioration together with dystonia and scoliosis. Most RTT girls lose mobility and develop more autonomic impairments, as oropharyngeal dysfunction, constipation and several cardiac abnormalities. Older patients often develop Parkinsonian features (Roze et al., 2007; Hagberg, 2005). Despite the great amount of physical debilitation and autonomic dysfunctions, some patients survive until 50-60 years of age.

### 3.1.2 Genetics of RTT

The restriction of the disease to females suggested an X-linked dominant mode of inheritance, with the mutation presumed to be lethal in males (Xiang et al., 1998). However, due to the fact that more than 99% of RTT cases are sporadic, it was hard to map the locus on the chromosome. From the use of genetic linkage studies on the rare family cases with RTT, the most probable location was indicated as Xq28 (Amir et al., 1999). Subsequently, following further gene sequencing analysis into plausible

disease-causing mutations of candidate genes in this locus, it was found that a mutation in *MECP2* was the cause of RTT (Amir et al., 1999). This was surprising, as MeCP2 had no previous known involvement in the CNS or brain development. It is important to mention that mutations in other genes, like Cyclin-dependent kinase-like 5 (*CDKL5*) (Mari et al., 2005) and Forkhead box protein G1 (*FOXG1*) (Ariani et al., 2008) may results in atypical Rett Syndrome.

After the discovery of *MECP2* mutations causing RTT, researchers wondered whether the disorder was indeed limited to girls. Following genetic testing, some male patients were found to display similar symptoms to those seen in classic RTT (Jan et al., 1999) or other disorders such as mental retardation and encephalopathy, but most of the boys with this mutation die within the first year of life. Although the clinical prognosis of male RTT patients is poor, the reason because male patients are underrepresented in RTT is not based on the likelihood of embryonic lethality. A study performed in sporadic RTT patients and the respective families showed that in 26 out of 27 cases, *de novo* mutations in the *MECP2* gene arose on the paternal X chromosome. (Trappe et al., 2001) According to this model, males are protected from X-linked dominant diseases because they do not inherit the mutated paternal X chromosome. Thus, two possible hypotheses may explain the high ratio female/male in RTT. The first one is based on the assumption that paternal germ cells have higher mutational incidence in the *MECP2* gene than the maternal ones. On the other hand, an alternative explanation considers that mutation incidence at paternal germ cells is normal, whereas a mutation at the *MECP2* gene may be detrimental for the oocytes that carry the mutated protein, decreasing the likelihood of being fertilized. Whatever the reason, the different epigenetic situation of the male (hypermethylated) and female (undermethylated) X chromosomes in germ cells may, however, be a key point for understanding the different behavior regarding the mutation rate of *MECP2*.

The major source of phenotypic variability in RTT patients is the pattern of X-chromosome inactivation. In females, only one of the two X chromosomes is active in each cell and the choice of which X chromosome is active is usually randomly

selected at the blastocyst stage of embryonic development. In this way, half of the cells will have the maternal X chromosome active and the other half the paternal X chromosome active. Intriguingly, cells expressing the wild-type *MECP2* allele divide faster or survive better than cells expressing the mutant allele, and that lead to a nonrandom pattern of XCI and such phenomenon, although not totally understood, results in improved prognosis of the RTT neurological phenotypes. Depending on the extent of such favorable skewing, some patients can be mildly affected or are even asymptomatic carriers of *MECP2* mutations. The latter cases are usually identified due to very rare cases of familial RTT where the carrier mothers pass the mutated *MECP2* to their offspring. An astonishing example of the dramatic consequence of X-chromosome inactivation in RTT is the case of two monozygotic RTT twins who manifest very different phenotypes (Dragich et al., 2000). This phenomenon has also been described in a RTT mouse model, where phenotypic severity of heterozygous female correlates with the degree of skewing (Young and Zoghbi, 2004). Finally, somatic mosaicism in females with *MECP2* mutations is another rare but important source of phenotypic variability (Bourdon et al., 2001).

### 3.1.3 MeCP2 mutations

Mutations in *MECP2* gene account for the more than 95% of classic RTT cases with the range of mutation types including missense, nonsense and frameshift mutations. More than 600 mutations have been described in RTT patients (RettBase: http://mecp2.chw.edu.au/mecp2/), and the majority is located in the exons coding for these domains (**Figure 8**). Frameshift mutations arise from the addition or loss of DNA bases, which change the reading frame of a gene. These types of mutation can occur over the entire length of the protein but are clustered at the C-terminal region of MeCP2 and usually result in a non-functional product. Nonsense mutations arise from a single nucleotide change, which prematurely signals the cell to stop building a protein. The majority of these occur within the MBD and TRD producing a shortened protein that might be functional or not. Missense mutations consist of a change in one

DNA base pair resulting in the substitution of one amino acid for another. Most of the mutations typically occur in the MBD with the rest of the protein left intact (Free et al. 2001; Yusufzai and Wolffe 2000).

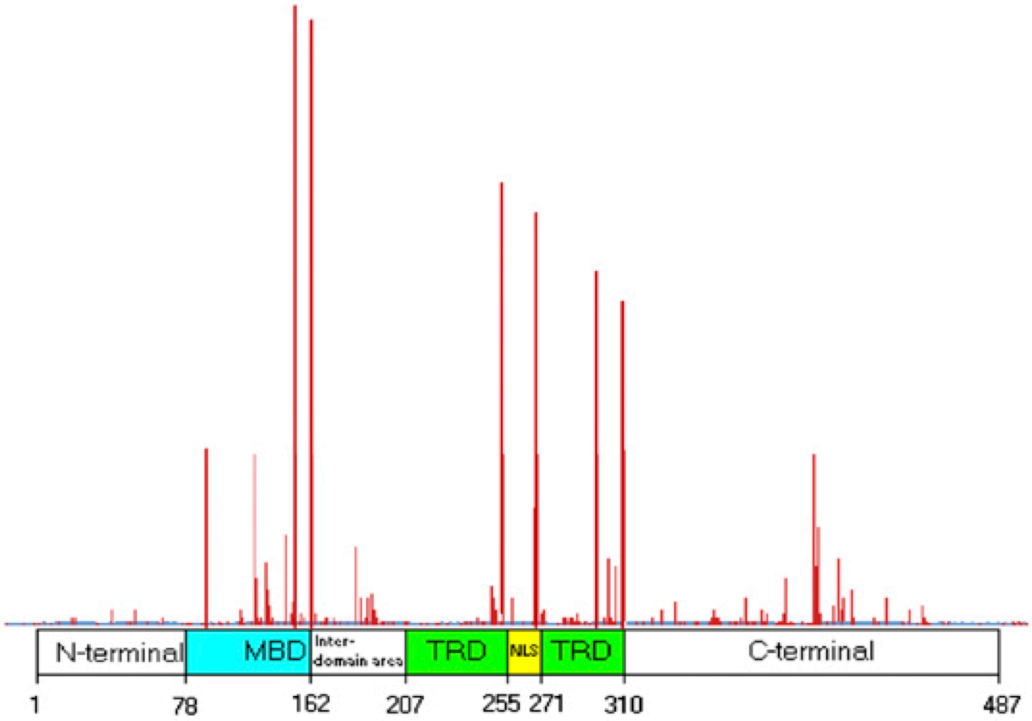


**Figure 8**: Frequency of pathogenic MeCP2 mutations causing RTT (from RettBASE). *Taken from Williamson and Christodoulou, 2006*

Around 70% of all cases are eight missense and nonsense mutations (R106, R133, T158, R168, R255, R270, R294 and R306) (**Table 3**). These are provoked by the deamination of methylated cytosines that generates C>T transitions, which in turn are responsible for the "hotspot" mutations. C-terminal deletions account for about 10% (Christodoulou et al. 2003; Moretti and Zoghbi 2006). Importantly, It was found that mutations in the NLS region or causing premature stop codons cause

| Nucleotide change | Amino acid change | Cases reported | Percentage (of pathogenic mutations reported) |
|---|---|---|---|
| 316C>T | R106W | 59 | 4.8% |
| 397C>T | R133C | 67 | 5.4% |
| 473C>T | T158M | 150 | 12.2% |
| 502C>T | R168X | 147 | 11.9% |
| 763C>T | R255X | 132 | 10.7% |
| 808C>T | R270X | 118 | 9.6% |
| 880C>T | R294X | 101 | 8.2% |
| 916C>T | R306C | 79 | 6.4% |
| 20-100bp deletions | | 120 | 9.7% |
| Total | | 1231 | 78.9% |

**Table 3**: frequency of most common *MECP2* mutations (from RettBASE)

more severe symptoms than missense mutations. In comparison, C-terminal deletions appear to produce a milder outcome since the protein retains a certain degree of functionality (Smeets et al., 2005). In conclusion, the type of mutation and the degree of X-chromosome skewing are the two major source of variability in the outcome of RTT.

### 3.1.4 Neuroanatomical defects of RTT

The study of the impact of RTT syndrome on brain structures has been carried on basically on two models: post-mortem human subjects and mouse models.

#### 3.1.4.1 RTT patients

Pioneering brain autopsies of RTT patients showed a smaller brain size (Schultz et al., 1993) with reduced neuronal soma size and increased packing density of cells in many brain regions including the cerebral cortex, hypothalamus and hippocampus (Armstrong et al., 1995). There have also been reports of impaired dendritic growth and complexity of pyramidal cells in the frontal and motor cortices (Armstrong et al., 1995), and reduced synaptic spine density in pyramidal neurons in the hippocampus (Chapleau et al., 2009) and frontal cortex (Belichenko et al., 1994). Presynaptically, loss of MeCP2 has been linked to impairment of the number of axonal boutons and

axonal arborization suggesting a decrease in the overall number of synapses in RTT brains (Belichenko et al., 2009). Consistent with this hypothesis, the density of several excitatory neurotransmitter including NMDA, AMPA and metabotropic glutamate receptors in the frontal cortex was reduced in older RTT patients but higher in younger patients (Blue et al., 1999). Interestingly, at the macroscopic level there appears to be no major neuronal degeneration or malformations present in the brains of RTT patients.

Neurochemical studies based on RTT patient data are relatively limited due to the small number of patients analyzed and the variability among them. However, cerebrospinal fluid (CSF) and brain tissues have been analyzed with respect to levels of various transmitters, receptors, and additional trophic factors. Abnormalities have been reported in most systems, including in acetylcholine (Wenk and Hauss-Wegrzyniak, 1999), dopamine (Zoghbi et al., 1989; Percy, 1992) serotonin (Segawa and Nomura, 1990), glutamate (Hamberger et al., 1992), and nerve growth factor (Lappalainen et al., 1996). In terms of therapeutic potential, much interest has surrounded the monoamine systems, since most of the observation made in post-mortem human samples have been confirmed in mouse models (Panayotis et al., 2011).

### 3.1.4.2 RTT mouse models

Most of the advances in the field have been obtained thanks to many animal models developed through genetic engineering. Several transgenic mice that possess altered MeCP2 expression have been generated to study the *in vivo* function of MeCP2 (reviewed in Ricceri et al., 2008). The three most widely used animal models of RTT syndrome are the Mecp2-Bird, Mecp2-Jae and Mecp2-T308A mouse models. The first two mouse models listed have been used to assess the effects caused by the complete absence of MeCP2 function, although the Mecp2-Jae allele still produces a truncated and modified protein product (Guy et al., 2001; Chen et al., 2001). The Mecp2-T308A mouse model was generated to study the effects of partial MeCP2 loss-

of-function and possesses a truncating nonsense mutation at amino acid position 308, which spares the MBD, TRD and NLS (Shahbazian et al., 2002). Though the majority of work in the field has focused on the loss of MeCP2 in male mice, RTT is typically a female-restricted disease and modeling such a disorder should take into account hypothetical confounding effects due to sex. The reason for the choice of the male as the primary subject of RTT research is because some evidence suggests that non-random X chromosome inactivation (XCI) may lead to phenotypic variability in female animals (Young and Zoghbi, 2004). However, our understanding of the disease would benefit from further efforts to elucidate the complete spectrum of behavioral abnormalities in Mecp2-deficient female mice (Katz et al., 2012).

The importance of mouse models is primarily reflected in the evidence that MeCP2 is critical for neuronal maturation but not for neurogenesis. For example, neural stem cells from the dentate gyrus (DG) of the hippocampus in knockout and WT animals were found to proliferate at the same rate and have a comparable cell density (Smrt et al., 2007).

Similar to the situation seen in RTT patients, Mecp2-mutant mice have smaller cortical neurons packed at a higher density than their wild-type littermates (Chen et al., 2001; Stearns et al., 2007). Moreover, pyramidal neurons in the cortex (Kishi and Macklis, 2004) as well as in hippocampal CA3 region, and granule cells of the dentate gyrus show reduced dendritic complexity (Ballas et al., 2009). In addition, Mecp2-Jae-mutant mice show a disorganized olfactory neuroepithelium indicative of delayed terminal differentiation (Matarazzo et al., 2004). However, dendritic branching in Layers III and V pyramidal neurons of the frontal cortex of male Mecp2-T308A mice was comparable with that in the controls (Moretti et al., 2006). In the Mecp2-Bird model, pyramidal neurons from the somatosensory cortex of 6-week-old mice show lower spine densities compared with wild-type controls (Fukuda et al., 2005). Newly generated granule cells in the dentate gyrus of 8-week-old Mecp2-Jae mutant mice also show impaired dendritic spine density and distribution (Smrt et al., 2007). In female heterozygous mice, the onset of this dendritic spine phenotype is delayed and more severe in Mecp2-lacking neurons than in Mecp2-expressing

neurons, suggesting both cell autonomous and non-cell autonomous effects (Belichenko et al., 2009a). The intensity of PSD-95 (an abundant postsynaptic protein) is also lower in Layer V pyramidal neurons of the motor cortex of Mecp2-Jae mice (Tropea et al., 2009). As for the presynaptic terminals, the motor cortex of Bird mice model show defects in axonal fasciculation (Belichenko et al., 2009b). Moreover, the intensity of VGLUT1 (a presynaptic protein) has been showed to be lower in the dendritic region of the hippocampal CA1 region from Mecp2-null mice. However, since the postsynaptic dendritic marker MAP-2 was not different, the authors interpreted that Mecp2 deletion caused a reduction in the number of mature synapses in area CA1, consistent with their results from dissociated neuronal cultures (Chao et al., 2010).

### 3.1.5 Synaptic defects in RTT

Neurophysiological and molecular studies in all the available models consistently report alterations in synaptic function, and notably, defects in synaptic plasticity. These data suggest that RTT might be regarded as a synaptopathy (disease of the synapse) and thus potentially amenable to rational therapeutic intervention.

The first physiological studies performed in mice showed subtle changes in neuronal electrical properties within cortical areas (Dani et al., 2005; Kline et al., 2010) and more pronounced changes in other regions such as the brain stem and locus ceruleus (Taneja et al., 2009; Medrihan et al., 2008). Clear changes in synaptic function have also been reported, including reduced synaptic plasticity (Guy et al., 2007; Moretti et al., 2006; Asaka et al., 2006; Nelson et al., 2006) and changes in basal inhibitory and excitatory synaptic transmission (Dani et al., 2005; Kline et al., 2010; Medrihan et al., 2008; Nelson et al., 2006; D'Cruz et al., 2010; Maliszewska-Cyna et al., 2010). Observations in hippocampal slices of Mecp2-null mice lead to consider that CA1 and CA3 regions are highly hyperexcitable (Calfa et al., 2011). Interestingly, synaptic plasticity (activity-dependent changes in the strength of synaptic communication) appeared normal in young Mecp2-mutant mice (Guy et al., 2007;

Asaka et al., 2006; Dani and Nelson, 2009) but showed impairment when tested in older mice on onset of overt RTT-like signs (Guy et al., 2007; Asaka et al., 2006). Moreover, the degree of impairment appears to correlate with the severity of the RTT-like neurological phenotype. The exact mechanisms driving the involvement of MeCP2 in regulating morphological and functional aspects of synaptic signaling remain to be identified. However, synaptic plasticity deficits are one of the most consistent findings and may provide important insights into RTT-like pathogenesis as well as serving as a target system for therapeutic interventions.

## 3.2 *MECP2* duplication disorder

One of the most intriguing facts about MeCP2 is that loss of function and gain in MeCP2 dosage result in clinically similar neurological syndromes. In fact, until 2010, more than 100 patients were identified with duplication of the Xq28 region of X-chromosome, which is the location of the *MECP2* gene. The different duplications found so far differ in terms of size, although the smallest duplication only contains the *IRAK1* and *MECP2* genes (del Gaudio et al., 2006; Ramocki et al., 2009). Interestingly, patients with a triplication of *MECP2* have also been identified. These patients showed a very poor prognosis, suggesting a correlation between MeCP2 dosage and severity of the disease (del Gaudio et al., 2006). Noteworthy, *MECP2* duplication syndrome is mostly inherited by patients through their carrier mothers, with *de novo* mutations being a small minority. Females are usually asymptomatic carriers due to complete skewing of the mutated X-chromosome, although a recent report showed that heterozygous females display some light neurological features including anxiety, depression, and an autistic-like phenotype (Ramocki et al, 2009)

Cardinal features of this X-linked neurodevelopmental syndrome includes infantile hypotonia, mild dysmorphic features (brachycephaly, large ears, midface hypoplasia and depressed nasal bridge), developmental delay, severe to profound mental retardation, and absent to minimal speech. Affected patients may manifest a

combination of variable phenotypes including recurrent infections, progressive spasticity with the lower limbs affected to a greater extent than the upper limbs, ataxia, autism or autistic features, and focal onset or generalized epilepsy.

### 3.2.1 Physiopathology of MeCP2 duplication disorder

Contrary to RTT, post-mortem analysis of brains with *MECP2* duplication are not available. Therefore, the anatomical and physiological observations regarding this disorder derive exclusively from mouse models. Two different mice have been described so far, that differs substantially on the approach used to generate them.

In order to generate the first mouse, researchers used a large genomic clone that contained the entire human *MECP2* locus (Collins et al, 2004). Four viable lines of Mecp2-overexpressing mice were created: Mecp2-TG1, Mecp2-TG3, Mecp2-TG11, and Mecp2-TG22 all with varying levels of protein expression. Interestingly, phenotype severity corresponded with protein level, as it was described also in patients (del Gaudio et al., 2006). Of the phenotypes reported from these mice, forepaw clasping, aggressiveness, hypoactivity, as well as kyphosis were observed. It was also found that the Mecp2-TG1 mice developed seizures that worsened with age and that corresponded to abnormal EEG patterns. Additional experiments were conducted on Mecp2-TG1 mice because of their viability and because the MeCP2 protein levels reflected that seen in clinical populations of *MECP2* duplication syndrome. Accurate behavioral characterization of Mecp2-TG1 mice demonstrated accelerated motor learning and enhanced contextual fear conditioning, with no obvious anxiety-like phenotype (Collins et al, 2004).

The second mouse was named Tau-Mecp2 because the transgenic Mecp2 gene was associated with Tau promoter, resulting in neuron-restricted expression. This mouse recapitulated aspects of *MECP2* duplication syndrome, including profound motor dysfunction characterized by side-to-side swaying, tremors, and gait ataxia (Luikenhuis et al, 2004). Moreover, another study reported that heightened anxiety-like phenotype *in Tau-Mecp2* mice was demonstrated by both elevated plus maze and

dark–light tests suggesting that MeCP2 overexpression is sufficient to recapitulate the anxiety phenotype observed in *MECP2* duplication syndrome patients (Na et al., 2012). Tau-Mecp2 mice also had deficits in motor learning reminiscent of impairments commonly observed in afflicted individuals. These results show that neuronal overexpression of MeCP2 has detrimental effects on learning and memory processes and produces an increased anxiety-like phenotype.

Unfortunately, to date, only few physiological analyses have been performed on mouse models of the *MECP2* duplication syndrome. For example, it has been demonstrated that excitatory/inhibitory (E/I) ratio is decreased in the cortex of Mecp2-TG1 mice (Collins et al., 2004). In addition, LTP is attenuated in hippocampal slices from Tau-Mecp2 mice compared to wild type animals (Na et al., 2012). Conversely, the Mecp2-TG1 mouse model showed increased LTP (Collins et al, 2004). Finally, It has been reported also an increase in mEPSC frequency and no changes in amplitude in primary hippocampal cultures compared to controls (Na et al., 2012).

All this findings are important to demonstrate that Mecp2 duplication mouse models recapitulate key features of the human-associated disorder, like motor dysfunction, heightened anxiety and learning and memory deficits.

## 3.3 Autism

MeCP2-related disorders are developmental diseases that belong to the spectrum of autism disorders (ASD) (Johnson and Myers, 2007). Although Autism and MeCP2-related disorders share similar progression and manifestations, autism differs from RTT with respect to its genetic basis. While RTT is caused by *MECP2* mutations, the genetic basis of autism is not fully clear and is proposed to involve multiple genes (Carney et al., 2003). It is important to know that autism disproportionately affects males, and for this reason some researchers have therefore searched for the involvement of X-linked loci (Jamain et al., 2003; Gauthier et al., 2005). Intriguingly, mutations in the *MECP2* regulatory elements, resulting in decreased expression of the protein, are commonly associated with autism

(Shibayama et al., 2004). Reduced expression of MeCP2 protein has been shown to occur frequently in the frontal cortex of autistic patients and is correlated with increased *MECP2* promoter DNA methylation (Nagarajan et al., 2006). The silencing of autism-related genes through promoter DNA hypermethylation is commonly associated with autism, and drugs that can demethylate promoters might be useful in activating these genes (Chiurazzi et al, 2003; Nguyen et al., 2010). In a recent study, it was shown that treatment with Decitabine cause reduced DNA demethylation of Mecp2 promoter and intron 1 and that leads to increased Mecp2 expression. These results represent a promising insight on the use of drugs for future therapeutic interventions of autism (Liyanage et al., 2013). In addition, *MECP2* mutations that are associated with classic RTT have been identified in a number of autistic females who do not meet the diagnostic criteria for RTT (Carney et al., 2003). This makes it difficult to determine if a *MECP2* mutation that is associated with autism diagnosis is a different disorder from RTT, or if both disorders are simply different representations on a spectrum associated with *MECP2* mutations. Finally, it is important to mention that *MECP2* mutations have also been reported in patients with mild cognitive and motor difficulties and early-onset schizophrenia (Adegbola et al., 2009).

# AIM OF THE STUDY

## Aim 1 (studies I and II)

In the last 15 years MeCP2 has been associated with the transcriptional regulation of a wide range of protein-coding genes (Tudor et al., 2002; Ballestar et al., 2005; Samaco et al., 2005; Nuber et al., 2005; Chahrour et al., 2008; Urdinguio et al., 2008; Ben-Shachar et al., 2009) and miRNAs (Urdinguio et al., 2010; Wu et al., 2010), with several of them being involved in important CNS functions. The way these MeCP2 targets have been discovered relied heavily on transcriptional profiling of RTT-mimicking samples through microarray-based technologies. The ultimate goal of this strategy is the identification of specific genes mis-regulated in RTT that can be targeted for therapeutic purposes. However, such studies often produced no interstudy reproducibility results due to many reasons, including the heterogeneity of samples, the lack of proper controls (especially for post-mortem human samples) and the statistical approach used to screen the cohort of candidate genes.

**HYPOTHESIS**

To date, no MeCP2-regulated gene has been successfully targeted in order to improve the severe symptoms of RTT. In the present Doctoral Thesis we sought to identify new MeCP2 targets through different approaches with the purpose of expanding the knowledge of the impaired biological pathways in RTT.

- In the first study we focused on a class of transcriptional regulators called long non-coding RNAs (lncRNAs). There is increased evidence that lncRNAs could play a fundamental role in the complexity of CNS, both in normal development and in diseases (Qureshi et al., 2010). For this reason we propose to investigate lncRNAs expression in the brain of the Mecp2-deficient mouse.
- In order to gain more resolution on transcriptomic changes that occur in RTT, in the second study we took advantage of RNA sequencing, a powerful high-throughput technique with the ability to detect very low amounts of transcript.

Therefore we propose the identification of new MeCP2 targets in the hippocampus and prefrontal cortex of the Mecp2-deficient mouse.

**OBJECTIVES**

- Discover lncRNAs involved in RTT physiopathology through transcriptional profiling of the Mecp2-null mouse.
- Uncover biological functions for lncRNAs mis-regulated in the RTT mouse model.
- Identify and validate new coding genes mis-regulated in the hippocampus and prefrontal cortex of a RTT mouse model.
- Determine the binding of MeCP2 to the newly identified targets through chromatin immunoprecipitation.
- Confirm the transcriptional impairment of coding-genes in primary neurons derived from Mecp2-null and control mice.

## Aim 2 (study III)

Duplications in the *MECP2* gene have been described in male patients with symptoms clearly resembling those of RTT (Ramocki et al., 2010). This intriguing finding is a clear evidence that precise dosage of MeCP2 is extremely important for the proper development of the brain.

**HYPOTHESIS**

Despite the creation of mouse models that exhibit increased expression of Mecp2, little is known about the effects of an increase of MeCP2 dosage in the early stages of embryo development. With this purpose, we proposed to investigate the consequence of MeCP2 over-expression in a well-known developmental model such as the chicken embryo.

**OBJECTIVES**

- Check the expression pattern of chicken MeCP2 (cMeCP2) in the developing spinal cord.
- Perform transient over-expression of human MeCP2 by *in ovo* electroporation.
- Investigate the effect of MeCP2 increased dosage in the proliferation of neuroblasts.
- Determine whether MeCP2 overexpression interfere with neuronal precursor differentiation and cell death.

# RESULTS

**DIRECTORS REPORT**

To who may concern, we authenticate that the PhD student PAOLO PETAZZI will present his PhD thesis by scientific publications. His contribution for each publication will be next pointed out.

**STUDY I**

**"Dysregulation of the long non-coding RNA transcriptome in a Rett syndrome mouse model"**

Paolo Petazzi, Juan Sandoval, Karolina Szczesna, Olga de la C Jorge, Laura Roa, Sergi Sayols, Antonio Gomez, Dori Huertas and Manel Esteller

**Contribution:** In this article, Paolo Petazzi was the responsible of experimental design, supervised by Dr Manel Esteller and Dra Dori Huertas. In addition, he performed all the experiments, and participated in data analysis and interpretation. The manuscript was written by Paolo Petazzi and revised by Dr Manel Esteller and Dra Dori Huertas.

**Journal:** RNA biology 2013 July 1; 10 (7), 1197. doi: 10.1158/1078- 0432.CCR-12-3518. doi: 10.4161/rna.24286. PMCID: PMC3849168. Impact Factor: 5,6

**STUDY II**

**"RNA-sequencing of a Rett syndrome mouse model reveals global impairment of immediate-early genes expression"**

Paolo Petazzi, Mauricio Sáez, Karolina Szczesna, Laura Roa, Olga C. Jorge, Antonio Gómez, Dori Huertas and Manel Esteller

**Journal:** *In preparation*

**Contribution:** Paolo Petazzi was in charge of the experimental design, and execution, supervised by Dr. Esteller and Dra. Huertas. He also performed the analysis and interpretation of the generated data, together with the manuscript writing.

**STUDY III**

**"An Increase in MECP2 Dosage Impairs Neural Tube Formation"**

Paolo Petazzi, Naiara Akizu, Alejandra García, Conchi Estarás, Alexia Martínez de Paz, Manuel Rodríguez-Paredes, Marian A. Martínez-Balbás, Dori Huertas and Manel Esteller

**Journal:** Neurobiology of Disease. 2014 march 13; DOI: 10.1016/j.nbd.2014.03.009. Impact Factor: 5.6

**Contribution:** In this study, Paolo Petazzi participated in the electroporation of chicken embryo and immunofluorescence experiments. He also collected and analyzed the data, together with manuscript writing, under Dr. Manel Esteller and Dra. Dori Huertas supervision.

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## STUDY I

# Dysregulation of the long non-coding RNA transcriptome in a Rett syndrome mouse model

Paolo Petazzi[1], Juan Sandoval[1], Karolina Szczesna[1], Olga [1], Laura Roa[1], Sergi Sayols[1], Antonio Gomez[1], Dori Huertas[1] and Manel Esteller[1,2,3]

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RNA biology 2013 July 1; 10 (7), 1197. doi: 10.1158/1078- 0432.CCR-12-3518.
doi: 10.4161/rna.24286. PMCID: PMC3849168. Impact Factor: 5,6

**(Annex I)**

## Abstract

Mecp2 is a transcriptional repressor protein that is mutated in Rett syndrome, a neurodevelopmental disorder that is the second most common cause of mental retardation in women. It has been shown that the loss of the Mecp2 protein in Rett syndrome cells alters the transcriptional silencing of coding genes and microRNAs. Herein, we have studied the impact of Mecp2 impairment in a Rett syndrome mouse model on the global transcriptional patterns of long non-coding RNAs (lncRNAs). Using a microarray platform that assesses 41,232 unique lncRNA transcripts, we have identified the aberrant lncRNA transcriptome that is present in the brain of Rett syndrome mice. The study of the most relevant lncRNAs altered in the assay highlighted the upregulation of the AK081227 and AK087060 transcripts in Mecp2-null mice brains. Chromatin immunoprecipitation demonstrated the Mecp2 occupancy in the 5′-end genomic loci of the described lncRNAs and its absence in Rett syndrome mice. Most importantly, we were able to show that the overexpression of AK081227 mediated by the Mecp2 loss was associated with the downregulation of its host coding protein gene, the gamma-aminobutyric acid receptor subunit Rho 2 (Gabrr2). Overall, our findings indicate that the transcriptional dysregulation of lncRNAs upon Mecp2 loss contributes to the neurological phenotype of Rett syndrome and highlights the complex interaction between ncRNAs and coding-RNAs.

**Introduction**

Epigenetic regulation, whose mechanisms involve chromatin remodeling by many different enzymes/complexes/molecular scaffolds that targets DNA methylation and histone code modifications,[1] is also mediated by non-coding RNAs (ncRNAs).[2, 3, 4, 5] ncRNAs include the most studied member microRNAs, but also include long non-coding RNAs (lncRNAs). LncRNAs are transcripts of at least 200 nucleotides transcribed from all over the genome, including intergenic regions, antisense, overlapping or intronic to protein-coding genes.[2, 3, 4, 5, 6] LncRNAs have a broad range of functions, such as enhancer-like activity,[7] establishment of repressive chromatin in genomic regions[8] or entire chromosomes,[9] intronic antisense transcripts capable of binding to histone modifiers thereby regulating the transcriptional output of the host gene,[6] alternative splicing and other post-transcriptional RNA modifications10 that determine the activity of our genome.

In this regard, the fine-tuning of gene expression is critical in the human central nervous system (CNS). It is widely known that higher order cognitive and behavioral function are a CNS prerogative sustained by huge and intricate cell networks acting both locally and globally.[11] One main aim of modern neurobiology is the ultimate understanding of the transcriptional programs that give rise to distinct neural networks, which are, in turn, formed by several neuronal and glial subtypes. Herein, alterations in the epigenetic modulation of gene expression could lead to several neurodevelopmental disorders, the most evident example represented by Rett syndrome (RTT).[12,13] RTT (OMIM 312750) is an X-linked neurodevelopmental disorder that affects females at a frequency of 1:10,000 live births. The girls appear normal until 6–18 mo of age, when they lose their acquired skills and develop autistic features and mental retardation along with the typical stereotypic hand movement.[14] Rett syndrome is the second leading cause of mental retardation in women after Down syndrome. Nearly 95% of typical RTT is due to mutations in the gene encoding the transcriptional regulator Methyl-CpG binding protein 2 (MECP2 in humans; Mecp2 in mice).[15,16] Mecp2 is a basic nuclear protein that acts mainly as a transcriptional repressor, binding preferentially to methylated DNA sequences.[17,18]

In human cancer cells, the binding of *MECP2* to the hypermethylated CpG islands of tumor suppressor genes[19, 20, 21] and microRNAs[22] is associated with transcriptional silencing. The loss of MECP2 in Rett syndrome patients and mice models is associated with a dysregulated pattern of coding-gene [23, 24, 25, 26, 27, 28] and microRNA expression.[29,30]

Herein, we investigate the role of lncRNAs in the physiopathology of Rett syndrome by comparing the transcriptome profiles of Mecp2-null mice brains[31] vs. wild-type animals. In a similar pattern to Rett syndrome patients, Mecp2-null mice do not show the RTT-like phenotype just after birth, but 4–5 wk later,[31] resembling the natural history of the disease in humans. The identified lncRNAs dysregulated in the Rett syndrome mice models provide important clues to understand the neurological phenotype of the disorder; furthermore, they illustrate the imbricate relationship between coding and ncRNA transcripts.

## Results

### Identification of candidate lncRNAs misregulated in RTT

We utilized a well-established mouse model of RTT that mimics the human disease[31] to perform lncRNA microarray expression analyses using pairs of wild-type (WT) and Mecp2-null (KO) 9-wk animals. RNA was extracted from total brain and hybridized to an lncRNA microarray platform. The Mouse long non-coding RNA array (Arraystar) contains more than 41,232 probes representing unique lncRNAs. The probes were designed according to NCBI RefSeq, UCSC, RNAdb2.0, NRED, Fantom3.0 and UCRs annotations. Two biological replicates were used for each sample and condition. Repeat sequences and ncRNAs shorter than 200 bp are not represented in the microarray. The lncRNA expression data obtained are freely available at the Gene Expression Omnibus database: www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=fvavvcewquegcfc&acc=GSE43689.

Unsupervised hierarchical clustering of the lncRNA microarray expression data indicates that the Mecp2-null mouse brains show a distinct pattern of lncRNA transcription in comparison to the wild-type animals (Fig. 1A). Among the 41,232

lncRNAs transcripts included in the microarray, we found 701 (1.7%) lncRNAs (Table S1) that had a different expression pattern in WT and Mecp2-null brain samples with a score of < 0.05 in the false discovery rate (FDR) test and a > 1.5-fold expression change (Fig. 1B). Among these significantly altered lncRNAs, overall downregulation of transcripts was the most common feature (520 of 701, 74%), while upregulation occurred in the remaining 26% (181 of 701) (Fig. 1B).

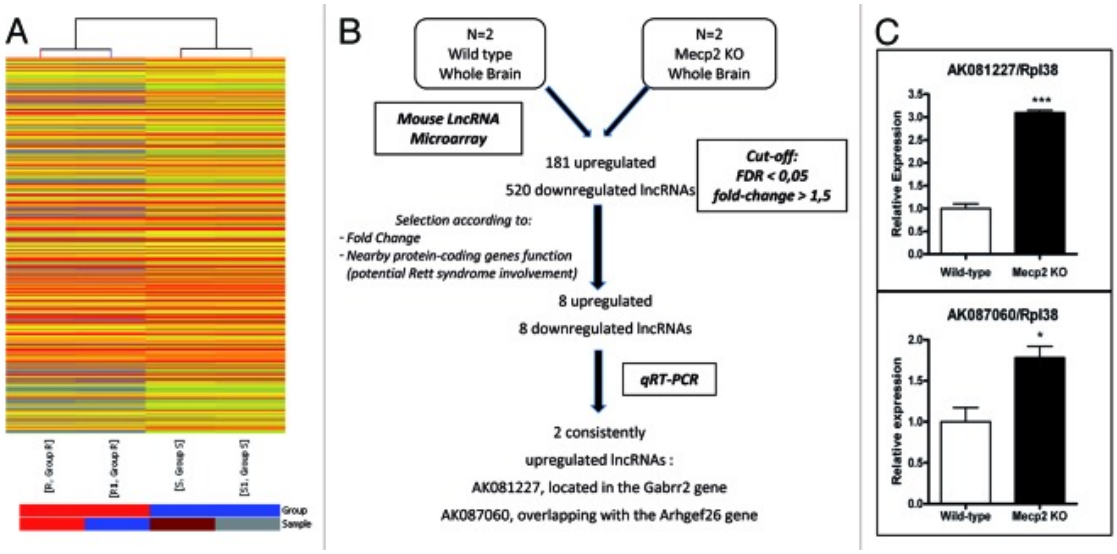


**Figure 1.** Dysregulation of the lncRNAs transcriptome in Mecp2-null mice brains. (**A**) Hierarchical clustering of the lncRNA microarray expression data shows a distinguishable gene expression profiling between wild-type (S, S1) and Mecp2-null (R, R1) mice brains. (**B**) Flow-chart used to identify candidate misregulated lncRNAs in the RTT mouse model. (**C**) qRT-PCR of AK081227 and AK087060 normalized with RPL38. Error bars represent SE. Five biological replicates were used for each condition. * p<0.05 *** p<0.001

### Validation of lncRNA candidates in RTT

For practical purposes to reduce the lncRNAs to be further studied and to enrich those potentially involved in RTT, we selected candidate transcripts with a fold expression change > 2 that were associated with an annotated protein-coding gene where a biological function in neurons or glial cells have been proposed according to the scientific literature and through GO term enrichment on “negative regulation of neuron differentiation” (p = 0,008), “nerve development” (p = 0,007), “dendrite regeneration” (p = 0,004), “regulation of nervous system development” (p

< 0,001), "regulation of excitatory postsynaptic membrane potential" (p < 0,001) and "positive regulation of synapse assembly" (p < 0,001). Using the above described criteria, we produced a short list of 16 lncRNAs: eight upregulated and eight downregulated in Mecp2-null mouse brains (Table S2). We then extracted brain RNA from five new pairs of wild-type and Mecp2-null 9-wk animals and analyzed the expression level of each one of the 16 candidate lncRNAs. The analyses were performed using quantitative PCR on reverse-transcribed RNA, three biological replicates per sample were developed and the statistical significance was assessed by two-tailed t-tests. Although for most cases, the expression analysis of each single candidate lncRNA matched the microarray data (data not shown), two lncRNAs exhibited a statistically significant difference: AK081227 (two-tailed t-test p < 0.0001) and AK087060 (two-tailed t-test p < 0.0248), both of them upregulated in Mecp2-null brain mouse (Fig. 1C).

**Mecp2 binding to the 5′-end genomic loci of the lncRNAs AK081227 and AK087060**

One important function of Mecp2 is to act as a transcriptional repressor that binds to the promoters located in the 5′-end genomic region of its target coding genes[19, 20, 21] and microRNAs.[22,29,30] Thus, we speculated whether Mecp2 was directly involved in regulating AK087060 and AK081227 gene expression by analyzing its capability of binding to their promoters. To this end, we performed chromatin immunoprecipitation (ChIP) experiments with formaldehyde-cross-linked nuclear extracts from WT and Mecp2 KO brains followed by semi-quantitative PCR. We immunoprecipitated the cross-linked chromatin with a histone H3 antibody to serve as an internal control of the ChIP assay (Fig. 2A–C). We also ensured that the Mecp2 antibody was specific for the protein and reliable in the ChIP assay by checking Mecp2 occupancy on the promoter of Xist, a previously reported lncRNA involved in X-inactivation whose promoter is occupied by Mecp2 in male wild-type mice (where Xist is silenced in the only X chromosome).[32] As expected, we found Mecp2 bound to the Xist promoter in wild-type, but not in Mecp2 KO mouse brain chromatin (Fig. 2A).

Once these controls were established, we proceeded to address the Mecp2 occupancy for the AK081227 and AK087060 5′-end genomic loci. The PCR primers for the ChIP assay were designed in a region of 500 bp upstream to the transcription start site (TSS) within the corresponding lncRNAs proximal promoters. Importantly, we found that AK081227 and AK087060 5′-end loci were occupied by the Mecp2 protein in wild-type mouse brains, while the *Mecp2* protein was absent from the 5′-end of the two lncRNAs in the Mecp2-null mouse brains that overexpress the AK087060 and AK081227 transcripts (Fig. 2B and C). DNA methylation differences in the two studied lncRNAs 5′-ends were studied by bisulfite genomic sequencing of multiple clones and were not detected in the brain of wild-type vs. Mecp2 KO samples (data not shown). These data support the role of Mecp2 as a transcriptional repressor of the transcripts located in the vicinity of its binding sites to DNA, herein regulating the expression of the lncRNAs AK081227 and AK087060.

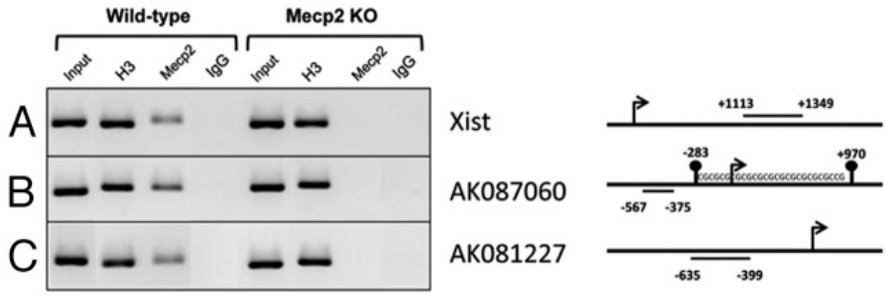


**Figure 2.** Mecp2 is bound to the 5'-end genomic loci of the lncRNAs AK081227 and AK087060 in wild-type mouse brain and lost in Mecp2-null mice. Semiquantitative chromatin immunoprecitation assay for a fraction of the total DNA (5%) (input), an antibody anti-H3 total (IP control), an antibody anti-Mecp2 and a negative control (mouse IgG) on the 5'-end genomic loci of the lncRNAs Xist (**A**), AK081227 (**B**) and AK087060 (**C**). the regions amplified by PCR were represented as straight lines.

## Upregulation of the lncRNA AK081227 in RTT is associated with downregulation of its host gene gamma-aminobutyric acid receptor subunit rho 2

One of the main challenges in research with lncRNAs is the identification of a

particular molecular or cellular function.[6] One possibility is that lncRNAs act locally regulating the expression levels of neighboring RNA transcripts.6 Herein, we have studied the possible effect of AK081227 and AK087060 in the activity of their associated protein-coding genes. To address this issue, we have analyzed the expression levels of the two identified lncRNAs by qRT-PCR in comparison to their corresponding host genes in four functional relevant brain regions (frontal cortex, hypothalamus, thalamus and cerebellum) from new pairs of wild-type and Mecp2-null 9-wk animals.

AK087060 is transcribed from 225 bp downstream of the first exon of the Arhgef26 gene (Fig. 3A). The *Arhgef26* protein is a Rho guanine nucleotide exchange factor (GEF) that has a role in the actin-driven endocytic process known as macropinocytosis that contributes to repulsive turning in the axons of neurons[33] and retinal cone growth.[34] Thus, a role for this protein in a neurological disorder, such as Rett syndrome, could be invoked. In this regard, in humans, the equivalent RNA transcript is originated 357 bp downstream of the first exon of the ARHGEF26 promoter with a 79% nucleotide homology to AK087060. We found that the observed upregulation of AK087060 in Mecp2 KO mice had a statistical association with an increase in the expression levels of its host gene Arhgef26 in the four studied brain regions (Pearson's correlation test = 0.41, p = 0.08) (Fig. 3B). Since this particular lncRNA is transcribed from just 225 bps downstream of the coding gene, common promoter regulatory mechanism for AK087060 and Arhgef26 seems an unlikely scenario. In addition, AK087060 could be a promoter-associated lncRNA.[6] These lncRNAs are transcribed around the TSS proximal region and are able to reclute other regulatory factors[6] that impact on the expression of the associated-coding gene, in this case Arhgef26.

The lncRNA/mRNA equation is completely different for AK081227. This lncRNA is transcribed from an intronic region of the GABA receptor subunit rho-2 (Gabrr2) gene (Fig. 3C). Many autistic and neurodevelopmental disorders, including Rett syndrome, have been linked to dysfunction in particular aspects of GABAergic inhibitory neurotransmission in the brain.[35,36] Most importantly, the expression of

another GABA receptor subunit member (GABRB3) is reduced in Rett syndrome.[24,37] Thus, the host gene of the identified lncRNA is a likely candidate to be altered in Rett syndrome. In this regard, in humans, the equivalent lncRNA has a 30% nucleotide homology to AK081227. Herein, we found that the upregulation of AK081227 in Mecp2 KO mice was statistically associated with a downregulation of the expression levels of its host gene Gabrr2 in the four studied brain regions (Pearson's correlation test = 0.44, p = 0.06) (Fig. 3D). Thus, the expression of the lncRNA AK081227 might act locally to interfere in the mRNA transcript levels of Gabrr2 and contribute to the neurological phenotype of Rett syndrome. In this regard, we have also recently identified another intronic lncRNA that finely regulates the expression of the host gene[38] in what might become a common theme in the complex interaction between ncRNAs and coding-RNAs.

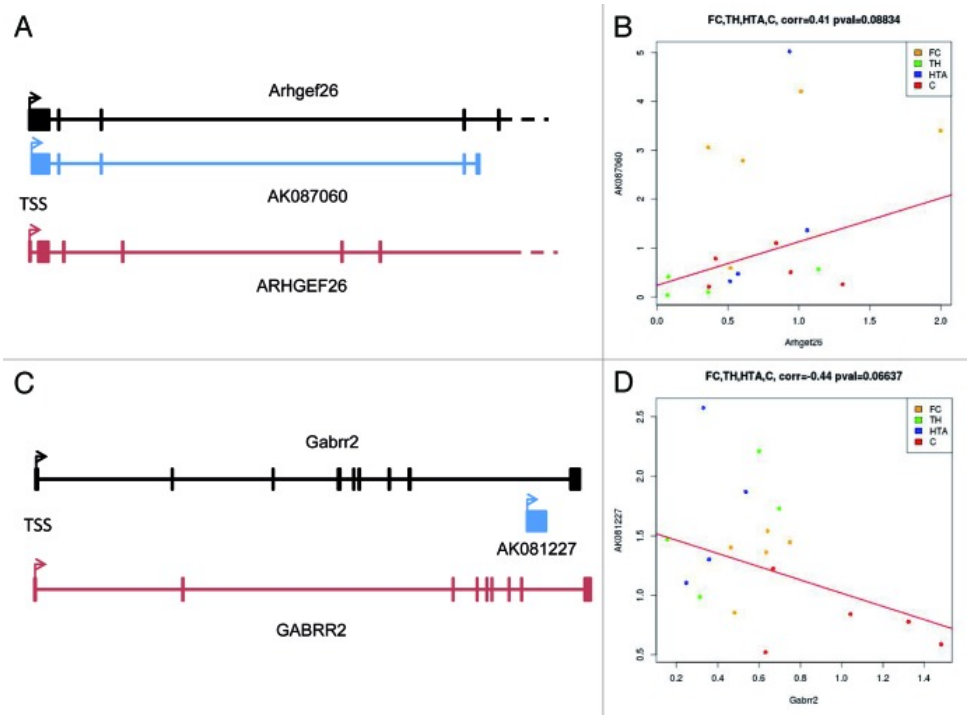


**Figure 3.** Genomic context and associated coding-genes for the identified lncRNAs. AK087060 originates 200 bps downstream from the transcription start site of the mRNA for the Arhgef26 gene (**A**) and its upregulation of AK087060 in Mecp2 KO mice is associated with an increase in the expression levels of the host gene in the four studied brain regions (Pearson's correlation test = 0.41, p = 0.08) (**B**). AK081227 is transcribed from the last intron of the Gabrr2 gene (**C**) and and its

upregulation of AK087060 in Mecp2 KO mice is associated with a downregulation in the expression levels of the host gene in the four studied brain regions (Pearson's correlation test = 0.44, p=0.06) (**D**).

**Discussion**

The current report represents the first lncRNA profiling in Rett syndrome, data that can be used to identify the transcripts from this class that are regulated by Mecp2 and that could explain the physiopathology of the disease. We observed a distinct lncRNA transcriptome between the brain of wild-type and Mecp2-null mice, where the expression of 701 lncRNAs was significantly different. Due to the limited information available about the function of each lncRNA, we selected only those whose higher fold expression change overlapped with protein-coding genes whose function was related to neurons or glia cells for further validation and study. In this last setting, the most relevant observation was that the release of the transcriptional silencing of the lncRNA AK081227 was associated with a downregulation of its host gene, Gabrr2. The importance of lncRNAs as *cis*-acting regulators is rapidly being recognized.[6] LncRNAs can guide chromatin change in *cis* in a co-transcriptional manner (tethered by RNA polymerase) or as a complementary target for small regulatory RNAs. Regarding our case, there is increasing knowledge that lncRNAs overlapping with introns of protein-coding genes, whether originating from splicing or produced by independent transcriptional units, may recruit several classes of coactivator (e.g., trithorax group proteins)[39] or corepressor (polycomb-group members like histone methyltransferase EZH2)[38] complexes and, therefore, act as guides for the establishment of activating or repressive histone marks all over the host gene. In this regard, the exact mechanisms regulating the inverse expression levels of the non-coding (AK081227) and coding (Gabrr2) transcripts are currently under investigation and should be the focus of further developments in this area.

The observation that the intronic lncRNA AK081227 upregulation in the Rett syndrome brain is associated with the depletion of its host coding gene Gabrr2 can have a relevant impact in the understanding of the described neurological disorder. At the physiological level, post-mortem analysis of RTT brains showed altered levels

of neurotransmitters such as glutamate and biogenic amines as well as changes in the abundance of some neurotransmitter receptors. In mouse models of RTT, analysis of spontaneous miniature excitatory and inhibitory postsynaptic currents indicated a shift in the excitatory/inhibitory balance, with increased excitatory and decreased inhibitory neurotransmission in the hippocampus and cortex.[40] Consistently, studies of Mecp2-knockout mouse models revealed abnormalities in long-term potentiation (LTP) and impaired synaptic plasticity.[41] Recent findings underline that Mecp2 deficiency in GABAergic neurons recapitulates most of the features displayed by Mecp2-null mice, including altered synaptic activity and plasticity.[42] Since GABA is the major inhibitory neurotransmitter in the brain, most of the synaptic defects seen in RTT could be a direct consequence of the Mecp2 loss in GABA neurons. Additionally, genes necessary for GABAergic function, like Dlx5 and GABRB3, have already been associated with RTT.[24,37] Gabrr2, as a member of GABA(C) receptor class, has been reported to be expressed in various brain regions.[43,44] The fact that we found Gabrr2 downregulated in frontal cortex is consistent with the dysfunction of GABAergic signaling seen in the frontal cortex of RTT patients.[45] Moreover, Gabrr2 was also downregulated in thalamus and hypothalamus, with the latter being one of the most affected region in RTT. As for the thalamus, a recent study showed that Mecp2 regulates GABAergic synapses differentially in excitatory and inhibitory neurons in the thalamus.[46]

Overall, our data provides the first hint that the lack of the transcriptional regulatory effect of Mecp2 in Rett syndrome leads to a dysregulation of lncRNA expression in the affected mice brain. Future data mining of the obtained lncRNA transcriptomes deposited in the public genomic databases could provide further clues about the impact of the altered lncRNAs on other transcripts; however, the neuropathological relevance of the inverse association between AK081227 (the intronic lncRNA) and Gabrr2 (the host coding-gene) already provides proof of principle for the existence of disrupted *cis*-regulated mechanisms in the disease.

**Materials and Methods**

**Animal model**

Four-week-old B6.129P2(C)-*Mecp2tm.11Bird* /J (stock number: 003890) heterozygous females (*Mecp2*+/−) were acquired from the Jackson Laboratory. In brief, the mutant strain was generated by replacing exons 3 and 4 of *Mecp2* in embryonic stem cells with the same exons flanked by loxP sites. Homozygous *Mecp2* lox/lox females were mated with mice with ubiquitous Cre expression to bring about gene disruption. The offspring from the crosses of *Mecp2*+/− females with C57BL/6J males were genotyped by PCR. Mice were kept under specific pathogen-free conditions in accordance with the recommendations of the Federation of European Laboratory Animal Science Associations. Lighting conditions (lights on from 08:00–20:00 h) and temperature (22°C) were kept constant. Animals were allowed ad libitum access to food and water and were inspected every day. Tissue samples were obtained from hemizygous *Mecp2*-null males (*Mecp2*-/y, KO) and their wild-type (WT) littermates after establishing RTT-like symptoms in the defective animals (at about 8–10 wk of age). Mice were euthanized in accordance with the Guidelines for Humane Endpoints for Animals Used in Biomedical Research. Tissues were frozen on dry ice immediately after removal and stored at -80°C until use.

**RNA extraction**

To extract RNA, frozen tissues were ground into powder with mortar and pestle and resuspended in Trizol reagent (Life technologies). The RNA purification was performed on the RNA-containing aqueous phase with RNeasy mini kit (Qiagen). After elution with RNase-free water and treatment with turbo DNase (Ambion), the RNA is ready for all kinds of applications. Quantification and quality check were performed with Nanodrop and Agilent 2100 Bioanalyzer (Agilent Technologies), respectively.

**Sample preparation and microarray analysis**

Briefly, 1 ug of total RNA was labeled with Cy3 using Agilent Quick Amp Labeling Kit and microarray hybridization was performed at 65°C for 17 h in

Agilent's SureHyb Hybridization Chambers. After being washed in an ozone-free environment, the slides were scanned using the Agilent DNA microarray scanner (part number G2505B). Data was extracted using Agilent Feature Extraction Software (version 10.5.1.1) and normalization was performed using the Agilent FE one-color scenario (mainly median normalization). Finally, four samples were hybridized, two biological replicates for each condition (Mecp2 KO mice and their wild-type littermates). False discovery rate (FDR) test was performed on lncRNA that pass the cut-off (t test < 0.05, fold change > 1.5).

**Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)**

Two ug of RNA for each sample were retro-transcribed to cDNA using random hexamers from ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis kit (Life Technologies). A negative sample (no ThermoScript enzyme) was performed to exclude DNA contamination. Primers for the reaction were designed according to Primer3 software and tested with a cDNA serial dilution to check amplification linearity and unique amplification product. Ppia and Rpl38 were used as house-keeping genes for normalization. 50 ng cDNA were used for each PCR reaction together with 5 ul 2X SYBR green PCR master mix (Life technologies), 250 nM of each primer and water up to 10 ul. Mouse primers sequences used were the following: Ppia Forward 5'-CAA ATG CTG GAC CAA ACA CAA-3' Reverse 5'-GTT CAT GCC TTC TTT CAC CTT-3'; Rpl38 Forward 5'-AGG ATG CCA AGT CTG TCA AGA-3' Reverse 5'-TCC TTG TCT GTG ATA ACC AGG G-3'; AK081227 Forward 5'-TCG GTC AGT GCA TTT GGG CTG T-3' Reverse 5'-TCG GTC CAC TGT CTC AGG AGT GC-3'; Gabrr2 Forward 5'-CAA GGG GAA CGA CGT GCG GA-3'; AK087060, Forward 5'-GAA CGA CGT GCG GA-3'.TGT ATG GCG TCC ATC TCT TCG G-3' and Reverse 5'-GTC CTC CTC TCT GCA ATT GCT TAG-3'; Arhgef26, Forward 5'-GGC CCT TGA TAT CGA CTC TGA TGA-3' and 5'-CTT TTC ACC GCG GAG AGC TGG-3'. Quantitative PCR was performed for 40 cycles on an ABI 7900HT sequence detection system (Life Technologies) under the thermal cycling conditions recommended by the manufacturer.

**Chromatin immunoprecipitation in brain tissues**

Frozen wild-type and Mecp2 KO male brains were reduced to powder with mortar and pestle. The pulverized brain tissues were cross-linked with 1% formaldehyde for 8 min and the reaction was blocked by adding glycine to a final concentration of 0.125 M. After washing two times with ice-cold PBS, cell pellets were resuspended in cell lysis buffer (HEPES 5mM, KCl 85 mM, NP40 0.5% pH 8.0) supplemented with protease inhibitor cocktail (Complete EDTA-free, Roche) and the lysate was homogenized with a douncer to facilitate cell membrane break. The nuclear pellet was then resuspended in Nuclei lysis buffer (TRIS-HCl 50 mM, EDTA 10 mM, SDS 1% pH 8.1) and subsequently sonicated with Bioruptor (Diagenode) for 30 min (30 sec ON, 30 sec OFF cycles). The chromatin size of the fragments obtained was 150–400 bp. Samples were diluted with Dilution buffer (SDS 0.01%, Triton X-100 1.1%, EDTA 1.2 mM, NaCl 165 mM, TRIS-HCl 16.7 mM pH 8.1). Magnetic beads were used for the pre-clearing of diluted chromatin (over-night at 4°C) and for incubation with anti-total H3 (ab1791, abcam) and anti-Mecp2 (m9317, sigma). Non-related mouse IgG antibody (12–371, Millipore) was used as a negative control. The Beads-Antibody complexes were then incubated with pre-cleared chromatin for 2 h at 4°C in agitation. The immune-complexes were washed: twice with low salt Buffer (TRIS-HCl 50 mM pH 8.0, NaCl 150 mM, SDS 0.1%, NP-40 1%, EDTA 1 mM, Deoxicolate Na 0.5%), twice with high Salt Buffer (TRIS-HCl 50 mM pH 8.0, NaCl 500 mM, SDS 0.1%, NP-40 1%, EDTA 1 mM, Deoxicolate Na 0.5%), twice with LiCl Buffer (TRIS-HCl 50 mM pH 8.0, LiCl 250 mM, SDS 0.1%, NP-40 1%, EDTA 1 mM, Deoxicolate Na 0.5%) and twice with TE Buffer (TRIS-HCl 10 mM pH 8.0, EDTA 0.25 mM). Cross-linked chromatin was then eluted from the magnetic beads by adding elution Buffer (NaHCO3 100 mM, SDS 1%). Samples were de-crosslinked overnight at 65°C and incubated with Proteinase K at 50 ug/ml final concentration for 1 h. Finally, DNA was purified with PCR purification kit (Qiagen). The primers used for ChIP

analysis were the following: AK081227 promoter, Forward 5'-TTG TCC CCA CTA AGAG ACA G-3' and Reverse 5'-CCT GTA CTC TGC TAT GCT TAC TC-3'; AK087060, Forward 5'-CTG TGT GAC TTT CAA ACA TAC AG-3' and Reverse 5'-CTT CAC TGG GCC ACT TGT G-3'; Xist, Forward 5'-CCT GTA CGA CCT AAA TGT CC-3' and Reverse 5'-GTA TTA GTG TGC GGT GTT GC-3'.

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**Supplementary information**

Supplementary Table 1 can be consulted here:
https://www.landesbioscience.com/journals/rnabiology/2013RNABIOL0035R-Sup.pdf

**Supplementary table 2**

**Upregulated lncRNAs**

| *Accession* | *p-value* | *FC* | *strand* | *Map* | *length* | *source* |
|---|---|---|---|---|---|---|
| AK148638 | 0,0279 | 7,13 | - | chr5: 67485667.. 67486864 | 1199 | Fantom |
| AK081227 | 0,0075 | 4,08 | + | chr4: 33179640.. 33180890 | 1250 | Fantom |
| AK084846 | 0,0133 | 3,67 | - | chr3: 119441639.. 119445686 | 4052 | Fantom |
| AK087060 | 0,0085 | 3,27 | + | chr3: 62142923.. 62183108 | 2309 | RNAdb |
| AK087668 | 0,0051 | 3,23 | + | chr7: 75374178.. 75377336 | 3156 | Fantom |
| AK162139 | 0,0155 | 2,91 | + | chr12: 85934321.. 85936391 | 2071 | Fantom |
| AK054306 | 0,0141 | 2,67 | - | chr2: 163462259.. 163463893 | 1635 | Fantom |
| AK032566 | 0,0344 | 2,18 | + | chr12: 110887814.. 110890438 | 2626 | NRED |

**Downregulated lncRNAs**

| *Accession* | *p-value* | *FC* | *strand* | *Map* | *length* | *source* |
|---|---|---|---|---|---|---|
| AK028061 | 0,0060 | 7,30 | - | chrX: 71275119.. 71278389 | 3270 | Fantom |
| AK080837 | 0,0029 | 4,54 | - | chrX: 72644392.. 72645693 | 1301 | Fantom |
| AK049444 | 0,0185 | 4,49 | + | chr1: 193091056.. 193112593 | 1452 | Fantom |
| AK158844 | 0,0364 | 3,57 | + | chr2: 97307589.. 97308837 | 1247 | Fantom |
| AK049486 | 0,0433 | 3,20 | + | chr9: 72736851.. 72738292 | 1441 | Fantom |
| AK156874 | 0,0355 | 3,12 | + | chr2: 155651880.. 155654616 | 2735 | Fantom_stringent |
| AK039889 | 0,0084 | 3,11 | - | chr4: 63642939.. 63643617 | 679 | Fantom |
| AK142911 | 0,0308 | 2,85 | + | chr10: 89843525.. 89845745 | 2223 | Fantom |

Supplementary Table 2

## STUDY II

# RNA-sequencing of a Rett syndrome mouse model reveals global impairment of immediate-early genes expression

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*In preparation*

**Abstract**

Rett syndrome (RTT) is a neurodevelopmental disease caused by mutations in the *MECP2* gene. Physiological and anatomical studies on various RTT models suggest that this disease may be regarded as a synaptopathy. The correct understanding of all the mechanisms by which the transcription factor MeCP2 so profoundly affect the mammalian brain is yet to be determined. The MeCP2 involvement in activity-dependent expression programs is a critical link between this protein and proper neuronal activity, which allows the correct maturation of connections in the brain. To investigate new MeCP2 target genes in a RTT mouse model we took advantage of RNA-sequencing, a high-throughput technique that allows in-depth gene expression analysis. We found several immediate-early genes (IEGs) up-regulated in the hippocampus and prefrontal cortex of the Mecp2-null mouse. Intriguingly, IEGs are the best known mediators of activity-dependent responses in neurons. Then, we showed a direct binding of the MeCP2 protein on the promoters of IEGs in wild-type animals, suggesting that MeCP2 have a repressive role in the regulation of these genes. Furthermore, we quantified the IEGs response to a stimulus both *in vivo* and *in vitro* and detected an aberrant expression pattern in Mecp2-deficient neurons for some of the IEGs analyzed. These data support the idea that proper IEGs expression is crucial for correct synaptic development and that MeCP2 has a key role in the regulation of IEGs.

**Introduction**

Rett syndrome (RTT), the major cause of mental disability in women after Down syndrome, is characterized by several cognitive and motor impairments acquired in early childhood (Chahrour and Zoghbi, 2007). At this stage of brain development, sensory experience is modulating excitatory synapse maturation and elimination, and promoting the development of inhibitory synapses (Flavell and Greenberg, 2008). Disruption of the normal process of experience-dependent synaptic development may lead to an imbalance between excitatory and inhibitory neurons, therefore resulting in cognitive impairment as in autism spectrum disorder (ASD), which includes RTT (Ebert and Greenberg, 2013). Consistent with the hypothesis that RTT arises as a disorder of neuronal maturation, the lack of MeCP2 does not seem to alter the proliferation rate and cell density of neural stem cells from the dentate gyrus (DG) of the hippocampus (Smrt et al., 2007). On the other hand, there are many evidences suggesting that impairments in both synapse maturation and function are involved in the RTT physiopathology (Banerjee et al., 2012). Primarily, autopsy studies have shown reduced dendritic branching and increased cell-packing density in RTT brains (Armstrong, 2005). Moreover, great effort has been put in characterizing RTT mouse models at the synaptic level. Among all the defects that have been reported so far are reductions in dendritic spine density (Tropea et al., 2009), decrease glutamatergic synapse number (Chao et al., 2007) and changes in the strength and/or number of GABAergic inhibitory synapses (Zhang et al., 2010).

Mutations of MeCP2 gene account for more than 90% of RTT cases. MeCP2 is an abundant nuclear protein that was initially considered a negative regulator of transcription, due to its high affinity to methylated cytosine (5mC) (Lewis et al., 1992; Nan et al., 1997). In the last decade several brilliant studies have begun to dismantle this hypothesis in favor of a multi-functional global regulator role for MeCP2. In fact, MeCP2 is able to modulate the expression (activating or repressing) of a wide range of genes by interacting with several partners (Bedogni et al., 2014)

and by being post-translationally modified at several residues (Ausió et al., 2014). Apart from the high affinity to 5mC, MeCP2 has been demonstrated to be the major 5-hydroxymethylcytosine (5hmC)-binding protein in the brain, and this recently discovered epigenetic mark is enriched in active genes (Mellén et al., 2012). In addition to that, MeCP2 can bind unspecific DNA through its highly conserved AT-hook domains (Baker et al., 2013). MeCP2 lacking one of the AT-hook domains appears impaired in its DNA binding and chromatin compaction capabilities and leads to the loss of ATRX protein localization at pericentric heterochromatin. Besides this variety of genomic substrates, MeCP2 may modify its function depending on the protein partner it is bound to. MeCP2 interactors encompass the transcriptional activator CREB (Chahrour et al., 2008), the NCoR/SMRT repressor complex (Lyst et al., 2013), the histone deacetylase HDAC1/2 (Nan et al., 1998), the regulator of alternative splicing YB1 (Young et al., 2005) and the DNA methyltransferase DNMT1 (Kimura and Shiota, 2003), among others.

Although there are several reports of transcriptomic profiling in RTT mouse models, the majority of them made use of microarray technologies. We thought it would be of interest to investigate expression changes using a high-throughput approach such as RNA sequencing technology, which allows more depth in the quantification of gene expression. Furthermore, here we have focused on two distinct brain regions, hippocampus and prefrontal cortex, with the aim of gaining new insights on the tissue-specific roles of MeCP2 in brain. In the present study, we have found that many immediate-early genes (IEGs), which are crucial players in neuronal plasticity and cognitive functions (Okuno, 2011), are mis-regulated in the brain of RTT mice. In addition, the lack of MeCP2 provokes an impaired response to stimuli of IEG expression *in vitro* and *in vivo*. Altogether our results contribute to understand the mechanisms by which MeCP2 is critically involved in synaptic plasticity, which in turn underlies higher cognitive abilities as well as learning and memory skills.

## Materials and Methods

### Animals

Experiments were performed on the B6.129P2(c)-Mecp2tm1+1Bird mouse model for RTT (Guy et al., 2001). Mice were kept under specific pathogen-free conditions in accordance with the recommendations of the Federation of European Laboratory Animal Science Associations. Lighting conditions (lights on from 08:00–20:00 h) and temperature (22°C) were kept constant. Animals were allowed ad libitum access to food and water and were inspected every day. Tissue samples were obtained from hemizygous Mecp2-null males and their wild-type (WT) littermates after establishing RTT-like symptoms in the defective animals (at about 7-8 wk of age). Mice were euthanized in accordance with the Guidelines for Humane Endpoints for Animals Used in Biomedical Research. Tissues were frozen on dry ice immediately after dissection and stored at −80 °C until use.

### RNA isolation

In order to extract RNA from frozen tissues, prefrontal cortices and hippocampi were ground into powder with mortar and pestle and resuspended in Trizol reagent (Life technologies). In the case of primary cultures, Trizol reagent was added directly to the wells. The RNA purification was performed on the RNA-containing aqueous phase with RNeasy mini kit (Qiagen). After elution with RNase-free water and treatment with turbo DNase (Ambion). Quantification and quality check were performed with Nanodrop and Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. For sequencing purpose, we used RNA samples with RNA integrity number (RIN) above 9.

### Next generation sequencing

RNAs from 5 male adult wild-type or Mecp2-null prefrontal cortices or hippocampi were pooled. The sequencing library of each RNA sample was prepared with the TrueSeq RNA Sample Preparation kit (Illumina) according to the protocol

provided by the manufacturer. Each sample was run in duplicate and subjected to 50 cycles of sequencing in an Illumina Hiseq2000 Sequencer.

The extraction of 76-bp length paired-end reads was achieved using CASAVA (Illumina Pipeline v.37). For each sample, reads with a quality score equal or more than Q30 passed filtering, and were used to generate a complete FASTQ file, which was then mapped to NCBI Mouse reference genome (mm9 build 37) using TopHat with default parameters. The resulting aligned reads were then sorted and indexed with SAMTOOLS program (Li et al., 2009), and analyzed with RseQCpackage to show the average insert fragment size, 140 bp (+/- 40 bp; SD). Bedtools suite (Quinlan et al., 2010) was used to report the count of alignments from BAM files that overlap intervals in the mm9 transcripts using a BED formatted file. The resulting raw counts were then used as an input for the DESeq Bioconductor package (Anders et al., 2010) under R statistical software (R Development Core Team, 2008), to find the differential expressed transcript between KO and wild type conditions for each tissue.

**Quantitative real-time PCR**

cDNA synthesis was performed with 200-1000 ng total RNA and random hexamers primers using the thermoscript KIT (Life Technologies). 1 µl aliquots of diluted cDNA (1:4) were amplified by the SYBR Green PCR Master Mix (Life Technologies) in a final volume of 10 µl. Real-time PCR reactions were performed in triplicate on an Applied Biosystems 7900HT Fast Real-Time PCR system. All primer pairs were designed with Primer 3 software and validated by gel electrophoresis to amplify specific single products. All data were normalized with respect to endogenous control: Tbp and Rpl38. Cycle thresholds for each indivdual PCR reaction were calculated with SDS software (Applied Biosystems). PCR cycles were divided into initial denaturation at 95ºC for 5 minutes, followed by 40 cycles of 95ºC, for 30 s, 60ºC, for 30 s and 72ºC for 30 s.

**Chromatin Immunoprecipitation**

The brain tissues were cross-linked with 1% formaldehyde for 8 min and the reaction was blocked by adding glycine to a final concentration of 0.125 M. After washing two times with ice-cold PBS, cell pellets were resuspended in cell lysis buffer (HEPES 5 mM, KCl 85 mM, NP40 0.5% pH 8.0) supplemented with protease inhibitor cocktail (Complete EDTA-free, Roche) and the lysate was homogenized with a douncer to facilitate cell membrane break. The nuclear pellet was then resuspended in Nuclei lysis buffer (TRIS-HCl 50 mM, EDTA 10 mM, SDS 1% pH 8.1) and subsequently sonicated with Bioruptor (Diagenode) for 30 min (30 sec ON, 30 sec OFF cycles). The chromatin size of the fragments obtained was 150–500 bp. Samples were diluted with Dilution buffer (SDS 0.01%, Triton X-100 1.1%, EDTA 1.2 mM, NaCl 165 mM, TRIS-HCl 16.7 mM pH 8.1). Magnetic beads were used for the pre-clearing of diluted chromatin (over- night at 4 °C) and for incubation with anti-MeCP2 (m9317, sigma). Non-related mouse IgG antibody (12–371, Millipore) was used as a negative control. The Beads-Antibody complexes were then incubated with pre-cleared chromatin for 2 h at 4°C in agitation. The immune-complexes were washed: twice with low salt Buffer (TRIS-HCl 50 mM pH 8.0, NaCl 150 mM, SDS 0.1%, NP-40 1%, EDTA 1 mM, Deoxicolate Na 0.5%), twice with high Salt Buffer (TRIS-HCl 50 mM pH 8.0, NaCl 500 mM, SDS 0.1%, NP-40 1%, EDTA 1 mM, Deoxicolate Na 0.5%), twice with LiCl Buffer (TRIS-HCl 50 mM pH 8.0, LiCl 250 mM, SDS 0.1%, NP-40 1%, EDTA 1 mM, Deoxicolate Na 0.5%) and twice with TE Buffer (TRIS-HCl 10 mM pH 8.0, EDTA 0.25 mM). Cross-linked chromatin was then eluted from the magnetic beads by adding elution Buffer ($NaHCO_3$ 100 mM, SDS 1%). Samples were de-crosslinked overnight at 65 °C and incubated with Proteinase K at 50 ug/ml final concentration for 1 h. Finally, DNA was purified with PCR purification kit (Qiagen).

**Chromatin accessibility assay**

Prefrontal cortices and hippocampi were homogenized on ice using a douncer in 20 volumes of buffer A (0.25 M sucrose, 5 mM $MgCl_2$, KCl 25 mM, Tris-HCl 20 mM

ph 7.5, 0.1 % Triton) and then incubated on ice during 5 min. After that, samples were centrifugated at 1000 *g* during 10 min at 4ºC. Pellets were resuspended with buffer A (without Triton) and Optiprep (Sigma) was added. Nuclei were then centrifuged at 3200 *g* for 20 minutes at 4ºC and the resulting pellets were finally resuspended in 1 volume buffer B (50 mM NaCl, 10 mM PIPES pH 6.8, 5 mM $MgCl_2$, 1 mM $CaCl_2$) and pre-warmed at 37ºC for 5 minutes. Digestions were performed using 2 units of MNase (Roche) per mg of tissue during 15 minutes. Reactions were blocked on ice with 5 mM EDTA. An aliquot of digested nuclei was taken and used as input. To obtain the chromatin fractions, nuclei were centrifugated at 8000 *g* for 10 minutes, supernatant corresponds to S1 phase. The digested DNAs were then mixed with Laemmli buffer and warmed at 65ºC. After that, proteins were precipitated at 4ºC with 120 mM KCl and the DNA-containing supernatant was used for Real-time PCR. 0.5 ng of DNA were used for each PCR. Qubit 2.0 Fluorometer and dsDNA Broad Range Assay reagents (Life Technologies) were used to quantify DNA.

**Primary hippocampal and cortical cultures**

Dissociated cortical and hippocampal neurons were prepared from newborn mice as previously described (Beaudoin et al., 2012). Cultures were maintained in Neurobasal A medium supplemented with B27, antibiotic-antimycotic and Glutamax™ (Life Technologies). Cytosine arabinoside (Sigma) was added to a 5 µm final concentration at 1 DIV to inhibit the proliferation of dividing non-neuronal cells. Neurons were plated at 100,000-200,000 per well in a 12-well plate. Half medium was replaced with an equal volume of fresh warm medium at 4 DIV. For neuronal stimulation, cultures were incubated with 50 µm forskolin (Sigma) at 11 DIV for 1 hour and then the media was replaced with a fresh one.

***In vivo* Kainic acid administration**

*in vivo* stimulation was induced by intraperitoneal administration of symptomatic mice 7-8 weeks of age with kainic acid (KA) (15 mg/kg) in PBS. Control animals received saline only. The efficiency of the treatment was assessed observing

seizures in KA-treated animals. Mice were sacrificed 1 h after injection and the brains were rapidly dissected.

## Results

### Overview of the RNA-seq results

We focused our attention on the Hippocampus (HIP) and Prefrontal cortex (PFC) because they are highly affected in RTT patients. It has been reported that pyramidal neurons in the prefrontal region of RTT patients display reduced dendritic branches and shorter spines (Armstrong, 2005; Belichenko et al., 1994). Moreover, both in PFC and HIP the neuronal soma size is reduced (Kaufmann and Moser, 2000).

Total RNAs derived from a pool of 5 samples from both wild-type and Mecp2-null PFC and HIP were sequenced. Each of the four pools was run in duplicate in order to assess technical reproducibility. About 86-89% of the total reads were uniquely aligned to the Mus musculus NCBI37/mm9 assembly among samples. We have first analyzed the distribution of the mapped reads on the genome and the samples were consistent among themselves (Fig. 1A). Of the uniquely mapped reads, about 87.5% aligned at the transcript exons and UTRs (88% and 86.8% in PFC wt and Mecp2-null; 87.7% and 87.7% in HIP WT and Mecp2-null), 6% at the intronic regions (5.5% and 6.7% in PFC WT and Mecp2-null; 5.9% and 5.9% in HIP WT and Mecp2-null) and the remaining 6.5% at the regions up- and down-stream of the transcript (6.5% and 6.6% in PFC WT and Mecp2-null; 6.4% and 6.4% in HIP WT and Mecp2-null) (Fig. 1A). The RNA-seq data obtained are freely available at the Gene Expression Omnibus (GEO) database: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=qtsbyccstlgrvut&acc=GSE60219.

Then, we examined the differential gene expression between Mecp2-null and wild-type samples. We applied a 1.5 fold-change threshold and 0.05 FDR cut-off to the raw list of differentially expressed genes. Finally, we found 1049 and 1154 mis-regulated genes in HIP and PFC, respectively (Fig. 1B-C). The ratio of up- and down-regulated genes was different between the two regions. In the HIP the ratio was

favorable to the less expressed genes, being 388 (37%) and 661 (63%) the up- and down-regulated genes, respectively. On the other hand, in the PFC there were slightly more up-regulated genes, 630 (55%), compared to the down-regulated ones, 523 (45%). In addition, we analyzed the common differentially expressed genes between HIP and PFC. Only a small fraction, 76 and 109, were up- and down-regulated, respectively, in both brain areas, suggesting a region-dependent role for MeCP2 in the regulation of gene expression (Fig. 1D-E).

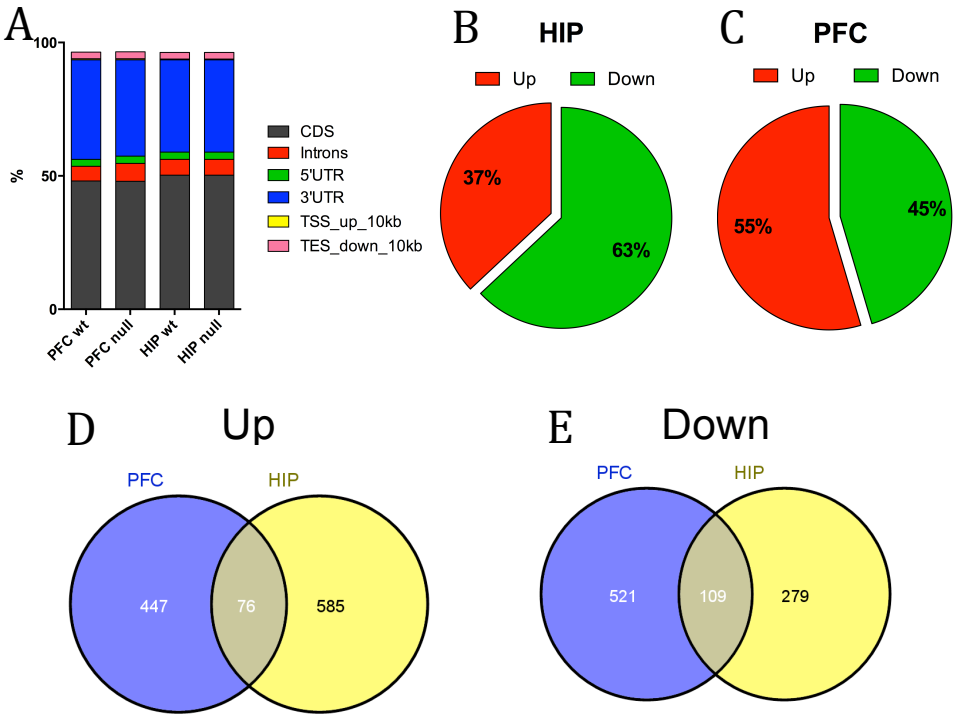


**Figure 1.** Summary of the sequencing results. **(A)** Percentage of mapped reads onto the genomic regions. The values on each column are the averages of the sequencing technical replicas. **(B)** and **(C)** pie chart displaying up- and down-regulated genes ratio in prefrontal cortex and hippocampus, respectively. **(D)** and **(E)** Venn's diagrams showing the common genes between PFC and HIP in down- and up-regulated genes, respectively.

Finally, to gain a functional overview of the gene expression differences we performed a gene ontology (GO) analysis on the genes lists. Both HIP and PFC up-regulated genes were enriched in neuronal function terms and, to a lesser extent,

signal transduction ones (Fig. 2A, C). The scenario was similar for the down-regulated genes but in this case we found many inflammatory, apoptosis, oxidative stress and immune system-related terms (Fig. 2B, D).

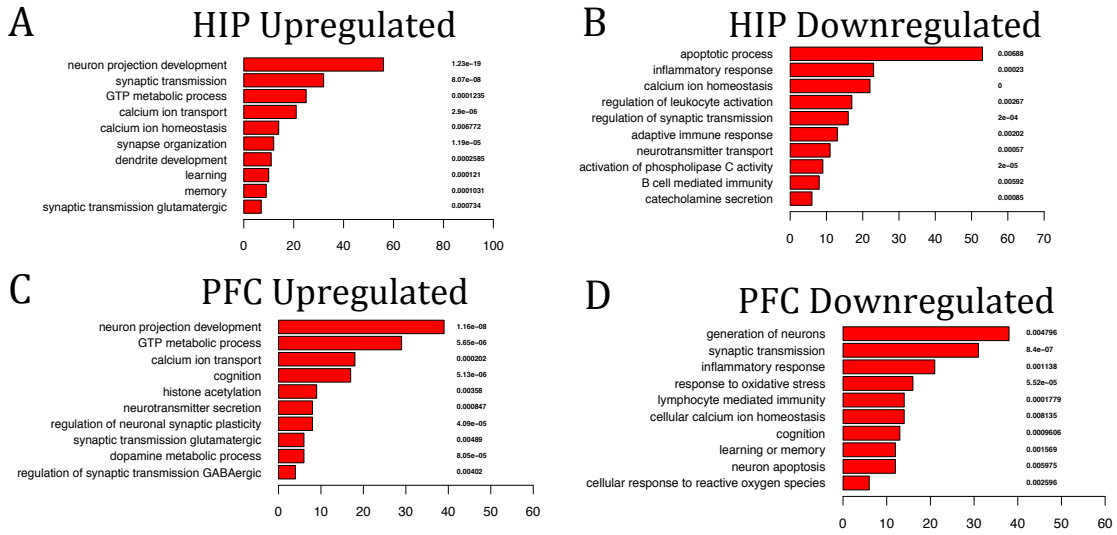


**Figure 2.** Enriched pathways significantly altered in Mecp2-null mice compared to control animals in the PFC **(A** and **B)** and HIP **(C** and **D)**. The bar at the bottom of each graph indicates the number of genes belonging to a Gene Ontology (GO) term. The p-value is indicated for each GO term.

### IEGs are up-regulated in MeCP2-null hippocampus and prefrontal cortex

The enrichment in neuronal function and signal transduction terms prompted us to examine accurately the up-regulated gene lists of PFC and HIP. We found several members of the immediate-early genes (IEGs) family up-regulated both in the PFC and HIP of the Mecp2-null mouse (Table 1). IEGs are the first genes rapidly and transiently induced by sensorial, emotional or cognitive stimuli. This activity-dependent gene family encodes several functionally different products such as secreted proteins (cytokines), cytoplasmic enzymes, ligand-dependent transcription factors and inducible transcription factors. IEGs act as signal mediator between the first and second messengers, normally extracellular factors (neurotransmitters) and cytoplasmic protein kinases, respectively, and the late neuronal downstream targets that directly regulate the extent of neuronal plasticity changes (Hughes and Dragunow, 1995). For this reason, we thought this class of gene could be of prime

interest for the pathophysiology of RTT. Among the IEGs up-regulated in the Mecp2-null brain we can found several members of the AP-1 complex (FOS, FOSB and JUNB), a transcription factor that regulates gene expression in response to a multitude of stimuli, such as growth factors and neurotransmitters, and two members of the Early growth response gene group (EGR1 and EGR2), another class of transcriptional regulators involved in neuronal response to a stimulus (Pérez-Cadahía et al., 2011). In addition, NR4A family members are also present (NR4A1 and NR4A3). This group of genes has been implicated with hippocampus-dependent memory formation and consolidation (Hawk and Abel, 2011). Finally the last two IEGs up-regulated in our RNA-seq were ARC, a post-synaptic protein involved in synaptic plasticity (Okuno, 2011) and NPAS4, a transcription factor that promotes the formation and maintenance of inhibitory synapses (Lin et al., 2008).

Fos, Fosb, Egr1, Egr2, Npas4, Nr4a1 and Arc were shared between the two brain regions, while Junb and Nr4a3 were present in the PFC but not in the HIP (Table 1). The magnitude of changes was moderate for all the genes, going from 4.92 of Egr2 to 1.59 fold-change of Egr1 in HIP. This is consistent with the majority of transcriptional screenings performed so far in RTT syndrome models that showed only subtle impairments in genes expression (Tudor et al., 2002; Urdinguio et al., 2008; Chahrour et al., 2008; Petazzi et al., 2013).

| | | | **Prefrontal cortex** | | **Hippocampus** | |
|---|---|---|---|---|---|---|
| ***Gene Symbol*** | ***Gene Name*** | ***Transcript ID*** | ***Fold-change*** | ***FDR*** | ***Fold-change*** | ***FDR*** |
| **Arc** | Activity-regulated cytoskeleton-associated protein | uc007wfo.1 | **3,57** | 6,36E-08 | **2,27** | 9,02E-18 |
| **Fos** | Proto-oncogene c-Fos | uc007oha.1 | **2,81** | 1,06E-31 | **2,55** | 4,38E-23 |
| **Junb** | Transcription factor jun-B | uc012ghn.1 | **2,49** | 8,03E-03 | **NO** | NO |
| **Egr2** | Early growth response protein 2 | uc007flx.1 | **2,46** | 4,14E-11 | **4,92** | 7,19E-27 |
| **Nr4a1** | Nuclear receptor subfamily 4 group A member 1 | uc007xsv.2 | **2,23** | 1,22E-12 | **2,03** | 4,89E-38 |
| **Npas4** | Neuronal PAS domain-containing protein 4 | uc008gbu.2 | **2,21** | 1,14E-14 | **3,86** | 1,37E-47 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Fosb** | Protein fosB | uc009flk.1 | **1,83** | 1,25E-07 | **1,95** | 2,10E-11 |
| **Nr4a3** | Nuclear receptor subfamily 4 group A member 3 | uc008suw.1 | **1,70** | 0,001 | **NO** | NO |
| **Egr1** | Early growth response protein 1 | uc008elt.1 | **1,61** | 3,68E-10 | **1,59** | 1,67E-21 |

**Table 1.** IEGs transcriptional changes in Prefrontal cortex and hippocampus

In order to confirm our results, we assessed the expression of our differential expressed candidates in independent samples. First we analyzed the expression of MeCP2 by qRT-PCR. As we expected, no Mecp2 transcript was detected in Mecp2-null PFC and HIP (Fig 3A-B). Consistent with the findings from the RNA-sequencing analysis in the HIP, qRT-PCR showed significant alterations in the expression of Fos, Egr2, Nr4a1, Npas4, Fosb and Egr1. Nevertheless, Junb, which was initially excluded from the differential expressed genes due to the low fold-change (1.4) was found to be up-regulated as well (Fig. 3C). In contrast, validation in PFC was only partially consistent with sequencing data, considering that 4 (Fos, Junb, Npas4, Fosb) out of 9 genes were confirmed to be up-regulated (Fig. 3D). No change was observed in the transcript levels of Arc in either of the two brain regions.

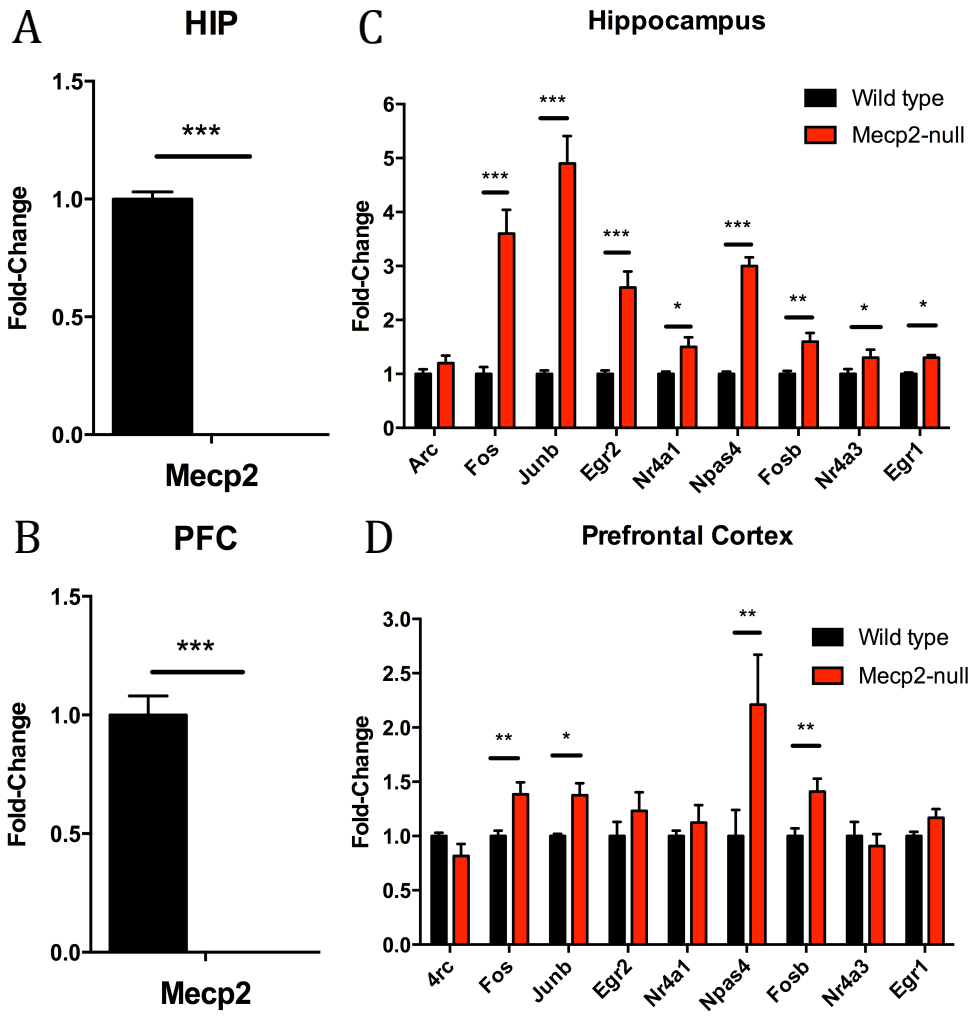


**Figure 3.** qRT-PCR validation of the IEG up-regulation in Mecp2-null mouse. **(A)** and **(B)** Mecp2 expression in the HIP and PFC, respectively. **(C)** and **(D)** IEGs expression in the HIP and PFC, respectively. (n = 5-6 for each condition, means ± SEM are represented). *p<0.05, **p<0.01, ***p<0.001 in Student's *t*-tests.

### MeCP2 has a potential repressive role on IEGs

Since we detected impairment in the expression of several member of the IEGs family in the Mecp2-null brain, our data suggest a potential repressive role of MeCP2 on these genes. Several times MeCP2 has been linked to transcriptional repression, from its discovery as a methyl-CpG binding protein (Lewis et al., 1992) to the

findings that MeCP2 is able to interact with HDAC2 and SIN3B (Nan et al., 1997) and NCoR/SMRT (Lyst et al., 2013) co-repressor complexes.

To determine a direct involvement of MeCP2 in the regulation of IEGs we analyzed the transcription start site (TSS) region of these genes by MeCP2 chromatin immunoprecipitation (ChIP) both in prefrontal cortex and hippocampus of wild type mice. Five regions along each IEGs TSS were selected for ChIP-qPCR analysis. MeCP2 binding was observed throughout all the promoter regions of the analyzed IEG (Fig 4A-H). However, IgG controls showed almost undetectable ChIP-qPCR products in both PFC and HIP (Fig 4A-H). Noteworthy, in every IEGs analyzed by ChIP, the level of MeCP2 occupancy on the TSS and proximal regions was higher in HIP than in PFC (Fig 4A-H), although they share the same binding pattern along the 4 kb genomic windows. Furthermore, in Fos, Junb, Nr4a1, Npas4, Fosb and Egr1, both PFC and HIP show a reduction of MeCP2 binding upon the regions associated with high CpG content (Fig 4A, B, D, E, F and H). The fact that MeCP2 is enriched within low CpG-content regions is likely due to the methylated status of CpG dinucleotides outside CpG islands (CGI). On the other hand, since these are actively transcribed genes, we expected the CGI to be unmethylated. Indeed, we checked the methylation of the CGIs surrounding the TSS in wild-type animals by bisulphite sequencing and we confirmed that were unmethylated both in PFC and HIP (Fig S1). To further prove that MeCP2 may be crucial for the proper regulation of these genes we wondered how the chromatin structure of the IEGs promoter would be in absence of MeCP2. To answer this question we performed a chromatin accessibility assay through micrococcal nuclease (MNase) digestion of nuclei. Nuclei were prepared from wild type and Mecp2-null animals for both PFC and HIP. After digestion with MNases, the chromatin fragments were subjected to quantitative PCR. The accessibility of Fos, Junb and Npas4 promoters was significantly increased in Mecp2-null HIP (Fig. 4I), indicating a more open chromatin state in absence of MeCP2. Taken together, these results demonstrate that MeCP2 binds to IEGs promoters tracking the methyl-CpG dinucleotide. Moreover, for most of the IEGs, MeCP2 occupancy upon the TSS is lower than up- and down-stream regions because of the un-methylated CGI located nearby

the TSS. Finally, in HIP, we were able to show that the lack of MeCP2 provokes an increase of MNase chromatin accessibility in Fos, Junb and Npas4 promoters, suggesting that MeCP2 may play a repressive role in the expression of IEGs in the brain.

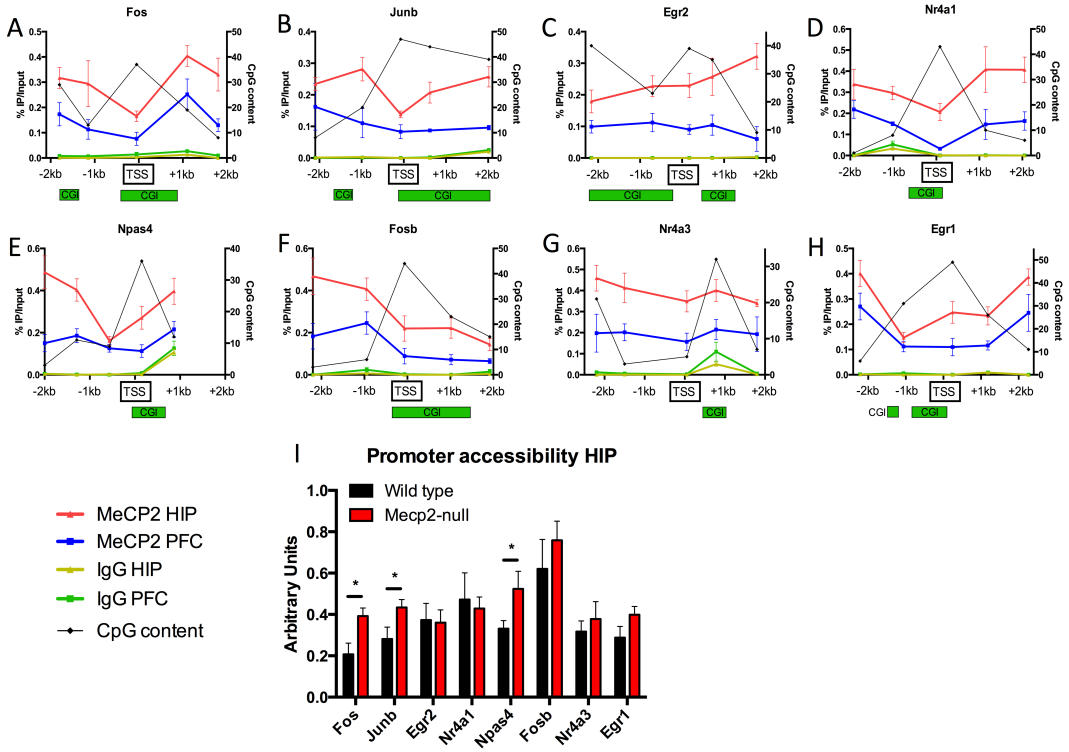


**Figure 4.** MeCP2 has a potential repressive role on IEGs. **(A-H)** Five regions along the IEGs transcription start site were analyzed with quantitative chromatin immunoprecipitation-PCR (qChIP-PCR). Green boxes at the bottom of each graph represent CpG islands (taken from UCSC genome browser http://genome-euro.ucsc.edu/cgi-bin/hgGateway?redirect=auto&source=genome.ucsc.edu). Values are expressed as percentage of MeCP2 or IgG enrichment over the Input (n = 3-4 for each condition, means ± SEM are represented) on the left y-axis and number of CpG dinucleotides in 500bp-windows on the right y-axis. **(I)** MNase accessibility assay of wild-type vs Mecp2-null hippocampi. MNase-digested DNA was subjected to quantitative PCR and normalized with input DNA (n = 3 for each condition, means ± SEM are represented). *p<0.05, ***p<0.001 in Student's *t*-tests.

## IEGs response to activation is mis-regulated in Mecp2-null neurons

Given that IEGs are activity-dependent genes and their expression is greatly enhanced after applying a stimulus (i.e. synaptic transmission in neurons), the best way to test the transcriptional impairment caused by the lack of MeCP2 is to activate

them in the proper biological system. With this aim, we cultured primary neurons derived from newborn wild type and Mecp2-null mice. Since we sought for a homogeneous model we performed both cortical and hippocampal neuron cultures so that it would resemble the neuronal population of the respective brain areas. In addition, our model allows us to focus exclusively on neurons, without the mis-leading contribution of glia. First, we checked the expression of Mecp2 on the unstimulated neurons after 11 DIV (days in vitro). As expected, both cortical and hippocampal Mecp2-null neurons showed no Mecp2 expression (Fig. 5A). Then, we treated our cultures with Forskolin, a well-known adenylate cyclase activator that provokes cAMP levels to rise, which in turn triggers the activation of the PKA pathway (Seamon et al., 1981).

Forskolin is widely used to elicit chemical long-term potentation (cLTP) (Sokolova et al., 2006) and modulate synaptic efficacy of excitatory glutamatergic synapses in the mammalian brain (Chavez-Noriega and Stevens, 1992). Therefore, to investigate IEGs expression in our cultures we performed a time-course with forskolin. Following 1 hour treatment with the drug, the medium was replaced with fresh one (without forskolin) and the recovery to the basal level was assessed. Fos, Junb, Egr2 and Npas4 display differential response patterns after forskolin treatment and withdrawal among different tissues and experimental conditions. In general, Fos, Junb, Egr2 and Npas4 expression levels in the Mecp2-null hippocampal neurons were higher than those of wild-type (Fig. 5B-E). Intriguingly, one hour after forskolin withdrawal, the expression of Fos, Junb, Egr2 and Npas4 continue to increase in Mecp2-null neurons, while in wild-type they did not change or even decrease. As a result, the expression levels of the four IEGs in wild type and Mecp2-null hippocampal neurons one hour after forskolin withdrawal are significantly different, suggesting that MeCP2 may be critical for the recovery of basal levels of IEGs expression after a stimulus. These data are particularly important, because they may contribute to the understanding of the mechanisms by which, in RTT, synaptic plasticity is reduced. The situation is the opposite in cortical neurons, where Fos, Junb, Egr2 and Npas4 are less expressed after forskolin withdrawal in Mecp2-null

samples (Fig. 5F-I), reaching the statistical significance one hour after the stimulus withdrawal.

In summary, our results show that MeCP2 is fundamental for the proper recovery of Fos, Junb, Egr2 and Npas4 basal expression levels in hippocampal neurons stimulated with forskolin. However, the same genes display decreased responsiveness in cortical neurons, suggesting that the MeCP2-mediated regulation of the kinetic of IEG expression is different between HIP and PFC.

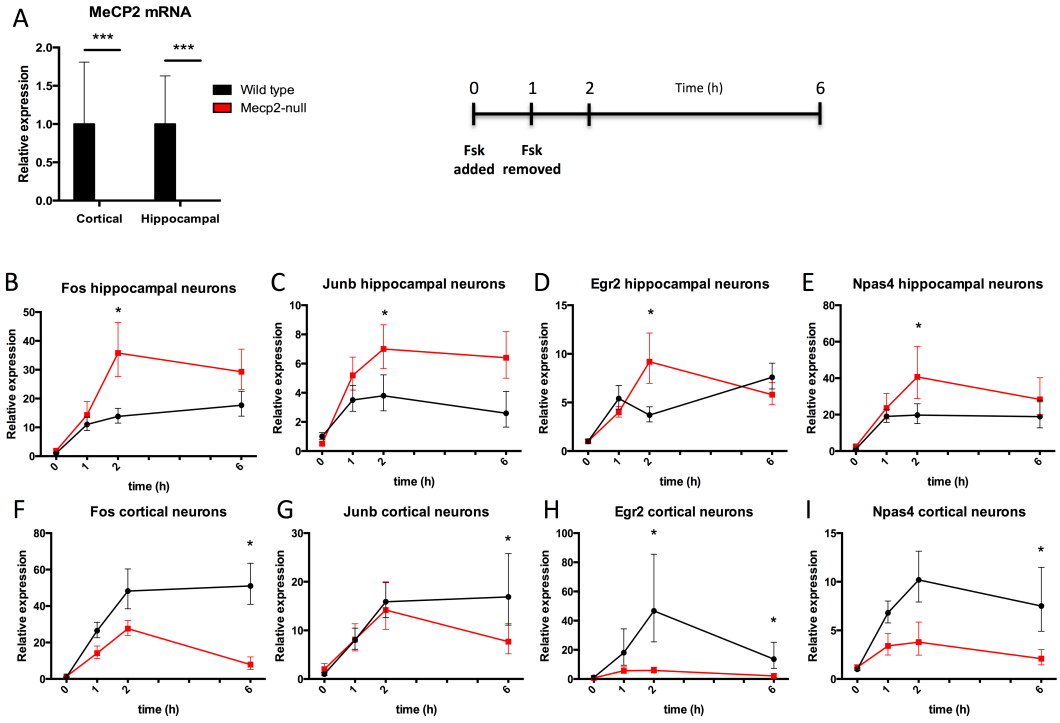


**Figure 5.** IEGs response to Forskolin is mis-regulated in Mecp2-null neurons. **(A)** Mecp2 expression in cortical and hippocampal neurons. Time-course of Fos, Junb, Egr2 and Npas4 transcript levels for both wild-type and Mecp2-null hippocampal **(B-E)** and cortical **(F-I)** neurons. All the expression data are relative to wild-type unstimulated values (n = 3-6 for each condition, means ± SEM are represented). *p<0.05, **p<0.01, in Student's *t*-tests.

## Junb is over-activated in Kainate-stimulated hippocampus *in vivo*

To ultimately prove the IEGs expression impairment in the brain of Mecp2-null mice, we took advantage of kainic acid (KA), a potent epileptogenic drug that induces excitatory neurons by activating kainate glutamate receptors and, to a minor extent,

AMPA channels (Ben-Ari and Cossart, 2000). Several studies showed that the main target of kainic acid, when administered *in vivo,* is the CA3 region of the hippocampus, due to the high amount of kainate receptors found in that region (Ben-Ari and Cossart, 2000). It is known that induction of seizures by KA triggers the expression of a myriad of genes, including IEGs (Liu et al., 2003).

With this aim, we treated wild-type and Mecp2-deficient animals with either KA or saline solution. Here we have focused only in the HIP for two reasons: first we have shown an aberrant response of Fos, Junb, Egr2 and Npas4 expression after a stimulus that may be consistent with a repressive role of MeCP2 in hippocampal neurons; second, KA effects on IEGs are milder in PFC (Fig S2) in comparison with HIP (Fig 6B). In order to exclude any artifact, we measured whether Mecp2 expression was affected by *in vivo* KA treatment in wild type HIP. No changes were detected in Mecp2 levels of transcript between KA- and saline-injected samples (Fig 6A). We next compared the effects of KA on Fos, Junb, Egr2 and Npas4 in WT and Mecp2-null mice. We observed a significant increase in Junb expression in the hippocampus of Mecp2-null animals treated with KA, when compared to treated WT samples (Fig. 6B). Although Egr2 expression displayed a similar mis-regulation (Fig. 6B), statistical significance was not reached ($p=0.0618$). Fos and Npas4 expression in HIP was not altered by KA in Mecp2-null mice. However, since we analyzed the transcript levels one hour after acute stimulus, it might still be possible that the recovery of basal expression levels for these genes is defectuos in Mecp2-null neurons. Altogether, This result confirms the data from primary neuronal cultures and reinforces the finding that activity-dependent induction of Junb is impaired in the hippocampus of a RTT mouse model.

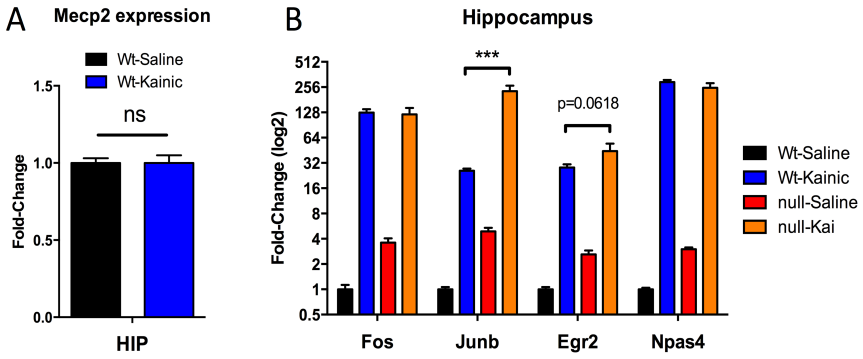


**Figure 6.** JunB is over-activated by kainic acid administration in Mecp-null hippocampus. **(A)** MeCP2 transcript levels of KA-treated mice versus untreated. (**B**) Effects of one-hour KA administration on Fos, Junb, Egr2 and Npas4 in wt and MeCP2-null mice (n = 5-6 for each condition, means ± SEM are represented). ***p<0.001 in Student's *t*-tests.

## Discussion

Several attempts have been made so far to identify new MeCP2 targets that could explain RTT pathophysiology. Transcriptional profiling on both pre- and post-symptomatic mutant mice provided few validated targets and the results often suffer from a lack of interlaboratory reproducibility (Tudor et al., 2002; Nuber et al., 2005; Urdinguio et al., 2008; Ben-Shachar et al., 2009). We provided here a transcriptomic study that takes advantage of high-throughput sequencing techniques on a Rett syndrome mouse model. In addition, we extended our analysis to two brain regions highly affected in RTT, the hippocampus and prefrontal cortex. Our sequencing data support a scenario in which MeCP2 plays as a master regulator able to both promote and repress gene expression. Furthermore, we showed that this MeCP2 feature is tissue-dependent, because very few genes were either up or down-regulated in both PFC and HIP.

GO analysis of the differentially expressed genes in the Mecp2-null mouse demonstrated that genes up and down-regulated in Mecp2-null samples are associated to different pathways. We reported a vast majority of oxidative stress and immune system-related GO terms in down-regulated genes of both PFC and HIP. This

observation is in line with several studies indicating a potential role for oxidative stress in RTT (De Felice et al., 2012). On the other hand, we detected no such terms in up-regulated genes. In fact, those genes which expression rises in absence of MeCP2 were related to synaptic function such as secondary messengers ($Ca^{2+}$ and GTP) and neurotransmitter pathways. Thus, our attention focused on a subset of up-regulated genes that were present in both PFC and HIP, the immediate-early genes (IEGs) family. There are several paper reporting mis-regulation of one or more members of this family in RTT (Kron et al., 2012; Su et al., 2012; Swanberg et al., 2009; Li et al., 2013; Ebert et al., 2013), but the different biological models used and the heterogeneous experimental condition applied make it difficult to properly review the topic. In this paper we provided evidences of a global impairment of IEGs expression in the Mecp2-null mouse. Validation of sequencing data by qRT-PCR confirmed the aberrant expression of Egr2, Nr4a1, Nr4a3 and Egr1 in the HIP and Fos, Junb, Npas4 and Fosb in both HIP and PFC of Mecp2-null mouse.

Consistent with the critical finding that MeCP2 binds throughout the whole genome tracking methylated cytosine (Skene et al., 2010), we confirm the binding of MeCP2 on the IEGs promoters with a de-enrichment in occupancy nearby the high-CpG-content regions that is very likely to be a consequence of unmethylated CpG islands. Moreover, we have shown that the MeCP2 occupancy pattern along a 4kb-region spanning the TSS is similar between PFC and HIP, although the level of MeCP2 binding was higher in the HIP in every IEG promoter analyzed. However, this last observation does not take into account intrinsic differences in tissue composition, for instance the neuron/glia ratio. For this reason, we cannot state that the level of MeCP2 binding on IEGs promoters correlates with the degree of repression of the same genes. We have demonstrated here a role for MeCP2 in the regulation of IEGs in light of its binding upon the regulatory regions of this activity-dependent class of genes. The finding that the HIP chromatin was more accessible to MNase digestion in the Mecp2-null brain suggests that loosening of chromatin is accompanied by IEGs up-regulation and further supports our hypothesis.

IEGs are activity-regulated genes that respond to a multitude of stimuli. In the case of neuronal IEGs the stimulus is represented by synaptic activity. Therefore, to better understand the nature of the IEGs impairment in our RTT model, neuronal activity needs to be synchronized by external means. One of the key findings of our study is that hippocampal and cortical neurons differ in the response to forskolin, an adenylate cyclase activator. Four IEGs (Fos, Junb, Egr2, Npas4) displayed altered expression in Mecp2-null cultured neurons. Precisely, this four IEG exhibited an aberrant kinetic of recovery to the basal state. It is noteworthy that IEGs activation constitutes a key step for long-term alterations that are necessary for plasticity and memory formation (Loebrich and Nedivi, 2009). Defects in the normal activation patterns of Fos, Fosb and Egr1 lead to impairment in sensory- and experience-dependent learning mechanisms (Okuno, 2011). In the case of RTT, it is known that overall neuronal activity is decreased in Mecp2 mutant. Therefore, our findings in hippocampus may seem a paradox, since Mecp2-deficient neurons display increased expression of IEGs. Our data may contribute to explain the results that Zhang et al. (2008) presented on the topic. In this study they identified an ambiguous state in Mecp2-null hippocampal slice preparation. While the intrinsic network activity was hyper-excitable, the local spontaneous post-synaptic excitatory activity was diminished from that of wild type. It seems plausible that this hyper-excitation of hippocampal neurons may be due to the increased responsiveness of IEGs. As for the reduced post-synaptic excitatory activity, it may be a consequence of Junb up-regulation. In fact, it has been shown that JUNB is a poor transactivator and may act to dampen the response to the more potent transactivators of this family (Young and Colburn, 2006), therefore reducing the transactivational power of the AP1 complex and consequently diminishing post-synaptic excitatory activity.

On the other hand, Fos, Junb, Egr2 and Npas4 expression is downregulated in cortical neurons derived from Mecp2-deficient animals. The divergence between sequencing and *in vitro* data in the case of cortical neurons can partially be explained with the fact that PFC is a concrete area of the adult brain, while cortical neurons derived form whole neonatal cortex. However, consistent with our in vitro results,

Kron et al. (2012) reported the reduced expression of Fos in forebrain cortices of Mecp2-null mice.

The transcription factor EGR2 has been associated with transcriptional activation in cortical cognitive functions associated with attention (DeSteno et al., 2008). This is particularly interesting because these cognitive features are often impaired in autism-spectrum disorder. Moreover, NPAS4 has been potentially implicated with schizophrenia (Lewis et al., 2005) and autism (Hussman; 2001), due to its critical involvement in maintaining a balance between excitation and inhibition by contributing to the formation of inhibitory synapses (Lin et al., 2008). FOS and JUNB are members of a large family of dimeric proteins complexes called AP-1 (activating protein 1). The main scenarios in which AP-1 members seem to play a role are memory formation and behavioral changes related to drug exposure (Pérez-Cadahía et al., 2011). Intriguingly, the composition of the dimer seems to be the major factor in the regulation of AP-1 in the nervous system.

With the purpose of investigating more in depth this IEGs induction impairment in Mecp2-deficient mice, we then analyzed Fos, Junb, Egr2 and Npas4 induction *in vivo.* Kainic acid- induced seizures provide a valuable tool to this aim, since the administration of this kainate receptor analog provokes a tremendous neuronal excitation, especially in the HIP, which in turn triggers the activation of several genes, most of them IEGs (Nedivi et al., 1993). We showed that Junb response triggered by kainic acid in Mecp2-null hippocampi was higher than that of control HIP. This represent a further confirmation of the putative role of IEGs expression defects in RTT physiopathology. In fact, if the response pattern of Junb is impaired in absence of MeCP2, we can expect that the proper expression of downstream genes may be dysregulated as well. To gain more insights on this topic, more efforts need to be put in the understanding of the IEGs contribution to RTT, with a close look on Junb. Finally, although the pathways activated by forskolin and kainic acid are partially overlapping, the fact that we observed no change in Fos, Egr2 and Npas4 expression in KA-induced wild type and Mecp2-null HIP demonstrates that the activation of each IEG is strictly pathway- and context-dependent.

This study presented evidences of a mis-regulation of immediate early genes in the nervous system of a RTT mouse model. Our results may contribute to shed new light on the mechanisms by which synaptic plasticity is impaired in Rett syndrome.

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**Supplementary Information**

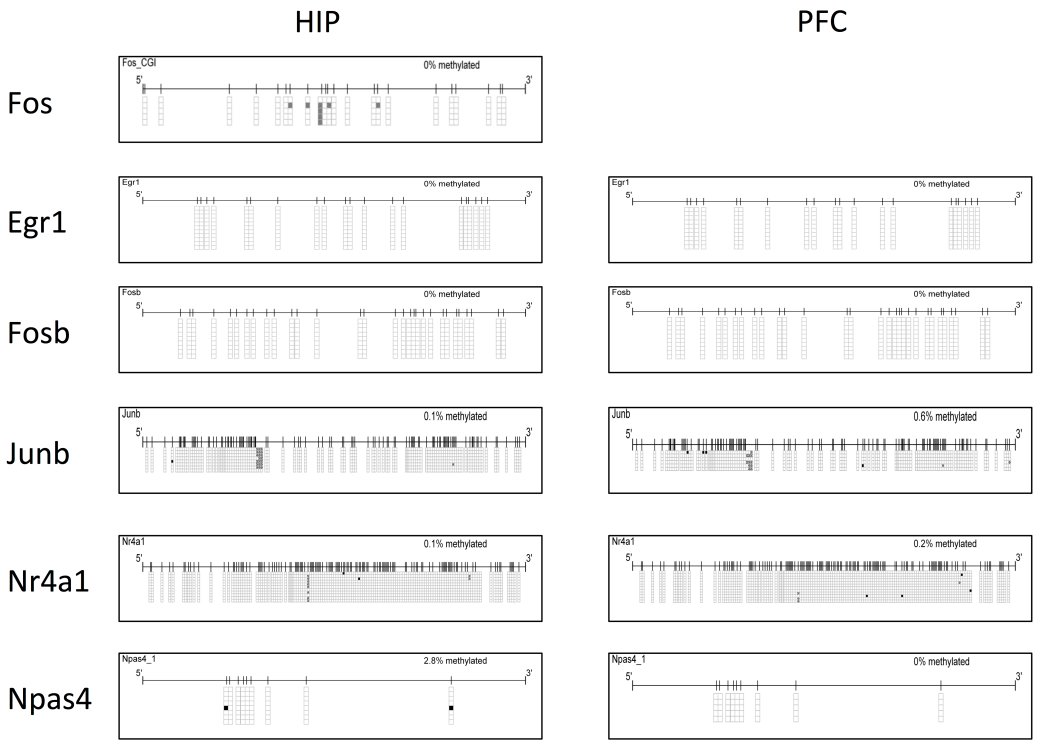


**Figure S1**. CpG islands of IEGs are not methylated. Sequencing of bisulphite-converted DNA coming from PFC and HIP. Every column represents a CpG nucleotide. Black squares account for methylated CpGs, whereas white ones represent un-methylated CpGs.

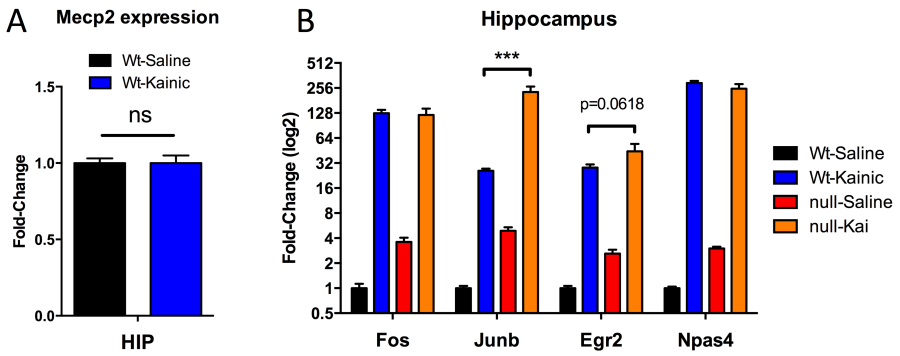


**Figure S2.** KA administration has a mild effect on prefrontal cortex. **(A)** MeCP2 transcript levels of KA-treated mice versus untreated. (**B**) Effects of one-hour KA administration on Fos, Junb, Egr2 and Npas4 in wt and MeCP2-null mice (n = 5-6 for each condition, means ± SEM are represented).

## STUDY III

## An Increase in MECP2 Dosage Impairs Neural Tube Formation

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**(Annex 2)**

**ABSTRACT**

Epigenetic mechanisms are fundamental for shaping the activity of the central nervous system (CNS). Methyl-CpG binding protein 2 (MECP2) acts as a bridge between methylated DNA and transcriptional effectors responsible for differentiation programs in neurons. The importance of MECP2 dosage in CNS is evident in Rett Syndrome and MECP2 duplication syndrome, which are neurodevelopmental diseases caused by loss-of-function mutations or duplication of the MECP2 gene, respectively. Although many studies have been performed on Rett syndrome models, little is known about the effects of an increase in MECP2 dosage. Herein, we demonstrate that MECP2 overexpression affects neural tube formation, leading to a decrease in neuroblast proliferation in the neural tube ventricular zone. Furthermore, an increase in MECP2 dose provokes premature differentiation of neural precursors accompanied by greater cell death, resulting in a loss of neuronal populations. Overall, our data indicate that correct MECP2 expression levels are required for proper nervous system development.

**INTRODUCTION**

During development, mitotically active precursors located in the neuroepithelium give rise to specialized neuronal and glial cells that define the adult nervous system. To maintain the brain's complexity, neurons originating from the neural tube undergo mitotic quiescence. Therefore, neuronal differentiation encompasses an elaborate developmental program in which neurogenic and antiproliferative signals work together to guarantee the differentiated state. This developmental step is mediated by genetic and epigenetic factors. Among the latter, chromatin remodelers (Clapier and Cairns, 2009), histone variants (Kamakaka and Biggins, 2005), histone post-translational modifications (Kouzarides, 2007) and DNA methylation (Miranda and Jones, 2007) are strongly involved in regulating the proliferation and differentiation of neural precursor cells. The importance of this regulation is highlighted by several neurological disorders caused by mutations in epigenetic genes, such as Rett syndrome (RTT), alpha thalassemia/mental retardation X-linked syndrome, Rubinstein–Taybi syndrome and Coffin–Lowry syndrome (Urdinguio et al., 2009).

Among the epigenetic regulators of the brain, methyl-CpG-binding proteins are responsible for reading the methylation code of DNA and therefore, for regulating gene transcription (Klose and Bird, 2006). In fact, a mutation in the best-known protein of this family, MECP2 (methyl-CpG binding protein 2), is responsible for RTT (Amir et al., 1999). MECP2 is a basic nuclear protein that acts mainly as a transcriptional repressor, preferentially binding to methylated DNA sequences (Klose et al., 2005 and Lewis et al., 1992). Although MECP2 is widely expressed, MECP2 levels are highest in the brain, principally in mature postmigratory neurons (Jung et al., 2003). MECP2 protein levels are low during embryogenesis and increase progressively during the postnatal period of neuronal maturation (Balmer et al., 2003 and Cohen et al., 2003). In addition to its necessary role in mature neuronal and glial cells, MECP2 has been implicated in neuronal specification during early embryogenesis in several species (Coverdale et al., 2004 and Stancheva et al., 2003). Moreover, MECP2 has been shown to promote neuronal differentiation of neural

stem cells while repressing astrocyte differentiation (Tsujimura et al., 2009).

The striking finding that MECP2 nucleotide mutations or duplications cause Rett syndrome or MECP2 duplication syndrome, respectively, suggests that careful regulation of this gene is necessary for correct brain development and function, as both overexpression and reduced expression are associated with neurodevelopmental disorders (Collins et al., 2004 and del Gaudio et al., 2006). Intriguingly, a loss of MECP2 function and an increase in MECP2 dosage lead to clinically similar neurological disorders (Van Esch et al., 2005). However, although many studies have been performed on MECP2 loss-of-function models, little is known about the biological consequences of MECP2 overexpression in either the adult or developing brain.

Using a well-known developmental model, the chick embryo neural tube, we sought to investigate the effects of human MECP2 overexpression on the proliferating progenitor cells of neurons and glia. Here, we show that MECP2 dosage is fundamental for proper neural tube development and demonstrate that MECP2 overexpression provokes premature differentiation of proliferating progenitor cells. This ectopic differentiation leads to cell-cycle exit and cell death, ultimately resulting in decreased neuronal populations.

## MATERIALS AND METHODS

### Plasmids

The human MECP2_e1 full-length coding sequence was cloned into pCIG vector (Megason and McMahon; 2002). The vector comprises CMV enhancer and beta-actin promoter, followed by multiple cloning sites, internal ribosomal entry site (IRES) and a nuclear-localized green fluorescent protein (GFP).

### Antibodies

The following primary antibodies were used: anti-MECP2 (Diagenode, custom); anti-BrdU (DSHB, G3G4); anti-phosphoH3S10 (Millipore, 05-806); anti-

neural β-tubulin III (Tuj1) (R&D systems, MAB1195); anti HuC/D (Life Technologies, A-21271); anti-N-cadherin (R&D systems, AF6426); anti-active Caspase 3 (BD pharmingen, 559565); anti-active Caspase 8 (Millipore, MAB10754) and anti-β-actin-peroxidase (Sigma, A3854)

**Chick in ovo electroporation**

Eggs from White-Leghorn chickens were incubated at 38.5 ºC and 70% humidity. Embryos were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1992). Chick embryos were electroporated with purified plasmid DNA at 2-3 μg/μl in $H_2O$ with 50 ng/ml of Fast Green. Plasmid DNA was injected into the lumen of HH10 neural tubes, electrodes were placed at both sides of the neural tube and finally, the embryos were electroporated by an IntracelDual Pulse (TSS-100) delivering five 50 ms square pulses of 20-25 V.

**mRNA extraction and RT-PCR**

RNA was extracted with Trizol reagent (Invitrogen) from dissected neural tubes according to the manufacturer's protocol. Single-stranded cDNA was synthesized with Thermoscript reverse-transcriptase and random hexamers (Life Technologies), and then subjected to PCR with the following primers: cMECP2, forward 5'-GGACCAGGAAGCTCAAACAGC-3' and reverse 5'-TTGGGGCTCTTGGCTTTCTTG-3'; Gapdh, forward 5'-CTGAATGGGAAGCTTACTG-3' and reverse, 5'-CATCATACTTGGCTGGTTTC-3'.

**Western Blotting**

Neural tubes were dissected from several embryos at the same stage, pooled together and total protein was extracted with Laemmli buffer. Equal amounts of protein (20 μg) were boiled for 10 minutes and β-mercaptoethanol was added to a 3% final concentration. Samples were then separated by electrophoresis on 10% SDS-PAGE gels, and transferred to nitrocellulose membranes. Membranes were blocked in 5% skim milk powder in PBS plus 0,1% Tween 20 for 1 h at room

temperature and then incubated overnight at 4ºC with primary antibodies. For MECP2 (1:2000) antibody, an anti-rabbit HRP-conjugated secondary antibody (1:10000) was used. Finally, complexes on the membrane were visualized using the ECL detection kit (Amersham).

**BrdU incorporation**

Bromodeoxyuridine (BrdU, 0.5 µg/µl) was injected into the chick embryo neural tube lumen 30 minutes before fixation. Before anti-BrdU antibody incubation (which was performed as described below), the sections were treated with HCl 2N for 30 minutes and washed with NaBorate 0.1 M (pH 8.5).

**Indirect immunofluorescence**

The collected brachial regions from embryos were fixed for 2 hours at 4 °C in 4% paraformaldehyde, rinsed with PBS, soaked in PBS 30% sucrose solution and embedded in either OCT or agarose for sectioning in a Leica Cryostat (CM 1900) or a Vibratome (VT1000). The sections were blocked at room temperature for at least 1 hour in 1% BSA (in PBS with 0.1% Triton X-100) before overnight incubation with primary antibodies at 4 °C. The sections were then incubated for 1,5 hours at room temperature with Alexa-conjugated goat secondary IgG antibodies (Life Technologies) and 0,1 ng/µl DAPI (Sigma). Images were captured on a Leica SP5 confocal microscope using a 40X oil-immersion objective and processed using Fiji software. MECP2 intensity was quantified using the Fiji software as following: each side of the neural tube (MECP2 EP and control) was selected as region of interest (ROI) and the total intensity of all pixels for each ROI was calculated and compared.

**Statistical Analysis**

Quantitative data were expressed as mean and standard error (s.e.). Significant differences between groups were tested by Student's *t*-test.

**RESULTS**

**Chicken MECP2 is expressed ubiquitously in the developing spinal cord**

MECP2 is present in all vertebrates and is highly conserved among mammals, while divergence between mammalian and amphibian or fish MeCP2 more extensive. However, the alignment of chicken MECP2 with mouse and human MECP2 shows that the protein is highly conserved throughout species as diverse as humans and chickens. Although cMECP2 mRNA and protein are only partially annotated, a large part of the sequence is highly similar to human MECP2. Particularly striking is the 96.8% sequence identity in a 125-amino-acid region. Significantly, the conserved region includes the methyl-CpG binding domain (MBD) (Weitzel et al., 1997) (Fig. 1a). The high degree of conservation compares well with the characterization of the MBD as an essential element for binding of MECP2 to heterochromatin as well as unmethylated four-way DNA junctions (Galvão and Thomas, 2005 and Nan et al., 1996). Hence, we wondered whether cMECP2 (previously known as ARBP) is expressed in the chicken embryo across different developmental stages. RT-PCR analysis of HH10, 20, 23 and 26 revealed that cMECP2 is indeed expressed in chick embryo (Fig. 1b) with greater expression seen at the HH20 stage. Since transcript presence does not always correlate with protein levels, we checked cMECP2 protein by western blot. Fig. 1c shows that cMECP2 is expressed at every tested developmental stage. Although in HH10 chick embryos the neural tube is formed mainly by the ventricular zone (VZ)—an epithelium composed entirely of mitotically active, multipotent neural precursor cells—from HH14 to 15 some of these neuroblasts exit the cell cycle and migrate laterally from the ventricular zone to the mantle zone (MZ), which is formed exclusively by post-mitotic, differentiating neurons and glia (Fig. 1d). Therefore, we wondered whether the MECP2 expression was restricted to the differentiating neurons or was global. To address this issue, we collected brachial sections of HH25 embryos and stained them with an anti-MECP2 antibody that recognizes the region shown in Fig. 1e. The chicken MECP2 partial annotated region shows high homology with the aminoacidic region of the mammalian MECP2 counterpart, thus, expecting that the antibody recognizes chicken MECP2 specifically (Weitzel et al., 1997). The results illustrate high expression of

cMECP2 in ventral and in more dorsal ventricular cells as well as in mantle cells (Fig. 1f, top panel). Thus, MECP2 is present both in differentiated neurons and in neural progenitors. Moreover, we found that cMECP2 localizes in the nucleus, as staining of MECP2 overlaps with DAPI (Fig. 1f, bottom panel).

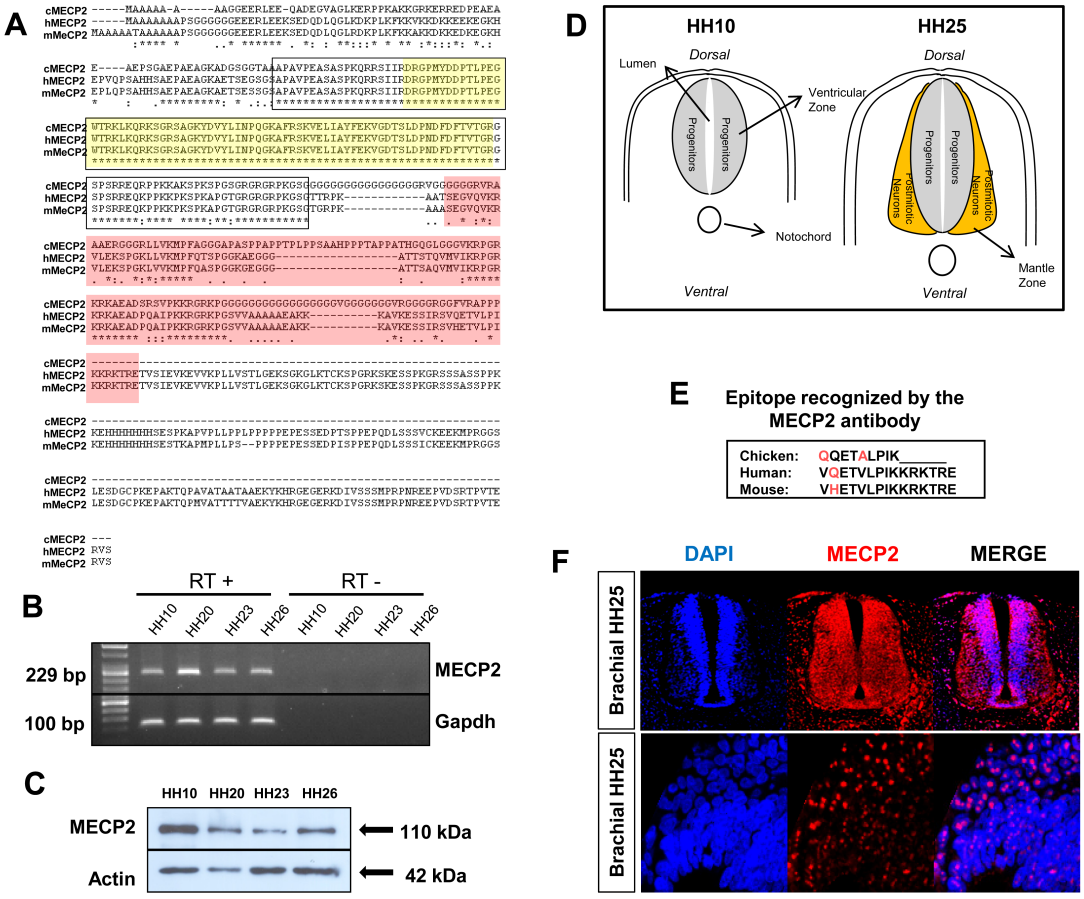


**Fig. 1.** cMECP2 is ubiquitously expressed in the developing spinal cord. **a** Protein sequences of chicken (Accession nº CAA74577), human (NP_001104262) and mouse (NP_001075448) MECP2 were aligned. The region inside the black box shows that the human sequence and the chicken sequence share 96.8% identity. MBD (yellow) and TRD (red) domains are highlighted. **b** RT-PCR on HH10, 20, 23 and 26 neural tube RNA extracts. Minus reverse transcriptase samples (-RT) are shown on the right. **c** Immunoblot on HH10, 20, 23 and 26 neural tubes. **d** Scheme showing the neural tube organization at HH10 and 25. **e** The cMeCP2 fragment is partly conserved with the human peptide recognized by the antibody. **f** HH25 embryos were dissected and brachial sections were stained for MECP2 (red) and DAPI (blue).

**MECP2 overexpression reduces neuroblast proliferation**

To investigate the role of a protein, lack and gain-of function studies are needed. The pCIG plasmid has been used in many gain-of-function studies to obtain new insights on genes function relevant for development, such as hJag1 (Neves et al., 2011), Wnt (Megason and McMahon, 2002), EZH2 (Akizu et al., 2010) and FGF (Martínez-Morales et al., 2011) among others, whose expression reached high levels when electroporated in ovo. Although different shRNA of the cMECP2 annotated region have been electroporated in chicken embryo, none of them worked (data not shown).

Humans and mice have two protein isoforms produced by the alternative splicing of the MECP2 gene with the MECP2E1 and E2 isoforms, differing only in their N-terminal sequences (Kriaucionis and Bird, 2004). It is known that MECP2E1-specific mutations alone are able to cause RTT (Gianakopoulos et al., 2012) and MECP2E1 displays 10 times more expression than E2 (Dragich et al., 2007). In addition, a recent study reported the differential distribution of MeCP2E1 within various brain regions in mice (Zachariah et al., 2012). With the aim to investigate the presence of different MECP2 isoforms in chicken we used a RT-PCR approach. Our exon-specific RT-PCR experiments based on the protein alignment between human and chicken and designed to amplify the sequence between Exons 1 and 3, failed to detect a second cMECP2 transcript (data not shown).

In order to analyze the effects of increased MECP2 dosage on the developing neural tube, we cloned MECP2_E1 full-length into a pCIG vector under the control of a CMV promoter. The additional expression of a nuclear-localized GFP from an internal ribosome entry site (IRES) enabled easy identification of transfected cells. In ovo injection of MECP2 expression plasmid into HH10 embryos and subsequent electroporation led to efficient and unilateral expression of this protein in the neural tube as it is shown in Fig. 2a, where the GFP channel colocalizes with the MECP2 red channel. Noteworthy, the pattern of nuclear localization of the endogenous protein is maintained upon MECP2 overexpression and the intensity of MECP2 signal is increased. Quantification of MECP2 intensity in electroporated (EP) neural tube

compared with pCIG EP reveals an average of 45-fold more MECP2 in the electroporated region (Fig. 2a, graph). Although at 24 hours post-electroporation (PE) the neural tubes did not show any evidence of altered phenotypes, at 48 (data not shown) and 72 hours PE the thickness and the structure of electroporated neural tubes were highly affected, compared to the non-electroporated side or to the empty vector. The most striking feature associated with the overexpression of MECP2 was the vastly reduced area occupied by the MZ, whose strong phenotype is appreciated in Figs. 2b, c, 3a, b and c.

To elucidate the mechanisms by which MECP2 overexpression so profoundly alters neural tube organization, we examined the proliferation rate of electroporated neural tubes. We took embryos at 72 h PE and processed them for bromodeoxyuridine (BrdU) staining, showing that MECP2 overexpression leads to an overall decrease in the number of proliferating BrdU positive cells (Fig. 2b). Noteworthy, the most affected part corresponds with higher levels of GFP (compare zoom squares of pCIG EP and MECP2 EP panels in Fig. 2b). In addition, in EP pCIG, GFP-labeled cells accumulated at the mantle zone due to the normal migration accompanying neuronal differentiation, while in EP MECP2 electroporated cells gathered mainly in the VZ, therefore making not possible to assess colocalization between GFP and BrdU. In order to quantify BrdU incorporation, BrdU labeled cells in control (pCIG) and MECP2 EP neural tubes were normalized with the total number of BrdU-positive cells in the respective non-EP side (graph of Fig. 2b). Results clearly indicate a reduction around 20% in BrdU levels as a result of MECP2 overexpression. We then wondered whether MECP2 overexpression affected also the levels of H3S10 phosphorylation, a histone mark that correlates with mitotically active cell populations. The H3S10p marker highlighted a mislocalization of actively dividing cells that normally reside close to the lumen. Again, higher levels of GFP coincide with disruption of H3S10p pattern (compare zoom square of pCIG EP and MECP2 EP panels in Fig. 2c). Quantification of anti-H3S10p immunostaining also showed that MECP2-electroporated neural tubes has a 20% decrease in the amount of mitotic cells than did the control neural tubes (Fig. 2c, graph). Collectively, these data

emphasize the importance of proper spatial–temporal MECP2 expression for ensuring correct proliferation of progenitor cells residing in the ventricular zone of the neural tube.

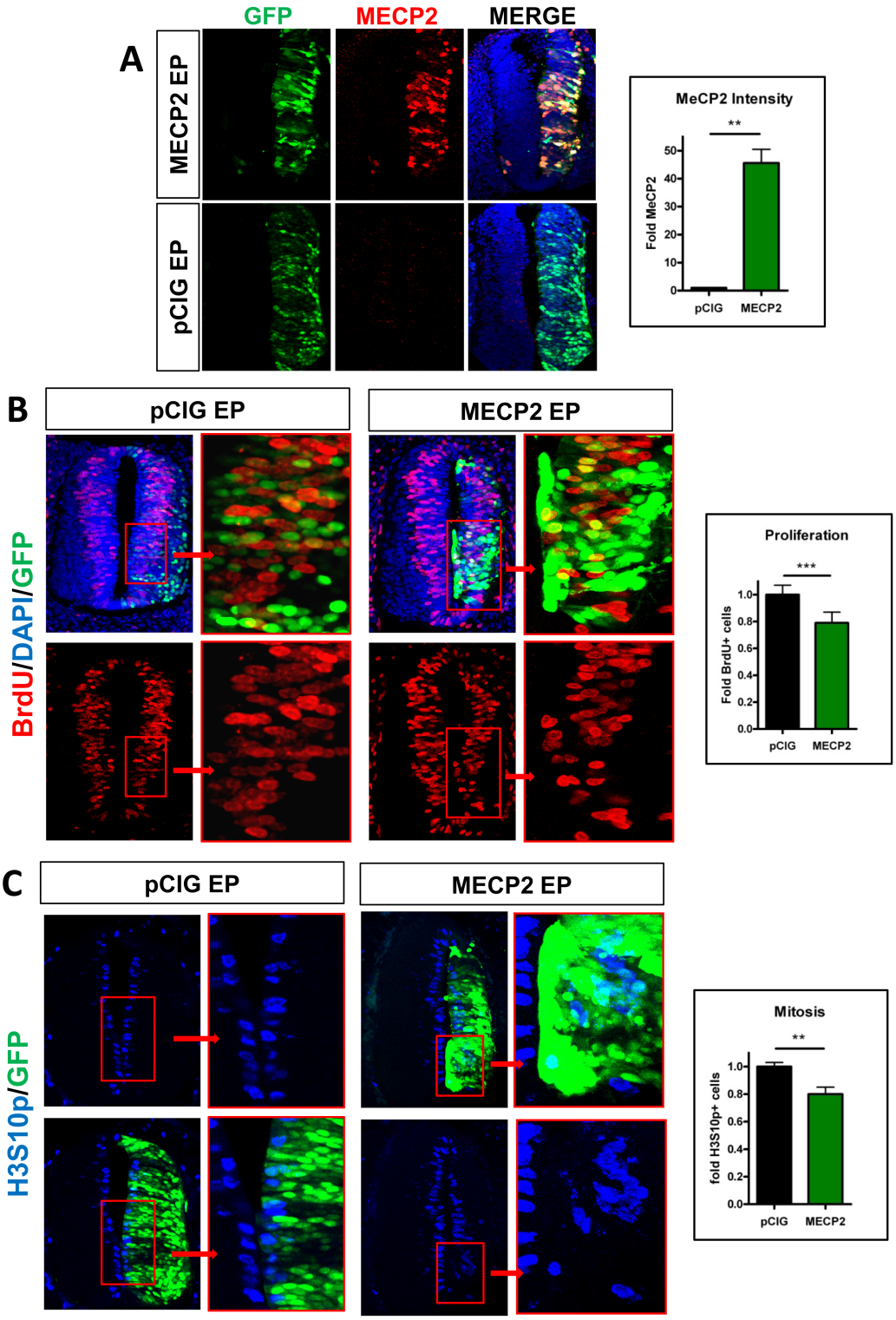


**Fig. 2.** Neuroblast proliferation is reduced upon MECP2 overexpression. HH10 embryos were electroporated with MECP2 or the empty vector in a bicistronic plasmid containing EGFP. All sections were taken from the brachial region. **a** MECP2 immunostaining (red) of embryos at

72 hours PE. The graph shows MECP2 area intensity of MECP2 EP neural tubes relative to the empty vector (pCIG). Intensities were quantified by Fiji software. Data shows mean of n=3 sections (from 3 different embryos). Error bars indicate s.e. *p<0,05. **b** BrdU immunostaining (red) of 72 hours PE embryos. The magnified red boxes show the most affected regions. The graph shows the number of BrdU positive cells found in MECP2 EP neural tubes relative to pCIG. Data show the mean of *n=9* sections (from three embryos). Error bars indicate s.e. ***p<0,001. **c** H3S10p immunostaining (blue). The magnified red boxes show the most affected regions. The graph shows the number of H3S10p positive cells found in MECP2 EP neural tubes relative to pCIG. Data for pCIG show the mean of *n=8* sections (from three embryos), for MECP2 *n=12* sections (from two embryos). Error bars indicate s.e. **p<0,01.

**MECP2 overexpression induces ectopic localization of differentiated neurons**

Given that a role for MECP2 in promoting neuronal differentiation of neural precursor cells has been proposed (Stancheva et al., 2003 and Tsujimura et al., 2009), we wondered whether the reduced proliferation of neural progenitor cells that we observed stemmed from premature induction of neurogenesis. To investigate this possibility, we took MECP2-electroporated neural tubes at 72 h PE, and then stained them with neural β-tubulin III (Tuj1), which is one of the earliest markers of neuronal commitment in primitive neuroepithelium. Fig. 3a show that MECP2 overexpression provokes a clear decrease in the amounts of differentiated neuronal population located at the mantle zone (compare zoom squares of pCIG control and MECP2 EP panels). Additionally, the same images of Tuj1 staining also show an ectopic localization of differentiated neurons in the MECP2-electroporated neural tubes. To confirm the phenotype caused by MECP2 overexpression, we immunostained with another marker, HuC/D, an RNA-binding protein specific to neuronal lineage. Again, when comparing MECP2 EP neural tubes with controls, depletion of differentiated cells is observed in MZ of MECP2 EP neural tubes (Fig. 3b, pCIG and MECP2 panels). This phenotype unequivocally shows an aberrant differentiation pattern for cells overexpressing MECP2, as it can be inferred by the presence of both GFP and Tuj1 in ectopically differentiated cells. Due to the non-nuclear localization of Tuj1 and HuC/D it has not been possible to quantify labeled cells of these markers. However, quantification of such qualitative markers was not

necessary given that the difference in the level of staining of the mantle zone was striking, as well as it was the presence of Tuj1-stained cells in the VZ, which is normally populated by proliferating cells.

Since Tuj1 staining was found in the ventricular zone, we decided to check for neuroepithelial polarity markers, such as the cadherin family of proteins. In particular, the pattern of N-cadherin (Cdh2), a transmembrane protein that mediates homophilic adhesion at the cell junctions, was analyzed by immunostaining in the MECP2-electroporated neural tubes (at 72 h PE). The results clearly demonstrate that MECP2 overexpression disrupts the N-cadherin expression pattern along the lamina of the neural tube (Fig. 3c, compare zoom squares of control and MECP2 EP panels). This indicates that an increase in MECP2 dosage leads to a decrease in neuroepithelial polarity markers. These results suggest that, in addition to exiting the cell cycle and suffering from compromised polarity, MECP2-overexpressing neural precursor cells do not reach terminal differentiation, as can be inferred by reduced number of differentiating neurons in the MZ (Figs. 3a, c MECP2 panel).

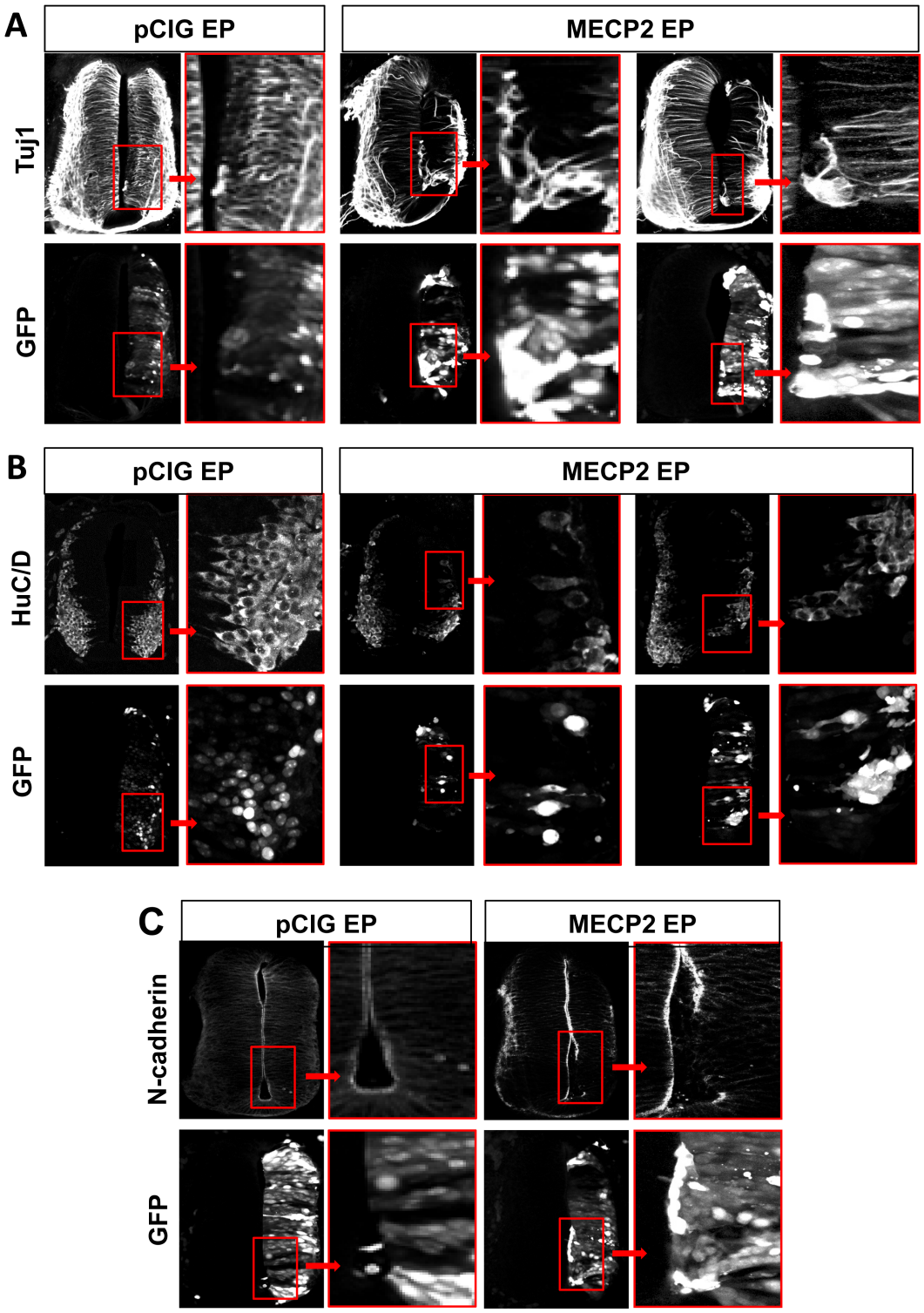


**Fig. 3.** MECP2 overexpression provokes ectopic localization of differentiated neurons. **a** Tuj1 immunostaining (gray, upper panel) of embryos at 72 hours PE. GFP is shown in the lower panel. For MECP2 EP, two embryos are shown. The magnified red box shows ectopically

differentiating neurons. **b** HuC/D immunostaining (gray, upper panel). For MECP2 EP, two embryos are shown. **c** N-cadherin immunostaining (gray, upper panel). The magnified red box shows disruption of the N-cadherin pattern by MECP2.

**MECP2 overexpression induces cell death**

The observations that at 72 h PE the number of Tuj1 labeled cells in MECP2-electroporated neural tubes is vastly reduced, led us to analyze the rate of apoptosis before the onset of the altered phenotype. To this end, we determined the cellular levels of active Caspase-3 and − 8, two well-known serine proteases that are activated during the early-intermediate stages of apoptosis (reviewed in Parrish et al., 2013). Caspase-8 is classified as an initiator caspase and it is one of the earliest signals in the cascade, while Caspase-3 act downstream and can be cleaved, and therefore activated, by Caspase-8. Control pCIG EP neural tubes show very low levels of Caspase-3 and − 8 labeled cells as expected (Figs. 4a and b pCIG EP panel, graph 4c and 4d). However, the number of Caspase-3 and Caspase-8 labeled cells in MECP2 EP compared with pCIG EP neural tubes is significantly higher both at 24 and 48 h PE (Figs. 4a and b MECP2 EP panel, graph 4c and 4d). To confirm these data we quantified cells undergoing cell death by counting Dapi-positive nuclei showing the characteristic condensed morphology. Fig. 4e shows an increase of pyknotic cells in MECP2 EP, both at 24 and 48 h PE compared with pCIG EP neural tubes. These results clearly indicate the presence of apoptotic cells upon MECP2 electroporation and can explain the aberrant phenotype.

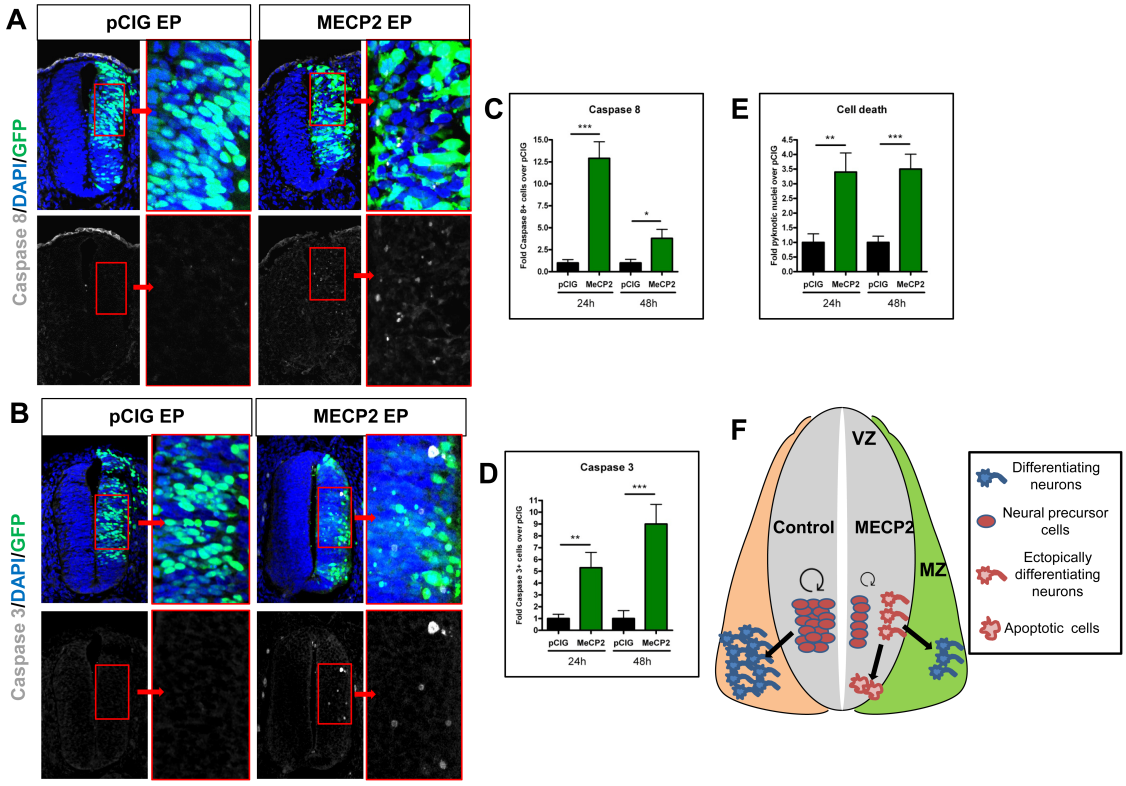


**Fig. 4.** MECP2 overexpression induces cell death. **a-b** HH10 embryos were electroporated with MECP2 or empty vector. Embryonic sections (at 24 and 48 hours PE) from brachial region were immunostained for Caspase 8 (**a**) (gray, lower panel) and Caspase 3 (**b**) (gray, lower panel). The red boxes in the MECP2 EP highlight the Caspase-positive spots. **c-d** The graphs show the number of Caspase 8 (**c**) and Caspase 3 (**d**) positive cells in MECP2 EP neural tubes relative to pCIG EP at 24 and 48 hours PE (pCIG 24 and 48h n=12 sections from 6 embryos; MECP2 24h n=21 from 10 embryos; MECP2 48h n=13 from 6 embryos). Error bars indicate s.e. *p<0,05; **p<0,01; ***p<0,001. **e** Quantification of cells showing pyknotic features in neural tubes at 24 and 48 hours PE (pCIG 24 and 48h n=12 sections from 6 embryos; MECP2 24h n=21 from 10 embryos; MECP2 48h n=13 from 6 embryos). Error bars indicate s.e. **p<0,01; ***p<0,001. **f** Model for MECP2 overexpression consequences in neural tube.

## DISCUSSION

To analyze the role of MECP2 in neurogenesis we have used a chicken model, however, we first checked whether our model was suitable for this purpose. First, expression of both cMECP2 transcript and protein has been detected in a wide window of developmental stages. Then, we have found that chicken MECP2 is functionally analogous to its mammalian counterpart since its nuclear localization

and the conservation of the region encompassing the methyl-CpG binding domain between human and chicken.

Our data also indicate that MECP2 overexpression causes neuroblasts to slow down proliferation, and that most of these neuroblasts die before they can reach terminal differentiation (Fig. 4f). Ectopic localization of differentiated neurons and reduced levels of polarity markers indicate that overexpression of MECP2 alone does not control the changes in polarity and migration that accompany neurogenesis. Neural cells die as a consequence of MECP2 overexpression, probably because they lack the additional spatial–temporal signals necessary for proper progression of neurogenesis. These results are in line with the lack of function studies from other models (Stancheva et al., 2003), indicating that the consequences of MECP2 overexpression do not represent toxic effects but specific ones.

Tsujimura et al. (2009) showed that MeCP2 overexpression in neural precursor cells (NPCs) promotes neuronal differentiation in adult mice. This work was based on the injection of embryo-derived NPCs in the brain or spinal cord of adult mice. We provided a more reliable study in which MECP2 ectopic expression has been induced in the chick neural tube without the need to deliver exogenous cells to the embryos. Our results are consistent with previous studies, which reported abnormally high levels of cell death in different in vitro systems overexpressing MECP2, relative to wild-type cells (Bracaglia et al., 2009 and Dastidar et al., 2012). Bracaglia et al. also reported that this pro-apoptotic effect disappears when the Rett syndrome-associated MECP2-R106W mutant, which is unable to bind to methylated DNA, is expressed—thereby implying that the MBD domain is essential for MECP2-induced apoptosis.

It is remarkable that Xenopus laevis MeCP2 was shown to regulate the number of neural precursor cells in the differentiating neuroectoderm of early Xenopus embryos ( Stancheva et al., 2003). In the absence of MeCP2 protein, the expression of Xenopus Hairy2a (a member of the Hes family of proteins, which are regulated by the Notch/Delta signaling pathway) was enhanced in embryos, which resulted in a lower number of differentiated neurons. Our results, together with the aforementioned

study, highlight the importance of MECP2 dosage, as both knock-out and overexpression of this protein results in a reduced number of differentiated neurons. In our case, reduction in total number of cells is not due only to apoptosis but in addition there is a proliferation problem.

Interestingly, our phenotype resembles the one produced by the genetic ablation of Notch1 (de la Pompa et al., 1997). Loss of Notch signaling results in premature onset of neurogenesis by neuroepithelial cells of the midbrain–hindbrain region of the neural tube. Notch1-deficient cells do not complete differentiation but instead are eliminated by apoptosis, resulting in a reduced number of neurons in the adult cerebellum (Lütolf et al., 2002).

The molecular mechanism responsible of this phenotype can be explained by the relevant interactions between MECP2 and other proteins. For example, the MECP2-associated kinase HIPK2 has been shown to regulate cell growth and apoptosis, both in vivo and in vitro (Bracaglia et al., 2009). In addition, MECP2 interacts with many co-factors crucial for both proliferation and differentiation, such as HDAC2 (MacDonald et al., 2010) and NCOR/SMRT (Ebert et al., 2013).

This is the first study that investigates the consequences of MECP2 gain-of-function in the nervous system of an in-vivo model in the early stages of development. In particular, we introduce the novel idea that high expression of MECP2 in mitotic cells leads to anti-proliferative and apoptotic effects. Several cases of increased MECP2 copy number have been reported in male patients with progressive neurodevelopmental delay phenotype (Friez et al., 2006, Lugtenberg et al., 2006, Meins et al., 2005 and Van Esch et al., 2005). Interestingly, a male patient with triplication of the MECP2 locus was described to have an even worse early-onset neurological phenotype at 3 months of age (del Gaudio et al., 2006), suggesting that the severity of an MECP2 overexpression phenotype is proportional to the copy number increase. In order to reinforce this hypothesis, researchers have studied a mouse model expressing seven times the wild-type levels of MeCP2 protein, reporting that it died by 3 weeks of age (Collins et al., 2004). Although the levels of MECP2 expression induced in the chicken neural tubes that we have described in the

present study are not fully representative of the physiological situation in patients with MECP2-related disorders, our results are in line with lack and gain of function studies that elucidate the importance of correct gene dosage in neuronal development and disorders.

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# RESULTS SYNTHESIS

**STUDY I**

**Dysregulation of the long non-coding RNA transcriptome in a Rett syndrome mouse model**

- We found 701 lncRNAs that had a different expression pattern in wild-type and Mecp2-null brain with a score of <0.05 in the false discovery rate (FDR) test and a >1.5-fold expression change. Among the altered lncRNAs, downregulation of transcripts was predominant (520 of 701, 74%), whereas upregulation occurred in the minority of differentially expressed genes (181 of 701, 26%).
- Following a selection of lncRNAs with a fold-change >2 that were associated with an annotated protein-coding gene involved in neuronal or glial functions, we validated two up-regulated lncRNAs, AK081227 and AK087060, in the Mecp2-null brain using qRT-PCR on independent samples.
- We showed that AK081227 and AK087060 promoters were occupied by the MeCP2 protein in wild-type mouse brains.
- We reported that the up-regulation of AK081227 in Mecp2-null mice was associated with a down-regulation of its host gene Gabrr2 in four brain regions (frontal cortex, hypothalamus, thalamus and cerebellum) (Pearson's correlation test = 0.44, p = 0.06).
- In the case of AK087060, we found that the up-regulation of this lncRNA was correlated with an increase in the expression of its host gene Arhgef26 in the four studied brain regions (Pearson's correlation test = 0.41, p = 0.08).

**STUDY II**

**RNA-sequencing of a Rett syndrome mouse model reveals global impairment of immediate-early genes expression**

- We sequenced the transcriptome of Mecp2-null and control mice and we detected 1049 and 1154 differentially expressed genes in HIP and PFC, respectively. The ratio of up- and down-regulated genes was different between the two regions. In the HIP the ratio was favorable to the less expressed genes,

being 388 (37%) and 661 (63%) the up- and down-regulated genes, respectively. On the other hand, in the PFC there were slightly more up-regulated genes, 630 (55%), compared to the down-regulated ones, 523 (45%). In addition we reported that only a small fraction of genes, 76 and 109, were up- and down-regulated, respectively, in both brain areas.

- Gene Ontology (GO) analysis of differentially expressed transcripts revealed that both HIP and PFC up-regulated genes were enriched in neuronal function terms and, to a lesser extent, signal transduction ones. The scenario was similar for the down-regulated genes but in this case we found many inflammatory, apoptosis, oxidative stress and immune system-related terms.
- We found several members of the immediate-early genes (IEGs) family to be up-regulated both in the PFC and HIP of Mecp2-null mouse. Consistent with the findings from the RNA-sequencing analysis in the HIP, qRT-PCR showed significant alterations in the expression of Fos, Junb, Egr2, Nr4a1, Npas4, Fosb and Egr1. Furthermore, Fos, Junb, Npas4 and Fosb were validated also in the PFC.
- We demonstrated the binding of MeCP2 upon the regulatory regions of IEGs. In both PFC and HIP wild-type brain, we observed a reduction of MeCP2 occupancy upon the regions associated with high CpG content of Fos, Junb, Nr4a1, Npas4, Fosb and Egr1 promoters. We also found that the HIP chromatin was more accessible to MNase digestion in the Mecp2-null brain.
- Then, we showed that four IEGs (Fos, Junb, Egr2, Npas4) displayed altered expression in Mecp2-null cultured neurons treated with forskolin. Precisely, this four IEG exhibited an aberrant kinetic of recovery to the basal state. One hour after forskolin withdrawal, Fos, Junb, Egr2 and Npas4 expression levels in the Mecp2-null hippocampal neurons continue to increase, while in wild-type they did not change or even decrease. The situation is the opposite in cortical neurons, where Fos, Junb, Egr2 and Npas4 are less expressed after forskolin withdrawal in Mecp2-null samples.

- Finally, we evaluated whether the IEGs response was impaired *in vivo* as well. Indeed, we observed a significant increase of Junb expression in the hippocampus of Mecp2-null animals treated with kainic acid, when compared to treated wild type mice.

**STUDY III**

**An increase in MeCP2 dosage impairs neural tube formation**

- We detected the expression of both chicken MeCP2 (cMECP2) transcript and protein in a wide window of developmental stages. In addition, we showed that nuclear localization and the sequence of the region encompassing the methyl-CpG binding domain are conserved between human and chicken.
- We found that the overexpression of MeCP2 in the neural tube of chicken embryos provokes an overall decrease in the number of proliferating BrdU-positive cells, with the most affected part being the ventricular zone. In addition, normal H3S10p pattern along the lumen is disrupted upon MeCP2 overexpression.
- Also, MeCP2 increase in dosage cause a clear decrease in the amounts of differentiated neuronal population located at the mantle zone, as it was demonstrated through immunostaining of neural tubes with TUJ1 and HUC/D, two neuronal-lineage restricted markers. Moreover, MeCP2 overexpression leads to a decrease of a neuroepithelial polarity marker such as N-cadherin.
- Finally, we showed that one of the possible explanations of our phenotype is the increased cell death occurring upon MeCP2 increase in dosage. We reported an increment of apoptotic cells in MeCP2-overexpressing neural tubes immunostained with Caspase-3 and -8. Furthermore, we described also an increase of pyknotic cells number in MeCP2 electroporated neural tubes.

# DISCUSSION

This thesis has been developed with the aim of investigating how the changes in MeCP2 dosage affect the correct development and maturation of CNS, with a special focus on the molecular impairment provoked by MeCP2 deregulation. Both loss- and gain-of MeCP2 function are detrimental for neuronal function, since RTT and *MECP2* duplication disorder arise from opposite changes in the expression of this protein.

In the first two studies, we tried to shed new light on the transcriptional impairment provoked by the loss of MeCP2. To this aim, we used two approaches: the increasing awareness of lncRNAs as crucial mediator of neuronal function led us to focus on the relationship between these epigenetic regulators and the transcription factor MeCP2; Furthermore, the availability of next-generation techniques prompted us to analyse deeply the transcriptome impairment in RTT.

On the other hand, overexpression of MeCP2 is far less studied than down-regulation. In particular, several open questions remain when the object of discussion is the role of MeCP2 in development. To address this, we analysed the effects of MeCP2 overexpression in neural tubes of chicken embryos. As a consequence, we focused on differentiation, proliferation and apoptotic markers in order to characterize the phenotype caused by MeCP2 overexpression in the developing nervous system.

**STUDY I**

**Dysregulation of the long non-coding transcriptome in a Rett syndrome mouse model**

Since the discovery of MeCP2 as the primary cause of Rett Syndrome (RTT), much effort has been spent on the identification of genes or pathways involved in the physiopathology of RTT. We proposed to expand our knowledge of MeCP2 targets by investigating an emerging class of epigenetic regulators such as long non-coding RNAs (lncRNAs). During the last decade, researchers started to recognize non-coding RNAs as fundamental players in shaping the mammalian brain. There is evidence of the importance of lncRNAs in higher cognitive and behavioural function evolution (Pollard et al., 2006).

Taking advantage of a well-studied mouse model that recapitulates key features of RTT (Guy et al., 2001), we analyzed the lncRNA transcriptome of the Mecp2-deficient mouse, and compared to that of its wild-type littermate. We were able to identify 701 differentially expressed lncRNAs between the brain of Mecp2-null and wild-type mice, all of them statistically significant. It is interesting to note that the majority of this transcripts (520) were down-regulated, suggesting a positive influence mediated by MeCP2 on the physiological expression of these lncRNAs. Our data find support in a previous work reporting disrupted miRNAs expression in the same RTT mouse model, where the overall down-regulation of miRNAs was the most common feature among the differential expressed transcripts (Urdinguio et al., 2010).

Many lncRNAs from our list were associated with known protein-coding genes and these relationships encompass different setting of interaction that ultimately may influence the lncRNA mechanism of action. lncRNAs may be transcribed in antisense, overlapping or intronic orientations relative to protein coding genes, and again from gene regulatory regions (reviewed in Qureshi and Mehler, 2012). In our case we screened our list by selecting those lncRNAs that were overlapping or intronic to protein-coding genes whose function was related to neurons or glia. Given this requirements for the identification of *bona fide* MeCP2 targets, we restricted our candidate by selecting only those lncRNAs with a fold expression change >2. Finally, we confirmed the impairment in the expression of two lncRNAs, AK081227 and AK087060, both of them up-regulated in the Mecp2-null mouse brain. Additionally, both the lncRNAs were found to be directly bound by MeCP2 in the promoter region, providing another evidence of MeCP2 as a role player in the regulation of this lncRNA.

Noteworthy, the lncRNAs AK081227 resides in an intron of the Gabrr2 gene. Intronic lncRNAs, whether originating from splicing or by independent transcriptional units, have been shown to recruit several classes of coactivator (Iglesias-Platas et al., 2012) or corepressor (Guil et al., 2012) complexes, therefore affecting the transcriptional state of the neighbour gene. In addition to this, we

described that the up-regulation of AK081227 was associated with the down-regulation of its host gene, Gabrr2, in several regions of the mouse brain. Intriguingly, the host gene of AK081227 is a subunit of the GABA receptor family. GABA is the major inhibitory neurotransmitter in the vertebrate brain and it mediates neuronal inhibition by binding to the GABA/benzodiazepine receptor. This finding may have a relevant impact in the understanding of Rett syndrome since there are already several reports of the disrupted GABA pathway in RTT. First, a recent study showed that Mecp2 deficiency in GABAergic neurons recapitulates most of the features displayed by Mecp2-null mice, including altered synaptic activity and plasticity (Chao et al., 2010). Secondly, various essential genes for GABAergic functions, like Dlx5 and GABRB3, have already been linked with RTT (Samaco et al., 2005; Hogart et al., 2007). Lastly, physiological studies of Mecp2-knockout animals revealed a shift in the excitatory/inhibitory balance, with increased excitatory and decreased inhibitory neurotransmission in the hippocampus and cortex (Chao et al., 2007). Moreover, more studies of RTT mouse models showed abnormalities in long-term potentiation (LTP) and impaired synaptic plasticity (Asaka et al, 2006). Our data provide a hint that the mechanisms by which the lack of MeCP2 is reflected on the GABaergic physiological impairment may be mediated by lncRNAs.

Our second impaired lncRNA, AK087060, is transcribed very close to the TSS of its host gene. These types of lncRNAs, known as promoter-associated, are usually transcribed around the TSS proximal region and are able to reclute other regulatory factors (Guil and Esteller, 2012) that impact on the expression of the associated-coding gene, in this case Arhgef26. The correlation between AK087060 and Arhgef26 expression was positive in the four studied brain regions. Even in this case, a role for this protein in a neurological disorder such as Rett syndrome could be invoked. The ARHGEF26 protein is a Rho guanine nucleotide exchange factor (GEF) that has a role in the actin-driven endocytic process known as macropinocytosis that contributes to repulsive turning (Kolpak et al., 2009) and retinal cone growth (Guo et al., 2012) in the axons of neurons. However, no associations between this gene (or its family) and

RTT have been discovered so far, suggesting the need of further investigation in this topic.

Overall, we reported here the transcriptional dysregulation of lncRNAs upon Mecp2 loss and we provided two examples of the complex interaction between lncRNAs and coding-RNAs. These results may contribute to the understanding of certain aspects of the physiopathology of RTT.

**STUDY II**

**RNA-sequencing of a Rett syndrome mouse model reveals global impairment of immediate-early genes expression**

The current therapeutic approach to RTT relies heavily on targeting factors that are downstream to MeCP2 function (Ricceri et al, 2012). In order to obtain those factors and subsequently target them with drugs or alternative therapies, transcriptional profiling on both pre- and post-symptomatic mutant mice have been used to identify MeCP2 targets (Tudor et al., 2002; Nuber et al., 2005; Urdinguio et al., 2008; Ben-Shachar et al., 2009). These studies produced few validated targets and the results often suffer from a lack of reproducibility. For this reason, we presented here a transcriptomic study that takes advantage of high-throughput sequencing techniques on a Rett syndrome mouse model. RNA-sequencing allows deeper analysis of the transcriptional landscape of cells compared with microarray technology. Moreover, we extended our analysis to two brain regions highly affected in RTT, the hippocampus (HIP) and prefrontal cortex (PFC).

Analysis of differential gene expression revealed a high number of dysregulated gene both in HIP and PFC. We detected more down-regulated genes in HIP than up-regulated, while in PFC the ratio up/down was balanced. Our sequencing data suggest a scenario in which MeCP2 plays as a master regulator able to both promote and repress gene expression. Furthermore, we showed that this MeCP2 feature is tissue-dependent, because very few genes were either up or down-regulated in both PFC and HIP.

Due to high amount of differentially expressed genes, we performed a Gene ontology analysis and we observed that genes up and down-regulated in Mecp2-null samples were associated to different pathways. A vast majority of oxidative stress and immune system-related GO terms were present in down-regulated genes of both PFC and HIP. This is in line with recent reports indicating a potential role for oxidative stress in RTT (De Felice et al., 2012). As for the up-regulated genes, the GO analysis pointed in the direction of genes related to synaptic function such as secondary messengers ($Ca^{2+}$ and GTP) and neurotransmitter pathways. For this reason, we focused our attention on a group of up-regulated genes that were present in both PFC and HIP, the immediate-early genes (IEGs) family. Although few members of this family has already been associated with RTT (Kron et al., 2012; Su et al., 2012; Swanberg et al., 2009; Li et al., 2013; Ebert et al., 2013), the different biological models used and the heterogeneous experimental condition applied does not allow taking a clear view on the IEG involvement in RTT. Finally, we were able to confirm the up-regulation of Egr2, Nr4a1, Nr4a3 and Egr1 in the HIP and Fos, Junb, Npas4 and Fosb in both HIP and PFC of Mecp2-null mouse.

The observation of the MeCP2 binding on the IEGs promoters is consistent with the critical finding that MeCP2 binds throughout the whole genome tracking methylated cytosine (Skene et al., 2010). Intriguingly, we detected a common pattern in most of the IEGs analyzed, a de-enrichment in occupancy nearby the high-CpG-content regions that is very likely to be a consequence of unmethylated CpG islands. In resume, we propose here a role for MeCP2 in the regulation of IEGs in light of its binding upon the promoter regions of this activity-dependent class of genes. As further support for our theory, the HIP chromatin was more accessible to MNase digestion in the Mecp2-null brain suggests that loosening of chromatin is accompanied by IEGs up-regulation.

The peculiarity of this class of genes is due to its responsiveness to external stimuli. In the case of neuronal IEGs the stimulus is represented by synaptic activity. Thus, to better understand the nature of the IEGs impairment in our RTT model, neuronal activity needs to be synchronized by external means. IEGs activation

constitutes a key step for long-term alterations that are necessary for plasticity and memory formation (Loebrich and Nedivi, 2009). Defects in the normal activation patterns of IEGs lead to impairment in sensory- and experience-dependent learning mechanisms (Okuno, 2011). One of the most challenging data presented here is the increased responsiveness of Fos, Junb, Egr2, Npas4 in hippocampal neurons. Strikingly, one hour after the withdrawal of the stimulating agent, Mecp2-deficient neurons derived from hippocampi displayed the higher up-regulation in respect of wild-type cells. This observation suggests that, in absence of MeCP2, the IEGs are over-activated in hippocampal neurons. Therefore, a first view at our data may argue against them, as it is current knowledge that the overall neuronal activity is decreased in Mecp2 mutant. Our data may find support on what Zhang et al. (2008) presented on the topic. In this study they identified an ambiguous state in Mecp2-null hippocampal slice preparation. While the local spontaneous post-synaptic excitatory activity was diminished from that of wild type, the intrinsic network activity was hyper-excitable. It seems plausible that this hyper-excitation of hippocampal neurons may be due to the altered responsiveness of IEGs that we described *in vitro.*

The four IEGs has already been linked with neurorelated disease, as all of them are ubiquitously expressed transcription factor that perform critical functions in the CNS:

- EGR2 has recently been associated with transcriptional activation in cortical cognitive functions associated with attention (DeSteno et al., 2008). This is particularly interesting because these cognitive features are often impaired in autism-spectrum disorders.
- NPAS4 has been potentially implicated with schizophrenia (Lewis et al., 2005) and autism (Hussman; 2001), due to its critical involvement in maintaining a balance between excitation and inhibition by contributing to the formation of inhibitory synapses (Lin et al., 2008).
- Both FOS and JUNB are members of a large family of dimeric proteins complexes called AP-1 (activating protein 1). The main scenarios in which AP-1 members seem to play a role are memory formation and behavioral changes related to

drug exposure (Pérez-Cadahía et al., 2011). Intriguingly, the composition of the dimer seems to be the major factor in the regulation of AP-1 in the nervous system. Several studies show that Junb is a poor transactivator and may act to dampen the response to the more potent transactivators of this family (Young and Colburn, 2006).

Given our results *in vitro* and with the purpose of investigating more in depth this IEGs induction impairment in Mecp2-deficient mice, we next analyzed Fos, Junb, Egr2 and Npas4 induction *in vivo.* Kainic acid induced seizures provide a valuable tool to this aim, since the administration of this kainate receptor analog provokes a tremendous neuronal excitation, especially in the hippocampus, which in turn triggers the activation of several genes, most of them IEGs (Nedivi et al., 1993). We showed that Junb response triggered by kainic acid in Mecp2-null hippocampi was higher than that of control HIP. This represent a further confirmation of the putative role of IEGs expression defects in RTT physiopathology. To gain more insights on this topic, more efforts are needed to understand the IEGs impairment contribution to RTT, with a close look on Junb. As a final remark, although the pathways activated by forskolin and kainic acid are partially overlapping, the fact that we observed no change in Fos, Egr2 and Npas4 expression in KA-induced wild type and null HIP demonstrates that the activation of each IEGs is strictly pathway- and context-dependent.

In conclusion, this study presented evidences of a mis-regulation of immediate early genes in the nervous system of a RTT mouse model. We proposed that such impairment might be at the bottom of the synaptic dysfunction reported in RTT. Given the fundamental role of IEGs in the regulation of activity-induced transcriptional programs, future therapies should be aimed to the re-establishemnt of the proper IEGs expression in RTT.

**COMMON DISCUSSION (STUDY I and II)**

We will discuss here the common points between study I and II:

- In both lncRNAs profiling and RNA sequencing, we observed a high number of genes misregulated in the Mecp2-null brain. However, the expression changes were subtle in both studies. This is consistent with several reports (Tudor et al., 2002; Nuber et al., 2005; Urdinguio et al., 2008; Chahrour et al., 2008; Ben-Shachar et al., 2009) reinforcing the hypothesis of MeCP2 as a global transcriptional regulator.
- In wild-type animals, all the misregulated genes and lncRNAs were bound by MeCP2 at least in the promoter region. Also, the MeCP2 occupancy seems to track the methylation of CpG, explaining the reduction of MeCP2 binding upon un-methylated CGIs. This observation further supports the hypothesis by which MeCP2 would act as a global and structural regulator.
- The lack of MeCP2 provokes changes in chromatin accessibility only in a fraction of genes, suggests that the site-specific function of MeCP2 heavily depends on other factors, such as PTMs or the association with activating/repressing partners.
- Our data strongly support the classification of RTT as a synaptopathy. Together with the existing evidences of impairment in synaptic features, we provide new MeCP2 target genes with an active role in neuronal functions. The fact that Gabrr2, the AK081227 host gene, was downregulated in the Mecp2-null mouse highlights the dysfunction of the neuronal inhibitory system. Furthermore, the global impairment in IEGs expression seen in the hippocampus of a RTT mouse model is strictly connected to defects in basal inhibitory and excitatory synaptic transmission (Calfa et al., 2011; Dani et al., 2005; Nelson et al, 2008).

**STUDY III**

**An increase in MeCP2 dosage impairs neural tube formation**

The aim of this study was to gain insights on the role of MeCP2 in the early stages of embryonic development, and especially in neurogenesis. To elucidate this

topic, we set a model in which robust overexpression of human MeCP2 was achieved in the chicken embryo neural tube. In order to avoid artefacts, we previously checked the expression of chicken MeCP2 and found that it is expressed in a wide window of developmental stage. Additionally, we reported evidences of similar function between chicken and mammalian MeCP2, since the nuclear localization and the high degree of conservation of the methyl-CpG binding among species (Weitzel et al., 1997).

The main point of our study is that MeCP2 overexpression causes neuroblasts to slow down proliferation, and that most of these MeCP2-overexpressing neuroblasts die before they can reach terminal differentiation. Ectopic localization of differentiated neurons and reduced levels of polarity markers indicate that overexpression of MeCP2 alone does not control the changes in polarity and migration that accompany neuronal differentiation. Our data agree with a report showing that MeCP2 overexpression in neural precursor cells (NPCs) promotes neuronal differentiation in adult mice (Tsujimura et al., 2009). This work was based on the injection of embryo-derived NPCs in the brain or spinal cord of adult mice. Here, we provided a more reliable study in which MeCP2 ectopic expression has been induced in the chick neural tube without the need to deliver exogenous cells to the embryos.

Remarkably, neural cells die as a consequence of MeCP2 overexpression, probably because they lack the additional spatial-temporal signals necessary for proper progression of neurogenesis. Consistent with this hypothesis a previous study reported abnormally high levels of cell death in different in vitro systems overexpressing MeCP2, relative to wild-type cells (Bracaglia et al., 2009; Dastidar et al., 2012). Bracaglia et al. also reported that this pro-apoptotic effect disappears when the Rett syndrome-associated MeCP2-R106W mutant, which is unable to bind to methylated DNA, is expressed—thereby implying that the MBD domain is essential for MeCP2-induced apoptosis.

It is noteworthy that *Xenopus laevis* MeCP2 was shown to regulate the number of neural precursor cells in the differentiating neuroectoderm of early *Xenopus*

embryos (Stancheva et al., 2003). In the absence of MeCP2 protein, the expression of *Xenopus* Hairy2a (a member of the Hes family of proteins, which are regulated by the Notch/Delta signaling pathway) was enhanced in embryos, which resulted in a lower number of differentiated neurons. Our results, together with the aforementioned study, highlight the importance of MeCP2 dosage, as both knock-out and overexpression of this protein results in a reduced number of differentiated neurons. In our case, reduction in total number of cells is not due only to apoptosis but in addition there is a proliferation problem.

This is the first study that investigates the consequences of MeCP2 gain-of-function in the nervous system of an in-vivo model in the early stages of development. In particular, we introduce the novel idea that high expression of MeCP2 in mitotic cells leads to anti-proliferative and apoptotic effects. Several cases of increased *MECP2* copy number have been reported in male patients with progressive neurodevelopmental delay phenotype (Friez et al., 2006; Lugtenberg et al., 2006; Meins et al., 2005; Van Esch et al., 2005). Interestingly, a male patient with triplication of the *MECP2* locus was described to have an even worse early–onset neurological phenotype at 3 months of age (del Gaudio et al., 2006), suggesting that the severity of the MeCP2 overexpression phenotype is proportional to the copy number increase. In order to reinforce this hypothesis, researchers have studied a mouse model expressing seven times the wild-type levels of MeCP2 protein, reporting that it had died by 3 weeks of age (Collins et al., 2004). Although the levels of MeCP2 expression induced in the chicken neural tubes that we have described in the present study are not fully representative of the situation in patients with *MECP2*-related disorders, our results are in line with lack and gain of function studies that elucidate the importance of correct gene dosage in neuronal development and disorders.

# CONCLUSIONS

Based on the findings of this PhD thesis, we can conclude:

**STUDY I**

- 701 lncRNAs are differentially expressed between wild-type and Mecp2-null brain.
- Two up-regulated lncRNAs, AK081227 and AK087060, are directly bound by MeCP2 at the promoter.
- The up-regulation of the lncRNA AK081227 in Mecp2 knock-out mice is associated with a down-regulation of its host gene Gabrr2 in four brain regions.
- The up-regulation of AK087060 is correlated with an increase in the expression of its host gene Arhgef26 in the four studied brain regions.

**STUDY II**

**RNA-sequencing of a Rett syndrome mouse model reveals global impairment of immediate-early genes expression**

- 1049 and 1154 transcripts are differentially expressed in hippocampus and prefrontal cortex, respectively, by performing RNA-seq.
- The immediate early genes (IEGs) Fos, Junb, Egr2, Nr4a1, Npas4, Fosb and Egr1 are up-regulated in the HIP. Furthermore, Fos, Junb, Npas4 and Fosb are up-regulated also in the PFC.
- In both PFC and HIP wild-type brain, the occupancy of MeCP2 upon the regions associated with high CpG content of Fos, Junb, Nr4a1, Npas4, Fosb and Egr1 promoters is reduced. In addition, Fos, Junb and Npas4 promoters are more accessible to MNase digestion in the HIP of Mecp2-null mice.
- Four IEGs (Fos, Junb, Egr2, Npas4) display an altered expression pattern in Mecp2-null cultured neurons treated with forskolin.

- Junb expression is significantly increased in the hippocampus of Mecp2-null animals treated with kainic acid, when compared to treated wild type mice.

**STUDY III**

**An increase in MeCP2 dosage impairs neural tube formation**

- Chicken MeCP2 (cMECP2) transcript and protein are expressed in a wide window of developmental stages.
- The overexpression of MeCP2 in the neural tube of chicken embryos provokes an overall decrease in the number of proliferating BrdU-positive cells. In addition, normal H3S10p pattern along the lumen is disrupted upon MeCP2 overexpression.
- MeCP2 high-dosage causes a clear decrease in the amounts of differentiated neuronal population located at the mantle zone. Moreover, MeCP2 overexpression leads to a decrease of a neuroepithelial polarity marker such as N-cadherin.
- Apoptosis is enhanced in MeCP2-overexpressing neural tubes

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# ANNEX I

RESEARCH PAPER

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# Dysregulation of the long non-coding RNA transcriptome in a Rett syndrome mouse model

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**Keywords:** non-coding RNA, Rett syndrome, Mecp2, mice, chromatin immunoprecipitation

Mecp2 is a transcriptional repressor protein that is mutated in Rett syndrome, a neurodevelopmental disorder that is the second most common cause of mental retardation in women. It has been shown that the loss of the Mecp2 protein in Rett syndrome cells alters the transcriptional silencing of coding genes and microRNAs. Herein, we have studied the impact of Mecp2 impairment in a Rett syndrome mouse model on the global transcriptional patterns of long non-coding RNAs (lncRNAs). Using a microarray platform that assesses 41,232 unique lncRNA transcripts, we have identified the aberrant lncRNA transcriptome that is present in the brain of Rett syndrome mice. The study of the most relevant lncRNAs altered in the assay highlighted the upregulation of the AK081227 and AK087060 transcripts in Mecp2-null mice brains. Chromatin immunoprecipitation demonstrated the Mecp2 occupancy in the 5'-end genomic loci of the described lncRNAs and its absence in Rett syndrome mice. Most importantly, we were able to show that the overexpression of AK081227 mediated by the Mecp2 loss was associated with the downregulation of its host coding protein gene, the gamma-aminobutyric acid receptor subunit Rho 2 (Gabrr2). Overall, our findings indicate that the transcriptional dysregulation of lncRNAs upon Mecp2 loss contributes to the neurological phenotype of Rett syndrome and highlights the complex interaction between ncRNAs and coding-RNAs.



## Introduction

Epigenetic regulation, whose mechanisms involve chromatin remodeling by many different enzymes/complexes/molecular scaffolds that targets DNA methylation and histone code modifications,[1] is also mediated by non-coding RNAs (ncRNAs).[2-5] ncRNAs include the most studied member microRNAs, but also include long non-coding RNAs (lncRNAs). LncRNAs are transcripts of at least 200 nucleotides transcribed from all over the genome, including intergenic regions, antisense, overlapping or intronic to protein-coding genes.[2-6] LncRNAs have a broad range of functions, such as enhancer-like activity,[7] establishment of repressive chromatin in genomic regions[8] or entire chromosomes,[9] intronic antisense transcripts capable of binding to histone modifiers thereby regulating the transcriptional output of the host gene,[6] alternative splicing and other post-transcriptional RNA modifications[10] that determine the activity of our genome.

In this regard, the fine tuning of gene expression is critical in the human central nervous system (CNS). It is widely known that higher order cognitive and behavioral function are a CNS prerogative sustained by huge and intricate cell networks acting both locally and globally.[11] One main aim of modern neurobiology is the ultimate understanding of the transcriptional programs that give rise to distinct neural networks, which are, in turn, formed by several neuronal and glial subtypes. Herein, alterations in the epigenetic modulation of gene expression could lead to several neurodevelopmental disorders, the most evident example represented by Rett syndrome (RTT).[12,13] RTT (OMIM 312750) is an X-linked neurodevelopmental disorder that affects females at a frequency of 1:10,000 live births. The girls appear normal until 6–18 mo of age, when they lose their acquired skills and develop autistic features and mental retardation along with the typical stereotypic hand movement.[14] Rett syndrome is the second leading cause of mental retardation in women after Down syndrome. Nearly 95% of typical RTT is due to mutations in the gene encoding the transcriptional regulator Methyl-CpG binding protein 2 (MECP2 in humans; Mecp2 in mice).[15,16] Mecp2 is a basic nuclear protein that acts mainly as a transcriptional repressor, binding preferentially to methylated DNA sequences.[17,18] In human cancer cells, the binding of *MECP2* to the hypermethylated CpG islands of tumor suppressor genes[19-21] and microRNAs[22] is associated with transcriptional silencing. The loss of MECP2 in Rett syndrome patients and mice models is associated with a dysregulated pattern of coding-gene[23-28] and microRNA expression.[29,30]

Herein, we investigate the role of lncRNAs in the physiopathology of Rett syndrome by comparing the transcriptome profiles of Mecp2-null mice brains[31] vs. wild-type animals. In a

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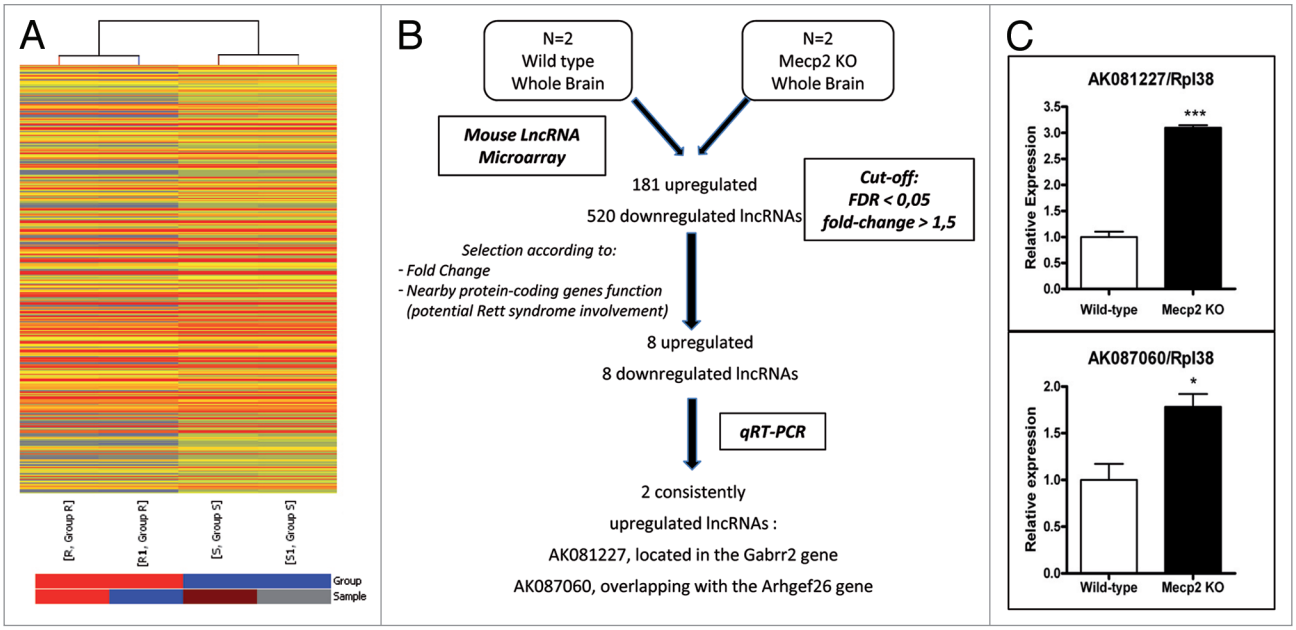


**Figure 1.** Dysregulation of the lncRNAs transcriptome in Mecp2-null mice brains. (**A**) Hierarchical clustering of the lncRNA microarray expression data shows distinguishable gene expression profiles between wild-type (S, S1) and Mecp2-null (R, R1) mice brains. (**B**) Flow-chart used to identify candidate misregulated lncRNAs in the RTT mouse model. (**C**) qRT-PCR of AK081227 and AK087060 normalized with RPL38. Error bars represent SE. Five biological replicates were used for each condition. * $P < 0.05$ *** $P < 0.001$.

similar pattern to Rett syndrome patients, Mecp2-null mice do not show the RTT-like phenotype just after birth, but 4–5 wk later,[31] resembling the natural history of the disease in humans. The identified lncRNAs dysregulated in the Rett syndrome mice models provide important clues to understand the neurological phenotype of the disorder; furthermore, they illustrate the imbricate relationship between coding and ncRNA transcripts.

## Results

**Identification of candidate lncRNAs misregulated in RTT.** We utilized a well-established mouse model of RTT that mimics the human disease[31] to perform lncRNA microarray expression analyses using pairs of wild-type (WT) and Mecp2-null (KO) 9-wk animals. RNA was extracted from total brain and hybridized to an lncRNA microarray platform. The Mouse long non-coding RNA array (Arraystar) contains more than 41,232 probes representing unique lncRNAs. The probes were designed according to NCBI RefSeq, UCSC, RNAdb2.0, NRED, Fantom3.0 ,and UCRs annotations. Two biological replicates were used for each sample and condition. Repeat sequences and ncRNAs shorter than 200 bp are not represented in the microarray. The lncRNA expression data obtained are freely available at the Gene Expression Omnibus database: www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=fvavvcewquegcfc&acc=GSE43689.

Unsupervised hierarchical clustering of the lncRNA microarray expression data indicates that the Mecp2-null mouse brains show a distinct pattern of lncRNA transcription in comparison to the wild-type animals (**Fig. 1A**). Among the 41,232 lncRNAs transcripts included in the microarray, we found 701 (1.7%) lncRNAs (**Table S1**) that had a different expression pattern in WT and Mecp2-null brain samples with a score of < 0.05 in the false discovery rate (FDR) test and a > 1.5-fold expression change (**Fig. 1B**). Among these significantly altered lncRNAs, overall downregulation of transcripts was the most common feature (520 of 701, 74%), while upregulation occurred in the remaining 26% (181 of 701) (**Fig. 1B**).

**Validation of lncRNA candidates in RTT.** For practical purposes to reduce the lncRNAs to be further studied and to enrich those potentially involved in RTT, we selected candidate transcripts with a fold expression change > 2 that were associated with an annotated protein-coding gene where a biological function in neurons or glial cells have been proposed according to the scientific literature and through GO term enrichment on "negative regulation of neuron differentiation" ($P = 0.008$), "nerve development" ($P = 0.007$), "dendrite regeneration" ($P = 0.004$), "regulation of nervous system development" ($P < 0.001$), "regulation of excitatory postsynaptic membrane potential" ($P < 0.001$) and "positive regulation of synapse assembly" ($P < 0.001$). Using the above described criteria, we produced a short list of 16 lncRNAs: eight upregulated and eight downregulated in Mecp2-null mouse brains (**Table S2**). We then extracted brain RNA from five new pairs of wild-type and Mecp2-null 9-wk animals and analyzed the expression level of each one of the 16 candidate lncRNAs. The analyses were performed using quantitative PCR on reverse-transcribed RNA, three biological replicates per sample were developed and the statistical significance was assessed by two-tailed t-tests. Although for most cases, the expression analysis of each single candidate lncRNA matched the microarray data (data not shown), two lncRNAs exhibited a statistically significant difference: AK081227 (two-tailed t-test $P < 0.0001$) and AK087060 (two-tailed t-test p <

0.0248), both of them upregulated in Mecp2-null brain mouse (**Fig. 1C**).

**Mecp2 binding to the 5'-end genomic loci of the lncRNAs AK081227 and AK087060.** One important function of Mecp2 is to act as a transcriptional repressor that binds to the promoters located in the 5'-end genomic region of its target coding genes[19-21] and microRNAs.[22,29,30] Thus, we speculated whether Mecp2 was directly involved in regulating AK087060 and AK081227 gene expression by analyzing its capability of binding to their promoters. To this end, we performed chromatin immunoprecipitation (ChIP) experiments with formaldehyde-cross-linked nuclear extracts from WT and Mecp2 KO brains followed by semi-quantitative PCR. We immunoprecipitated the cross-linked chromatin with a histone H3 antibody to serve as an internal control of the ChIP assay (**Fig. 2A–C**). We also ensured that the Mecp2 antibody was specific for the protein and reliable in the ChIP assay by checking Mecp2 occupancy on the promoter of Xist, a previously reported lncRNA involved in X-inactivation whose promoter is occupied by Mecp2 in male wild-type mice (where Xist is silenced in the only X chromosome).[32] As expected, we found Mecp2 bound to the Xist promoter in wild-type, but not in Mecp2 KO mouse brain chromatin (**Fig. 2A**).

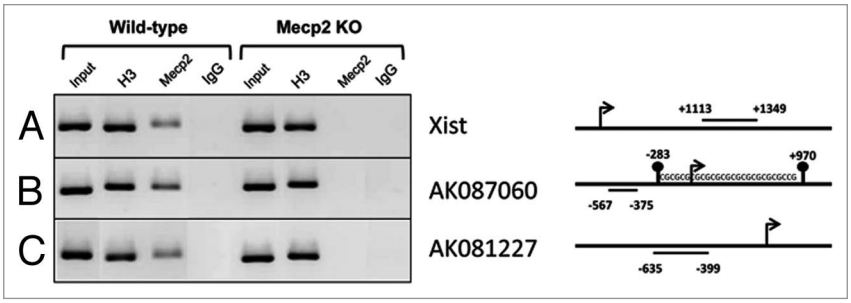


**Figure 2.** Mecp2 is bound to the 5'-end genomic loci of the lncRNAs AK081227 and AK087060 in wild-type mouse brain and lost in Mecp2-null mice. Semiquantitative chromatin immunoprecitation assay for a fraction of the total DNA (5%) (input), an antibody anti-H3 total (IP control), an antibody anti-Mecp2 and a negative control (mouse IgG) on the 5'-end genomic loci of the lncRNAs Xist (**A**), AK081227 (**B**), and AK087060 (**C**). The regions amplified by PCR were represented as straight lines.

Once these controls were established, we proceeded to address the Mecp2 occupancy for the AK081227 and AK087060 5'-end genomic loci. The PCR primers for the ChIP assay were designed in a region of 500 bp upstream to the transcription start site (TSS) within the corresponding lncRNAs proximal promoters. Importantly, we found that AK081227 and AK087060 5'-end loci were occupied by the Mecp2 protein in wild-type mouse brains, while the *Mecp2* protein was absent from the 5'-end of the two lncRNAs in the Mecp2-null mouse brains that overexpress the AK087060 and AK081227 transcripts (**Fig. 2B and C**). DNA methylation differences in the two studied lncRNAs 5'-ends were studied by bisulfite genomic sequencing of multiple clones and were not detected in the brain of wild-type vs. Mecp2 KO samples (data not shown). These data support the role of Mecp2 as a transcriptional repressor of the transcripts located in the vicinity of its binding sites to DNA, herein regulating the expression of the lncRNAs AK081227 and AK087060.

**Upregulation of the lncRNA AK081227 in RTT is associated with downregulation of its host gene gamma-aminobutyric acid receptor subunit rho 2.** One of the main challenges in research with lncRNAs is the identification of a particular molecular or cellular function.[6] One possibility is that lncRNAs act locally regulating the expression levels of neighboring RNA transcripts.[6] Herein, we have studied the possible effect of AK081227 and AK087060 in the activity of their associated protein-coding genes. To address this issue, we have analyzed the expression levels of the two identified lncRNAs by qRT-PCR in comparison to their corresponding host genes in four functional relevant brain regions (frontal cortex, hypothalamus, thalamus and cerebellum) from new pairs of wild-type and Mecp2-null 9-wk animals.

AK087060 is transcribed from 225 bp downstream of the first exon of the Arhgef26 gene (**Fig. 3A**). The *Arhgef26* protein is a Rho guanine nucleotide exchange factor (GEF) that has a role in the actin-driven endocytic process known as macropinocytosis that contributes to repulsive turning in the axons of neurons[33] and retinal cone growth.[34] Thus, a role for this protein in a neurological disorder, such as Rett syndrome, could be invoked. In this regard, in humans, the equivalent RNA transcript is originated 357 bp downstream of the first exon of the ARHGEF26 promoter with a 79% nucleotide homology to AK087060. We found that the observed upregulation of AK087060 in Mecp2 KO mice had a statistical association with an increase in the expression levels of its host gene Arhgef26 in the four studied brain regions (Pearson's correlation test = 0.41, $P = 0.08$) (**Fig. 3B**). Since this particular lncRNA is transcribed from just 225 bps downstream of the coding gene, common promoter regulatory mechanism for AK087060 and Arhgef26 seems an unlikely scenario. In addition, AK087060 could be a promoter-associated lncRNA.[6] These lncRNAs are transcribed around the TSS proximal region and are able to reclute other regulatory factors[6] that impact on the expression of the associated-coding gene, in this case Arhgef26.

The lncRNA/mRNA equation is completely different for AK081227. This lncRNA is transcribed from an intronic region of the GABA receptor subunit rho-2 (Gabrr2) gene (**Fig. 3C**). Many autistic and neurodevelopmental disorders, including Rett syndrome, have been linked to dysfunction in particular aspects of GABAergic inhibitory neurotransmission in the brain.[35,36] Most importantly, the expression of another GABA receptor subunit member (GABRB3) is reduced in Rett syndrome.[24,37] Thus, the host gene of the identified lncRNA is a likely candidate to be altered in Rett syndrome. In this regard, in humans, the equivalent lncRNA has a 30% nucleotide homology to AK081227. Herein, we found that the upregulation of AK081227 in Mecp2 KO mice was statistically associated with a downregulation of the expression levels of its host gene Gabrr2 in the four studied brain regions (Pearson's correlation test = 0.44, $P = 0.06$) (**Fig. 3D**). Thus, the expression of the lncRNA AK081227 might act locally to interfere in the mRNA transcript levels of Gabrr2 and contribute to the neurological phenotype of Rett syndrome.

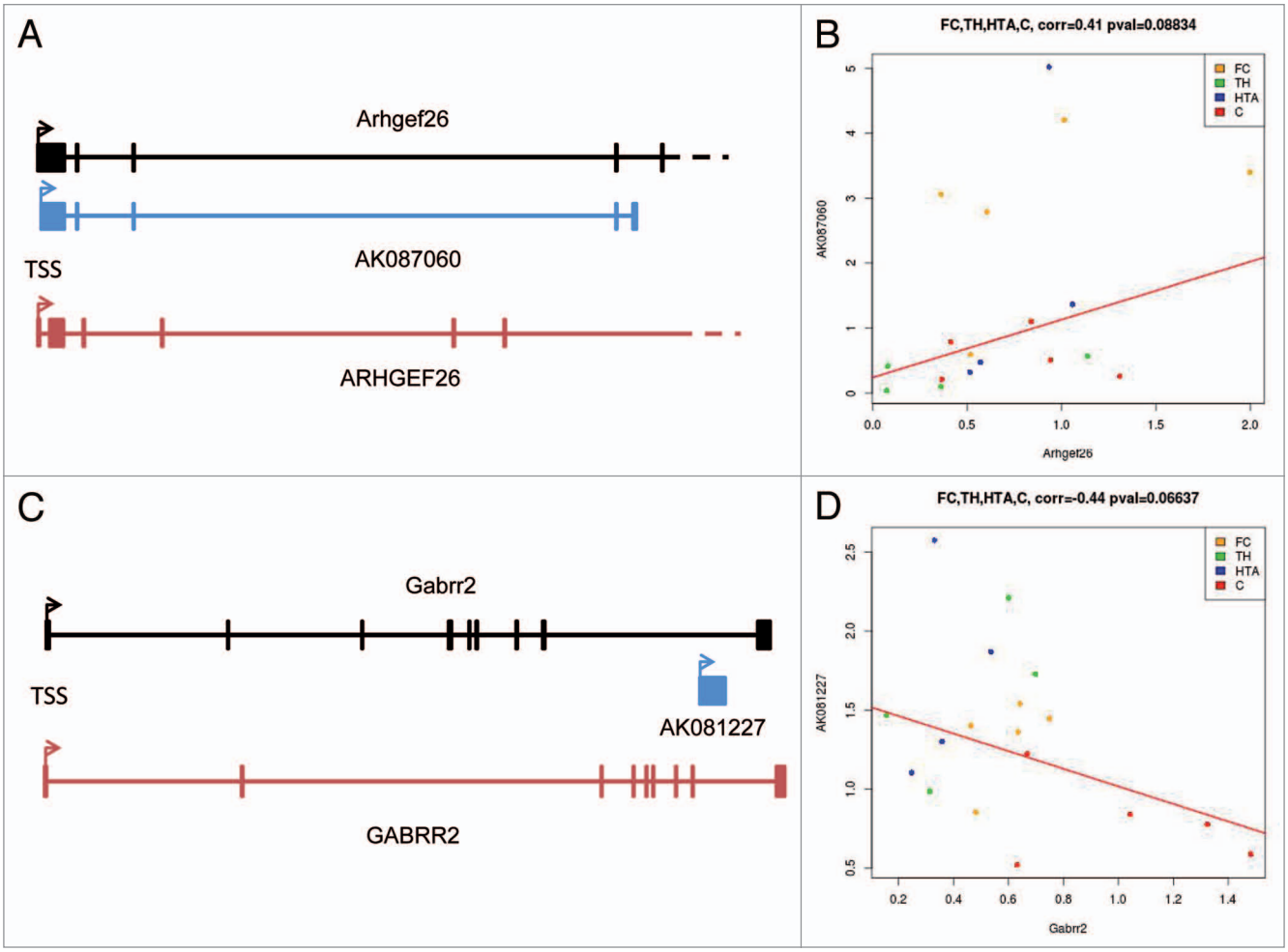


**Figure 3.** Genomic context and associated coding-genes for the identified lncRNAs. (**A**) AK087060 originates 200 bps downstream from the transcription start site of the mRNA for the Arhgef26 gene. Genomic organization of the human gene is also shown (ARHGEF26). (**B**) Upregulation of AK087060 in Mecp2 KO mice is associated with an increase in the expression levels of the host gene in the four studied brain regions (Pearson's correlation test = 0.41, $P = 0.08$). (**C**) AK081227 is transcribed from the last intron of the Gabrr2 gene. Genomic organization of the human gene is also shown (GABRR2). (**D**) Upregulation of AK087060 in Mecp2 KO mice is associated with a downregulation in the expression levels of the host gene in the four studied brain regions (Pearson's correlation test = 0.44, $P = 0.06$). Orange dots, frontal cortex; green dots, thalamus; blue dots, hipothalamus; red dots, cerebellum.



In this regard, we have also recently identified another intronic lncRNA that finely regulates the expression of the host gene[38] in what might become a common theme in the complex interaction between ncRNAs and coding-RNAs.

## Discussion

The current report represents the first lncRNA profiling in Rett syndrome, data that can be used to identify the transcripts from this class that are regulated by Mecp2 and that could explain the physiopathology of the disease. We observed a distinct lncRNA transcriptome between the brain of wild-type and Mecp2-null mice, where the expression of 701 lncRNAs was significantly different. Due to the limited information available about the function of each lncRNA, we selected only those whose higher fold expression change overlapped with protein-coding genes whose function was related to neurons or glia cells for further validation and study. In this last setting, the most relevant observation was that the release of the transcriptional silencing of the lncRNA AK081227 was associated with a downregulation of its host gene, Gabrr2. The importance of lncRNAs as *cis*-acting regulators is rapidly being recognized.[6] LncRNAs can guide chromatin change in *cis* in a co-transcriptional manner (tethered by RNA polymerase) or as a complementary target for small regulatory RNAs. Regarding our case, there is increasing knowledge that lncRNAs overlapping with introns of protein-coding genes, whether originating from splicing or produced by independent transcriptional units, may recruit several classes of coactivator (e.g., trithorax group proteins)[39] or corepressor (polycomb-group members like histone methyltransferase EZH2)[38] complexes and, therefore, act as guides for the establishment of activating or repressive histone marks all over the host gene. In this regard, the exact mechanisms regulating the inverse expression levels of the non-coding (AK081227) and coding (Gabrr2) transcripts are currently under investigation and should be the focus of further developments in this area.

The observation that the intronic lncRNA AK081227 upregulation in the Rett syndrome brain is associated with the depletion of its host coding gene Gabrr2 can have a relevant impact in the understanding of the described neurological disorder. At the physiological level, post-mortem analysis of RTT brains showed altered levels of neurotransmitters such as glutamate and biogenic amines as well as changes in the abundance of some neurotransmitter receptors. In mouse models of RTT, analysis of spontaneous miniature excitatory and inhibitory postsynaptic currents indicated a shift in the excitatory/inhibitory balance, with increased excitatory and decreased inhibitory neurotransmission in the hippocampus and cortex.[40] Consistently, studies of Mecp2-knockout mouse models revealed abnormalities in long-term potentiation (LTP) and impaired synaptic plasticity.[41] Recent findings underline that Mecp2 deficiency in GABAergic neurons recapitulates most of the features displayed by Mecp2-null mice, including altered synaptic activity and plasticity.[42] Since GABA is the major inhibitory neurotransmitter in the brain, most of the synaptic defects seen in RTT could be a direct consequence of the Mecp2 loss in GABA neurons. Additionally, genes necessary for GABAergic function, like Dlx5 and GABRB3, have already been associated with RTT.[24,37] Gabrr2, as a member of GABA(C) receptor class, has been reported to be expressed in various brain regions.[43,44] The fact that we found Gabrr2 downregulated in frontal cortex is consistent with the dysfunction of GABAergic signaling seen in the frontal cortex of RTT patients.[45] Moreover, Gabrr2 was also downregulated in thalamus and hypothalamus, with the latter being one of the most affected region in RTT. As for the thalamus, a recent study showed that Mecp2 regulates GABAergic synapses differentially in excitatory and inhibitory neurons in the thalamus.[46]

Overall, our data provides the first hint that the lack of the transcriptional regulatory effect of Mecp2 in Rett syndrome leads to a dysregulation of lncRNA expression in the affected mice brain. Future data mining of the obtained lncRNA transcriptomes deposited in the public genomic databases could provide further clues about the impact of the altered lncRNAs on other transcripts; however, the neuropathological relevance of the inverse association between AK081227 (the intronic lncRNA) and Gabrr2 (the host coding-gene) already provides proof of principle for the existence of disrupted *cis*-regulated mechanisms in the disease.

## Materials and Methods

**Animal model.** Four-week-old B6.129P2(C)-*Mecp2tm.11Bird*/J (stock number: 003890) heterozygous females (*Mecp2*+/–) were acquired from the Jackson Laboratory. In brief, the mutant strain was generated by replacing exons 3 and 4 of *Mecp2* in embryonic stem cells with the same exons flanked by loxP sites. Homozygous *Mecp2* lox/lox females were mated with mice with ubiquitous Cre expression to bring about gene disruption. The offspring from the crosses of *Mecp2*+/– females with C57BL/6J males were genotyped by PCR. Mice were kept under specific pathogen-free conditions in accordance with the recommendations of the Federation of European Laboratory Animal Science Associations. Lighting conditions (lights on from 08:00–20:00 h) and temperature (22°C) were kept constant. Animals were allowed ad libitum access to food and water and were inspected every day. Tissue samples were obtained from hemizygous *Mecp2*-null males (*Mecp2*-/y, KO) and their wild-type (WT) littermates after establishing RTT-like symptoms in the defective animals (at about 8–10 wk of age). Mice were euthanized in accordance with the Guidelines for Humane Endpoints for Animals Used in Biomedical Research. Tissues were frozen on dry ice immediately after removal and stored at –80 °C until use.

**RNA extraction.** To extract RNA, frozen tissues were ground into powder with mortar and pestle and resuspended in Trizol reagent (Life technologies). The RNA purification was performed on the RNA-containing aqueous phase with RNeasy mini kit (Qiagen). After elution with RNase-free water and treatment with turbo DNase (Ambion), the RNA is ready for all kinds of applications. Quantification and quality check were performed with Nanodrop and Agilent 2100 Bioanalyzer (Agilent Technologies), respectively.

**Sample preparation and microarray analysis.** Briefly, 1 ug of total RNA was labeled with Cy3 using Agilent Quick Amp Labeling Kit and microarray hybridization was performed at 65 °C for 17 h in Agilent's SureHyb Hybridization Chambers. After being washed in an ozone-free environment, the slides were scanned using the Agilent DNA microarray scanner (part number G2505B). Data was extracted using Agilent Feature Extraction Software (version 10.5.1.1) and normalization was performed using the Agilent FE one-color scenario (mainly median normalization). Finally, four samples were hybridized, two biological replicates for each condition (Mecp2 KO mice and their wild-type littermates). False discovery rate (FDR) test was performed on lncRNA that pass the cut-off (t test < 0.05, fold change > 1.5).

**Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR).** Two ug of RNA for each sample were retro-transcribed to cDNA using random hexamers from ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis kit (Life Technologies). A negative sample (no ThermoScript enzyme) was performed to exclude DNA contamination. Primers for the reaction were designed according to Primer3 software and tested with a cDNA serial dilution to check amplification linearity and unique amplification product. Ppia and Rpl38 were used as house-keeping genes for normalization. 50 ng cDNA were used for each PCR reaction together with 5 ul 2X SYBR green PCR master mix (Life technologies), 250 nM of each primer and water up to 10 ul. Mouse primers sequences used were the following: Ppia Forward 5'-CAA ATG CTG GAC CAA ACA CAA-3' Reverse 5'-GTT CAT GCC TTC TTT CAC CTT-3'; Rpl38 Forward 5'-AGG ATG CCA AGT CTG TCA AGA-3' Reverse 5'-TCC TTG TCT GTG ATA ACC AGG G-3'; AK081227 Forward 5'-TCG GTC AGT GCA TTT GGG CTG T-3' Reverse 5'-TCG GTC CAC TGT CTC AGG AGT GC-3'; Gabrr2 Forward 5'-CAA GGG GAA CGA CGT GCG GA-3'; AK087060, Forward 5'-GAA CGA CGT GCG GA-3'.TGT ATG GCG TCC ATC TCT TCG G-3' and Reverse 5'-GTC CTC CTC TCT GCA ATT GCT TAG-3'; Arhgef26, Forward 5'-GGC CCT TGA TAT CGA CTC TGA TGA-3' and 5'-CTT

TTC ACC GCG GAG AGC TGG-3'. Quantitative PCR was performed for 40 cycles on an ABI 7900HT sequence detection system (Life Technologies) under the thermal cycling conditions recommended by the manufacturer.

**Chromatin immunoprecipitation in brain tissues.** Frozen wild-type and Mecp2 KO male brains were reduced to powder with mortar and pestle. The pulverized brain tissues were cross-linked with 1% formaldehyde for 8 min and the reaction was blocked by adding glycine to a final concentration of 0.125 M. After washing two times with ice-cold PBS, cell pellets were resuspended in cell lysis buffer (HEPES 5 mM, KCl 85 mM, NP40 0.5% pH 8.0) supplemented with protease inhibitor cocktail (Complete EDTA-free, Roche) and the lysate was homogenized with a douncer to facilitate cell membrane break. The nuclear pellet was then resuspended in Nuclei lysis buffer (TRIS-HCl 50 mM, EDTA 10 mM, SDS 1% pH 8.1) and subsequently sonicated with Bioruptor (Diagenode) for 30 min (30 sec ON, 30 sec OFF cycles). The chromatin size of the fragments obtained was 150–400 bp. Samples were diluted with Dilution buffer (SDS 0.01%, Triton X-100 1.1%, EDTA 1.2 mM, NaCl 165 mM, TRIS-HCl 16.7 mM pH 8.1). Magnetic beads were used for the pre-clearing of diluted chromatin (overnight at 4 °C) and for incubation with anti-total H3 (ab1791, abcam) and anti-Mecp2 (m9317, sigma). Non-related mouse IgG antibody (12–371, Millipore) was used as a negative control. The Beads-Antibody complexes were then incubated with pre-cleared chromatin for 2 h at 4°C in agitation. The immune-complexes were washed: twice with low salt Buffer (TRIS-HCl 50 mM pH 8.0, NaCl 150 mM, SDS 0.1%, NP-40 1%, EDTA 1 mM, Deoxicolate Na 0.5%), twice with high Salt Buffer (TRIS-HCl 50 mM pH 8.0, NaCl 500 mM, SDS 0.1%, NP-40 1%, EDTA 1 mM, Deoxicolate Na 0.5%), twice with LiCl Buffer (TRIS-HCl 50 mM pH 8.0, LiCl 250 mM, SDS 0.1%, NP-40 1%, EDTA 1 mM, Deoxicolate Na 0.5%) and twice with TE Buffer (TRIS-HCl 10 mM pH 8.0, EDTA 0.25 mM). Cross-linked chromatin was then eluted from the magnetic beads by adding elution Buffer ($NaHCO_3$ 100 mM, SDS 1%). Samples were de-crosslinked overnight at 65 °C and incubated with Proteinase K at 50 ug/ml final concentration for 1 h. Finally, DNA was purified with PCR purification kit (Qiagen). The primers used for ChIP analysis were the following: AK081227 promoter, Forward 5'-TTG TCC CCA CTA AGAG ACA G-3' and Reverse 5'-CCT GTA CTC TGC TAT GCT TAC TC-3'; AK087060, Forward 5'-CTG TGT GAC TTT CAA ACA TAC AG-3' and Reverse 5'-CTT CAC TGG GCC ACT TGT G-3'; Xist, Forward 5'-CCT GTA CGA CCT AAA TGT CC-3' and Reverse 5'-GTA TTA GTG TGC GGT GTT GC-3'.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### Supplemental Material

Supplemental material may be found here:
http://www.landesbioscience.com/journals/rnabiology/article/24286/

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# ANNEX II

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# An increase in MECP2 dosage impairs neural tube formation

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ABSTRACT

Epigenetic mechanisms are fundamental for shaping the activity of the central nervous system (CNS). Methyl-CpG binding protein 2 (MECP2) acts as a bridge between methylated DNA and transcriptional effectors responsible for differentiation programs in neurons. The importance of MECP2 dosage in CNS is evident in Rett Syndrome and MECP2 duplication syndrome, which are neurodevelopmental diseases caused by loss-of-function mutations or duplication of the MECP2 gene, respectively. Although many studies have been performed on Rett syndrome models, little is known about the effects of an increase in MECP2 dosage. Herein, we demonstrate that MECP2 overexpression affects neural tube formation, leading to a decrease in neuroblast proliferation in the neural tube ventricular zone. Furthermore, an increase in MECP2 dose provokes premature differentiation of neural precursors accompanied by greater cell death, resulting in a loss of neuronal populations. Overall, our data indicate that correct MECP2 expression levels are required for proper nervous system development.



## Introduction

During development, mitotically active precursors located in the neuroepithelium give rise to specialized neuronal and glial cells that define the adult nervous system. To maintain the brain's complexity, neurons originating from the neural tube undergo mitotic quiescence. Therefore, neuronal differentiation encompasses an elaborate developmental program in which neurogenic and antiproliferative signals work together to guarantee the differentiated state. This developmental step is mediated by genetic and epigenetic factors. Among the latter, chromatin remodelers (Clapier and Cairns, 2009), histone variants (Kamakaka and Biggins, 2005), histone post-translational modifications (Kouzarides, 2007) and DNA methylation (Miranda and Jones, 2007) are strongly involved in regulating the proliferation and differentiation of neural precursor cells. The importance of this regulation is highlighted by several neurological disorders caused by mutations in epigenetic genes, such as Rett syndrome (RTT), alpha thalassemia/mental retardation X-linked syndrome, Rubinstein–Taybi syndrome and Coffin–Lowry syndrome (Urdinguio et al., 2009).

Among the epigenetic regulators of the brain, methyl-CpG-binding proteins are responsible for reading the methylation code of DNA and therefore, for regulating gene transcription (Klose and Bird, 2006). In fact, a mutation in the best-known protein of this family, MECP2 (methyl-CpG binding protein 2), is responsible for RTT (Amir et al., 1999). MECP2 is a basic nuclear protein that acts mainly as a transcriptional repressor, preferentially binding to methylated DNA sequences (Klose et al., 2005; Lewis et al., 1992). Although MECP2 is widely expressed, MECP2 levels are highest in the brain, principally in mature postmigratory neurons (Jung et al., 2003). MECP2 protein levels are low during embryogenesis and increase progressively during the postnatal period of neuronal maturation (Balmer et al., 2003; Cohen et al., 2003). In addition to its necessary role in mature neuronal and glial cells, MECP2 has been implicated in neuronal specification during early embryogenesis in several species (Coverdale et al., 2004; Stancheva et al., 2003). Moreover, MECP2 has been shown to promote neuronal differentiation of neural stem cells while repressing astrocyte differentiation (Tsujimura et al., 2009).

The striking finding that MECP2 nucleotide mutations or duplications cause Rett syndrome or MECP2 duplication syndrome, respectively, suggests that careful regulation of this gene is necessary for correct brain development and function, as both overexpression and reduced expression are associated with neurodevelopmental disorders (Collins et al., 2004; del Gaudio et al., 2006). Intriguingly, a loss of MECP2 function and an increase in MECP2 dosage lead to clinically similar neurological disorders (Van Esch et al., 2005). However, although many studies have been performed on MECP2 loss-of-function models, little

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is known about the biological consequences of MECP2 overexpression in either the adult or developing brain.

Using a well-known developmental model, the chick embryo neural tube, we sought to investigate the effects of human MECP2 overexpression on the proliferating progenitor cells of neurons and glia. Here, we show that MECP2 dosage is fundamental for proper neural tube development and demonstrate that MECP2 overexpression provokes premature differentiation of proliferating progenitor cells. This ectopic differentiation leads to cell-cycle exit and cell death, ultimately resulting in decreased neuronal populations.

## Materials and methods

### *Plasmids*

The human MECP2_e1 full-length coding sequence was cloned into pCIG vector (Megason and McMahon, 2002). The vector comprises CMV enhancer and beta-actin promoter, followed by multiple cloning sites, internal ribosomal entry site (IRES) and a nuclear-localized green fluorescent protein (GFP).

### *Antibodies*

The following primary antibodies were used: anti-MECP2 (Diagenode, custom); anti-BrdU (DSHB, G3G4); anti-phosphoH3S10 (Millipore, 05–806); anti-neural β-tubulin III (Tuj1) (R&D systems, MAB1195); anti HuC/D (Life Technologies, A-21271); anti-N-cadherin (R&D systems, AF6426); anti-active Caspase 3 (BD pharmingen, 559565); anti-active Caspase 8 (Millipore, MAB10754) and anti-β-actin-peroxidase (Sigma, A3854)

### *Chick in ovo electroporation*

Eggs from White-Leghorn chickens were incubated at 38.5 °C and 70% humidity. Embryos were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1992). Chick embryos were electroporated with purified plasmid DNA at 2–3 μg/μl in $H_2O$ with 50 ng/ml of Fast Green. Plasmid DNA was injected into the lumen of HH10 neural tubes, electrodes were placed at both sides of the neural tube and finally, the embryos were electroporated by an IntracelDual Pulse (TSS-100) delivering five 50 ms square pulses of 20–25 V.

### *mRNA extraction and RT-PCR*

RNA was extracted with a Trizol reagent (Invitrogen) from dissected neural tubes according to the manufacturer's protocol. Single-stranded cDNA was synthesized with Thermoscript reverse-transcriptase and random hexamers (Life Technologies), and then subjected to PCR with the following primers: cMECP2, forward 5′-GGACCAGGAAGCTCAAAC AGC-3′ and reverse 5′-TTGGGGCTCTTGGCTTTCTTG-3′; Gapdh, forward 5′-CTGAATGGGAAGCTTACTG-3′ and reverse, 5′-CATCATACTTGGCTGG TTTC-3′.

### *Western blotting*

Neural tubes were dissected from several embryos at the same stage, pooled together and total protein was extracted with a Laemmli buffer. Equal amounts of protein (20 μg) were boiled for 10 min and β-mercaptoethanol was added to a 3% final concentration. Samples were then separated by electrophoresis on 10% SDS-PAGE gels, and transferred to nitrocellulose membranes. Membranes were blocked in 5% skim milk powder in PBS plus 0,1% Tween 20 for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies. For MECP2 (1:2000) antibody, an anti-rabbit HRP-conjugated secondary antibody (1:10,000) was used. Finally, complexes on the membrane were visualized using an ECL detection kit (GE Healthcare Life Sciences).

### *BrdU incorporation*

Bromodeoxyuridine (BrdU, 0.5 μg/μl) was injected into the chick embryo neural tube lumen 30 min before fixation. Before anti-BrdU antibody incubation (which was performed as described below), the sections were treated with HCl 2 N for 30 min and washed with NaBorate 0.1 M (pH 8.5).

### *Indirect immunofluorescence*

The collected brachial regions from embryos were fixed for 2 h at 4 °C in 4% paraformaldehyde, rinsed with PBS, soaked in a PBS 30% sucrose solution and embedded in either OCT or agarose for sectioning in a Leica Cryostat (CM 1900) or a Vibratome (VT1000). The sections were blocked at room temperature for at least 1 h in 1% BSA (in PBS with 0.1% Triton X-100) before overnight incubation with primary antibodies at 4 °C. The sections were then incubated for 1.5 h at room temperature with Alexa-conjugated goat secondary IgG antibodies (Life Technologies) and 0.1 ng/μl DAPI (Sigma). Images were captured on a Leica SP5 confocal microscope using a 40× oil-immersion objective and processed using a Fiji software. MECP2 intensity was quantified using the Fiji software as the following: each side of the neural tube (MECP2 EP and control) was selected as a region of interest (ROI) and the total intensity of all pixels for each ROI was calculated and compared.

### *Statistical analysis*

Quantitative data were expressed as mean and standard error (s.e.). Significant differences between groups were tested by Student's *t*-test.

## Results

### *Chicken MECP2 is expressed ubiquitously in the developing spinal cord*

MECP2 is present in all vertebrates and is highly conserved among mammals, while divergence between mammalian and amphibian or fish MeCP2 more extensive. However, the alignment of chicken MECP2 with mouse and human MECP2 shows that the protein is highly conserved throughout species as diverse as humans and chickens. Although cMECP2 mRNA and protein are only partially annotated, a large part of the sequence is highly similar to human MECP2. Particularly striking is the 96.8% sequence identity in a 125-amino-acid region. Significantly, the conserved region includes the methyl-CpG binding domain (MBD) (Weitzel et al., 1997) (Fig. 1a). The high degree of conservation compares well with the characterization of the MBD as an essential element for binding of MECP2 to heterochromatin as well as unmethylated four-way DNA junctions (Galvão and Thomas, 2005; Nan et al., 1996). Hence, we wondered whether cMECP2 (previously known as *ARBP*) is expressed in the chicken embryo across different developmental stages. RT-PCR analysis of HH10, 20, 23 and 26 revealed that cMECP2 is indeed expressed in chick embryo (Fig. 1b) with greater expression seen at the HH20 stage. Since transcript presence does not always correlate with protein levels, we checked cMECP2 protein by western blot. Fig. 1c shows that cMECP2 is expressed at every tested developmental stage. Although in HH10 chick embryos the neural tube is formed mainly by the ventricular zone (VZ)—an epithelium composed entirely of mitotically active, multipotent neural precursor cells—from HH14 to 15 some of these neuroblasts exit the cell cycle and migrate laterally from the ventricular zone to the mantle zone (MZ), which is formed exclusively by post-mitotic, differentiating neurons and glia (Fig. 1d). Therefore, we wondered whether the MECP2 expression was restricted to the differentiating neurons or was global. To address this issue, we collected brachial sections of HH25 embryos and stained

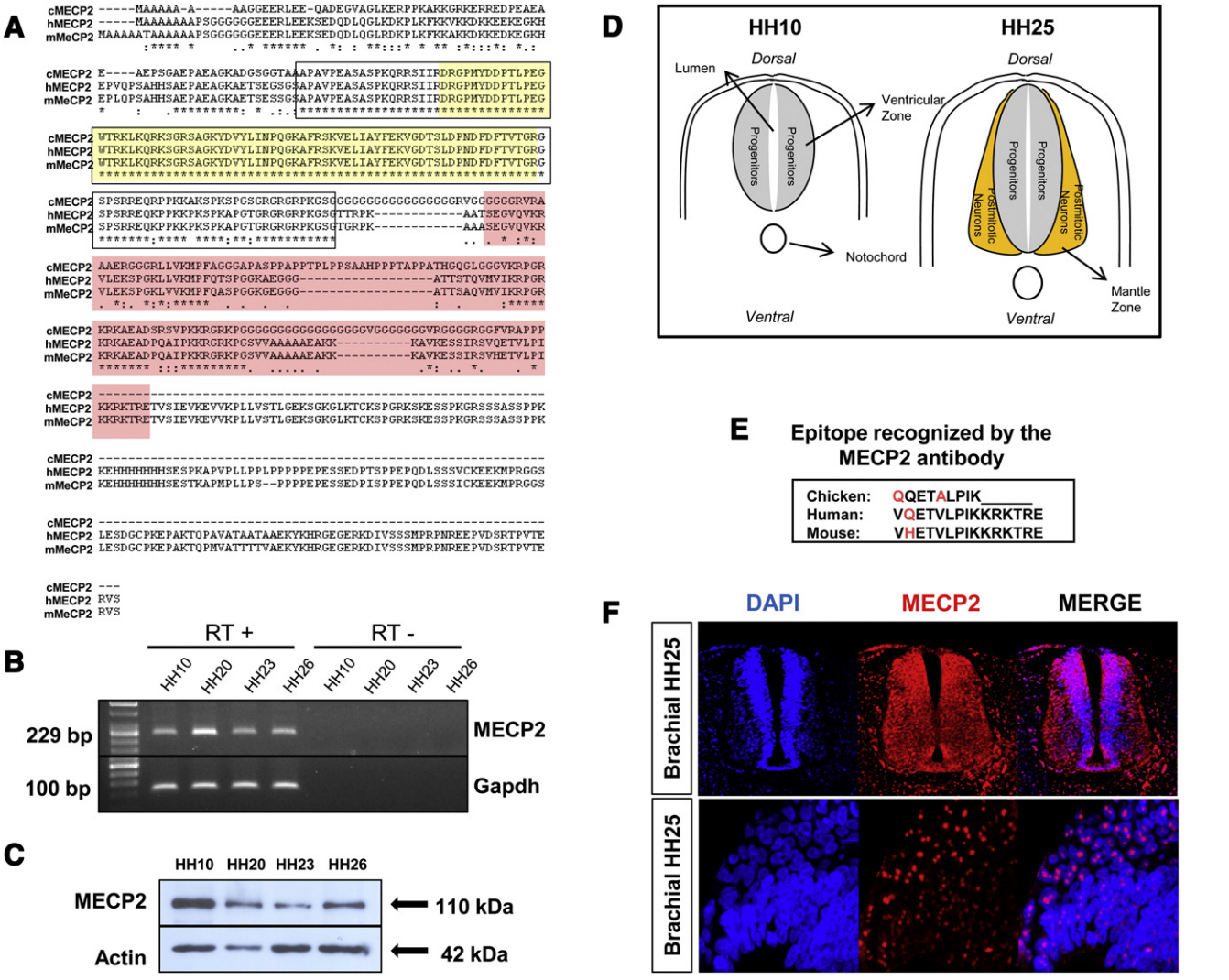


**Fig. 1.** cMECP2 is ubiquitously expressed in the developing spinal cord. (a) Protein sequences of chicken (Accession no. CAA74577), human (NP_001104262) and mouse (NP_001075448) MECP2 were aligned. The region inside the black box shows that the human sequence and the chicken sequence share 96.8% identity. MBD (yellow) and TRD (red) domains are highlighted. (b) RT-PCR on HH10, 20, 23 and 26 neural tube RNA extracts. Minus reverse transcriptase samples (−RT) are shown on the right. (c) Immunoblot on HH10, 20, 23 and 26 neural tubes. (d) Scheme showing the neural tube organization at HH10 and 25. (e) The cMeCP2 fragment is partly conserved with the human peptide recognized by the antibody. (f) HH25 embryos were dissected and brachial sections were stained for MECP2 (red) and DAPI (blue).

them with an anti-MECP2 antibody that recognizes the region shown in Fig. 1e. The chicken MECP2 partial annotated region shows high homology with the aminoacidic region of the mammalian MECP2 counterpart, thus, expecting that the antibody recognizes chicken MECP2 specifically (Weitzel et al., 1997). The results illustrate high expression of cMECP2 in ventral and in more dorsal ventricular cells as well as in mantle cells (Fig. 1f, top panel). Thus, MECP2 is present both in differentiated neurons and in neural progenitors. Moreover, we found that cMECP2 localizes in the nucleus, as staining of MECP2 overlaps with DAPI (Fig. 1f, bottom panel).

*MECP2 overexpression reduces neuroblast proliferation*

To investigate the role of a protein, lack and gain-of function studies are needed. The pCIG plasmid has been used in many gain-of-function studies to obtain new insights on genes function relevant for development, such as hJag1 (Neves et al., 2011), Wnt (Megason and McMahon, 2002), EZH2 (Akizu et al., 2010) and FGF (Martínez-Morales et al., 2011) among others, whose expression reached high levels when electroporated in ovo. Although different shRNA of the cMECP2 annotated region have been electroporated in chicken embryo, none of them worked (data not shown).

Humans and mice have two protein isoforms produced by the alternative splicing of the MECP2/MECP2 gene with the MECP2E1 and E2 isoforms, differing only in their N-terminal sequences (Kriaucionis and Bird, 2004). It is known that MECP2E1-specific mutations alone are able to cause RTT (Gianakopoulos et al., 2012) and MECP2E1 displays 10 times more expression than E2 (Dragich et al., 2007). In addition, a recent study reported the differential distribution of MeCP2E1 within various brain regions in mice (Zachariah et al., 2012). With the aim to investigate the presence of different MECP2 isoforms in chicken we used a RT-PCR approach. Our exon-specific RT-PCR experiments based on the protein alignment between human and chicken and designed to amplify the sequence between Exons 1 and 3, failed to detect a second cMECP2 transcript (data not shown).

In order to analyze the effects of increased MECP2 dosage on the developing neural tube, we cloned MECP2_E1 full-length into a pCIG vector under the control of a CMV promoter. The additional expression of a nuclear-localized GFP from an internal ribosome entry site (IRES) enabled easy identification of transfected cells. In ovo injection of MECP2 expression plasmid into HH10 embryos and subsequent electroporation led to efficient and unilateral expression of this protein in the neural tube as it is shown in Fig. 2a, where the GFP channel colocalizes with the MECP2 red channel. Noteworthy, the pattern of nuclear localization

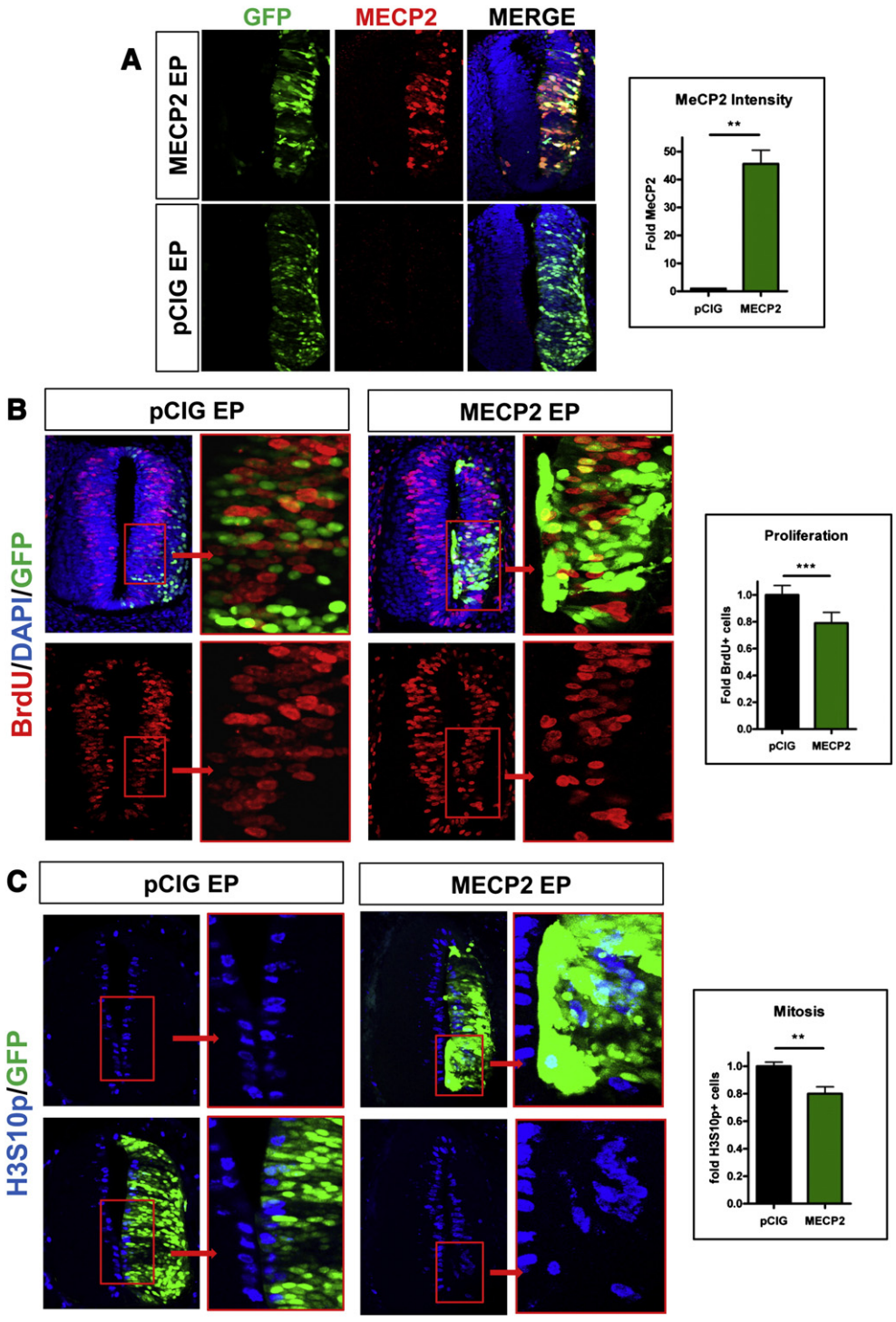


**Fig. 2.** Neuroblast proliferation is reduced upon MECP2 overexpression. HH10 embryos were electroporated with MECP2 or the empty vector in a bicistronic plasmid containing EGFP. All sections were taken from the brachial region. (a) MECP2 immunostaining (red) of embryos at 72 h PE. The graph shows MECP2 area intensity of MECP2 EP neural tubes relative to the empty vector (pCIG). Intensities were quantified by a Fiji software. Data shows mean of n = 3 sections (from 3 different embryos). Error bars indicate s.e. $^{*}p < 0.05$. (b) BrdU immunostaining (red) of 72 h PE embryos. The magnified red boxes show the most affected regions. The graph shows the number of BrdU positive cells found in MECP2 EP neural tubes relative to pCIG. Data show the mean of $n = 9$ sections (from three embryos). Error bars indicate s.e. $^{***}p < 0.001$. (c) H3S10p immunostaining (blue). The magnified red boxes show the most affected regions. The graph shows the number of H3S10p positive cells found in MECP2 EP neural tubes relative to pCIG. Data for pCIG show the mean of $n = 8$ sections (from three embryos), for MECP2 $n = 12$ sections (from two embryos). Error bars indicate s.e. $^{**}p < 0.01$.

of the endogenous protein is maintained upon MECP2 overexpression and the intensity of MECP2 signal is increased. Quantification of MECP2 intensity in electroporated (EP) neural tube compared with pCIG EP reveals an average of 45-fold more MECP2 in the electroporated region (Fig. 2a, graph). Although at 24 hours post-electroporation (PE) the neural tubes did not show any evidence of altered phenotypes, at 48 (data not shown) and 72 hours PE the thickness and the structure of electroporated neural tubes were highly affected, compared to the non-electroporated side or to the empty vector. The most striking feature associated with the overexpression of MECP2 was the vastly reduced area occupied by the MZ, whose strong phenotype is appreciated in Figs. 2b, c, 3a, b and c.

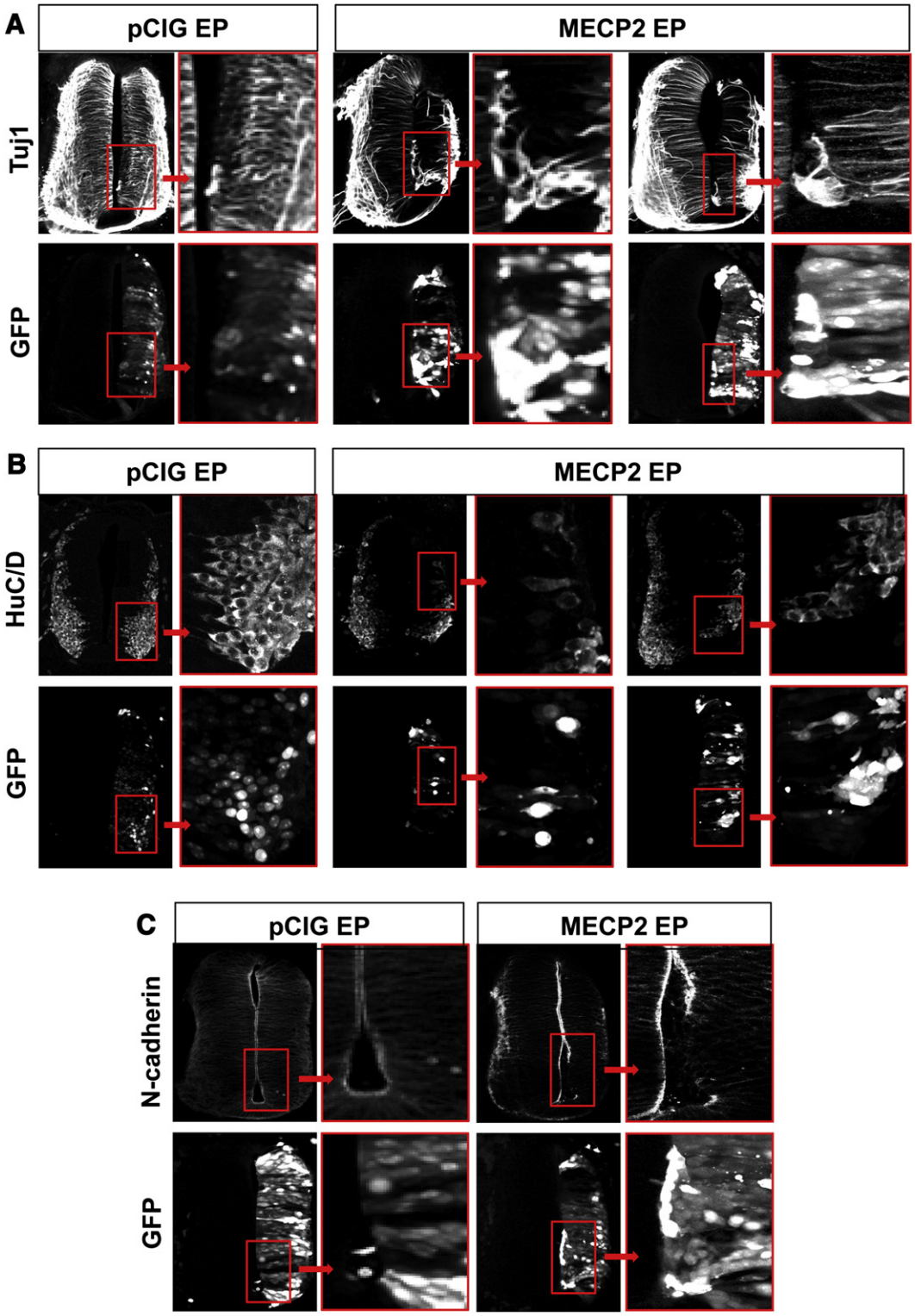


**Fig. 3.** MECP2 overexpression provokes ectopic localization of differentiated neurons. a Tuj1 immunostaining (gray, upper panel) of embryos at 72 h PE. GFP is shown in the lower panel. For MECP2 EP, two embryos are shown. The magnified red box shows ectopically differentiating neurons. b HuC/D immunostaining (gray, upper panel). For MECP2 EP, two embryos are shown. c N-cadherin immunostaining (gray, upper panel). The magnified red box shows disruption of the N-cadherin pattern by MECP2.

To elucidate the mechanisms by which MECP2 overexpression so profoundly alters neural tube organization, we examined the proliferation rate of electroporated neural tubes. We took embryos at 72 h PE and processed them for bromodeoxyuridine (BrdU) staining, showing that MECP2 overexpression leads to an overall decrease in the number of proliferating BrdU positive cells (Fig. 2b). Noteworthy, the most affected part corresponds with higher levels of GFP (compare zoom squares of pCIG EP and MECP2 EP panels in Fig. 2b). In addition, in EP pCIG, GFP-labeled cells accumulated at the mantle zone due to the normal migration accompanying neuronal differentiation, while in EP MECP2 electroporated cells gathered mainly in the VZ, therefore making not possible to assess colocalization between GFP and BrdU. In order to quantify BrdU incorporation, BrdU labeled cells in control (pCIG) and MECP2 EP neural tubes were normalized with the total number of

BrdU-positive cells in the respective non-EP side (graph of Fig. 2b). Results clearly indicate a reduction around 20% in BrdU levels as a result of MECP2 overexpression. We then wondered whether MECP2 overexpression affected also the levels of H3S10 phosphorylation, a histone mark that correlates with mitotically active cell populations. The H3S10p marker highlighted a mislocalization of actively dividing cells that normally reside close to the lumen. Again, higher levels of GFP coincide with disruption of H3S10p pattern (compare zoom square of pCIG EP and MECP2 EP panels in Fig. 2c). Quantification of anti-H3S10p immunostaining also showed that MECP2-electroporated neural tubes has a 20% decrease in the amount of mitotic cells than did the control neural tubes (Fig. 2c, graph). Collectively, these data emphasize the importance of proper spatial–temporal MECP2 expression for ensuring correct proliferation of progenitor cells residing in the ventricular zone of the neural tube.

*MECP2 overexpression induces ectopic localization of differentiated neurons*

Given that a role for MECP2 in promoting neuronal differentiation of neural precursor cells has been proposed (Stancheva et al., 2003; Tsujimura et al., 2009), we wondered whether the reduced proliferation of neural progenitor cells that we observed stemmed from premature induction of neurogenesis. To investigate this possibility, we took MECP2-electroporated neural tubes at 72 h PE, and then stained them with neural β-tubulin III (Tuj1), which is one of the earliest markers of neuronal commitment in primitive neuroepithelium. Fig. 3a show that MECP2 overexpression provokes a clear decrease in the amounts of differentiated neuronal population located at the mantle zone (compare zoom squares of pCIG control and MECP2 EP panels). Additionally, the same images of Tuj1 staining also show an ectopic localization of differentiated neurons in the MECP2-electroporated neural tubes. To confirm the phenotype caused by MECP2 overexpression, we immunostained with another marker, HuC/D, an RNA-binding protein specific to neuronal lineage. Again, when comparing MECP2 EP neural tubes with controls, depletion of differentiated cells is observed in MZ of MECP2 EP neural tubes (Fig. 3b, pCIG and MECP2 panels). This phenotype unequivocally shows an aberrant differentiation pattern for cells overexpressing MECP2, as it can be inferred by the presence of both GFP and Tuj1 in ectopically differentiated cells. Due to the non-nuclear localization of Tuj1 and HuC/D it has not been possible to quantify labeled cells of these markers. However, quantification of such qualitative markers was not necessary given that the difference in the level of staining of the mantle zone was striking, as well as it was the presence of Tuj1-stained cells in the VZ, which is normally populated by proliferating cells.

Since Tuj1 staining was found in the ventricular zone, we decided to check for neuroepithelial polarity markers, such as the cadherin family of proteins. In particular, the pattern of N-cadherin (Cdh2), a transmembrane protein that mediates homophilic adhesion at the cell junctions, was analyzed by immunostaining in the MECP2-electroporated neural tubes (at 72 h PE). The results clearly demonstrate that MECP2 overexpression disrupts the N-cadherin expression pattern along the lamina of the neural tube (Fig. 3c, compare zoom squares of control and MECP2 EP panels). This indicates that an increase in MECP2 dosage leads to a decrease in neuroepithelial polarity markers. These results suggest that, in addition to exiting the cell cycle and suffering from compromised polarity, MECP2-overexpressing neural precursor cells do not reach terminal differentiation, as can be inferred by reduced number of differentiating neurons in the MZ (Figs. 3a, c MECP2 panel).

*MECP2 overexpression induces cell death*

The observations that at 72 h PE the number of Tuj1 labeled cells in MECP2-electroporated neural tubes is vastly reduced, led us to analyze the rate of apoptosis before the onset of the altered phenotype. To this end, we determined the cellular levels of active Caspase-3 and −8, two well-known serine proteases that are activated during the early-intermediate stages of apoptosis (reviewed in Parrish et al., 2013). Caspase-8 is classified as an initiator caspase and it is one of the earliest signals in the cascade, while Caspase-3 act downstream and can be cleaved, and therefore activated, by Caspase-8. Control pCIG EP neural tubes show very low levels of Caspase-3 and −8 labeled cells as expected (Figs. 4a and b pCIG EP panel, graph 4c and 4d). However, the number of Caspase-3 and Caspase-8 labeled cells in MECP2 EP compared with pCIG EP neural tubes is significantly higher both at 24 and 48 h PE (Figs. 4a and b MECP2 EP panel, graph 4c and 4d). To confirm these data we quantified cells undergoing cell death by counting Dapi-positive nuclei showing the characteristic condensed morphology. Fig. 4e shows an increase of pyknotic cells in MECP2 EP, both at 24 and 48 h PE compared with pCIG EP neural tubes. These results clearly indicate the presence of apoptotic cells upon MECP2 electroporation and can explain the aberrant phenotype.

## Discussion

To analyze the role of MECP2 in neurogenesis we have used a chicken model, however, we first checked whether our model was suitable for this purpose. First, expression of both cMECP2 transcript and protein has been detected in a wide window of developmental stages. Then, we have found that chicken MECP2 is functionally analogous to its mammalian counterpart since its nuclear localization and the conservation of the region encompassing the methyl-CpG binding domain between human and chicken.

Our data also indicate that MECP2 overexpression causes neuroblasts to slow down proliferation, and that most of these neuroblasts die before they can reach terminal differentiation (Fig. 4f). Ectopic localization of differentiated neurons and reduced levels of polarity markers indicate that overexpression of MECP2 alone does not control the changes in polarity and migration that accompany neurogenesis. Neural cells die as a consequence of MECP2 overexpression, probably because they lack the additional spatial–temporal signals necessary for proper progression of neurogenesis. These results are in line with the lack of function studies from other models (Stancheva et al., 2003), indicating that the consequences of MECP2 overexpression do not represent toxic effects but specific ones.

Tsujimura et al. (2009) showed that MeCP2 overexpression in neural precursor cells (NPCs) promotes neuronal differentiation in adult mice. This work was based on the injection of embryo-derived NPCs in the brain or spinal cord of adult mice. We provided a more reliable study in which MECP2 ectopic expression has been induced in the chick neural tube without the need to deliver exogenous cells to the embryos. Our results are consistent with previous studies, which reported abnormally high levels of cell death in different in vitro systems overexpressing MECP2, relative to wild-type cells (Bracaglia et al., 2009; Dastidar et al., 2012). Bracaglia et al. also reported that this pro-apoptotic effect disappears when the Rett syndrome-associated MECP2-R106W mutant, which is unable to bind to methylated DNA, is expressed—thereby implying that the MBD domain is essential for MECP2-induced apoptosis.

It is remarkable that *Xenopus laevis* MeCP2 was shown to regulate the number of neural precursor cells in the differentiating neuroectoderm of early *Xenopus* embryos (Stancheva et al., 2003). In the absence of MeCP2 protein, the expression of *Xenopus* Hairy2a (a member of the Hes family of proteins, which are regulated by the Notch/Delta signaling pathway) was enhanced in embryos, which resulted in a lower number of differentiated neurons. Our results, together with the aforementioned study, highlight the importance of MECP2 dosage, as both knock-out and overexpression of this protein results in a reduced number of differentiated neurons. In our case, reduction in total number of cells is not due only to apoptosis but in addition there is a proliferation problem.

Interestingly, our phenotype resembles the one produced by the genetic ablation of Notch1 (de la Pompa et al., 1997). Loss of Notch signaling results in premature onset of neurogenesis by neuroepithelial cells

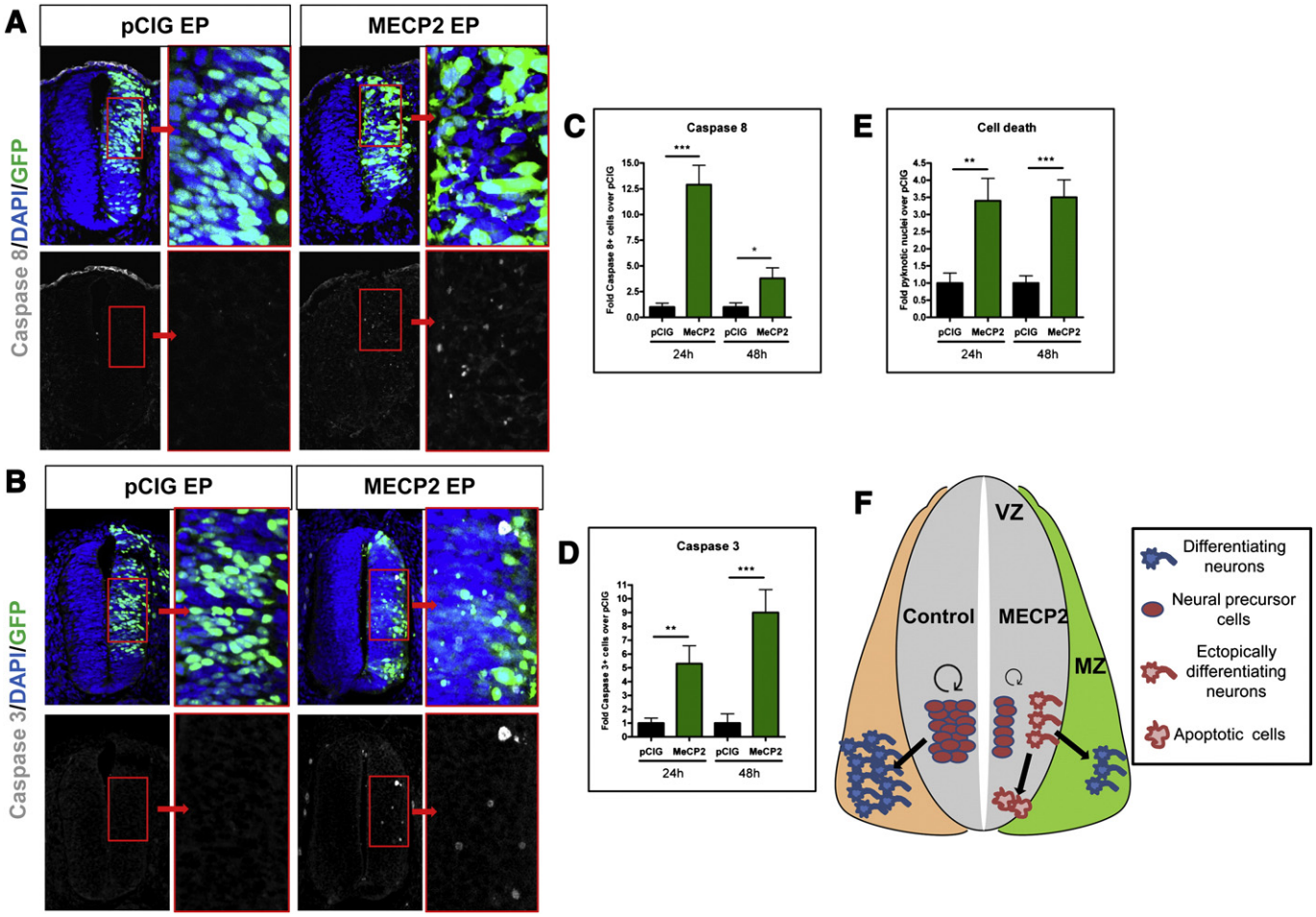


**Fig. 4.** MECP2 overexpression induces cell death. (a–b) HH10 embryos were electroporated with MECP2 or empty vector. Embryonic sections (at 24 and 48 h PE) from brachial region were immunostained for Caspase 8 (a) (gray, lower panel) and Caspase 3 (b) (gray, lower panel). The red boxes in the MECP2 EP highlight the Caspase-positive spots. c–d The graphs show the number of Caspase 8 (c) and Caspase 3 (d) positive cells in MECP2 EP neural tubes relative to pCIG EP at 24 and 48 h PE (pCIG 24 and 48 h n = 12 sections from 6 embryos; MECP2 24 h n = 21 from 10 embryos; MECP2 48 h n = 13 from 6 embryos). Error bars indicate s.e. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (e) Quantification of cells showing pyknotic features in neural tubes at 24 and 48 h PE (pCIG 24 and 48 h n = 12 sections from 6 embryos; MECP2 24 h n = 21 from 10 embryos; MECP2 48 h n = 13 from 6 embryos). Error bars indicate s.e. $^{**}p < 0.01$; $^{***}p < 0.001$. (f) Model for MECP2 overexpression consequences in neural tube.

of the midbrain–hindbrain region of the neural tube. Notch1-deficient cells do not complete differentiation but instead are eliminated by apoptosis, resulting in a reduced number of neurons in the adult cerebellum (Lütolf et al., 2002).

The molecular mechanism responsible of this phenotype can be explained by the relevant interactions between MECP2 and other proteins. For example, the MECP2-associated kinase HIPK2 has been shown to regulate cell growth and apoptosis, both in vivo and in vitro (Bracaglia et al., 2009). In addition, MECP2 interacts with many co-factors crucial for both proliferation and differentiation, such as HDAC2 (MacDonald et al., 2010) and NCOR/SMRT (Ebert et al., 2013).

This is the first study that investigates the consequences of MECP2 gain-of-function in the nervous system of an in-vivo model in the early stages of development. In particular, we introduce the novel idea that high expression of MECP2 in mitotic cells leads to anti-proliferative and apoptotic effects. Several cases of increased MECP2 copy number have been reported in male patients with progressive neurodevelopmental delay phenotype (Friez et al., 2006; Lugtenberg et al., 2006; Meins et al., 2005; Van Esch et al., 2005). Interestingly, a male patient with triplication of the MECP2 locus was described to have an even worse early-onset neurological phenotype at 3 months of age (del Gaudio et al., 2006), suggesting that the severity of an MECP2 overexpression phenotype is proportional to the copy number increase. In order to reinforce this hypothesis, researchers have studied a mouse model expressing seven times the wild-type levels of MeCP2 protein, reporting that it died by 3 weeks of age (Collins et al., 2004). Although the levels of MECP2 expression induced in the chicken neural tubes that we have described in the present study are not fully representative of the physiological situation in patients with MECP2-related disorders, our results are in line with lack and gain of function studies that elucidate the importance of correct gene dosage in neuronal development and disorders.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Acknowledgments

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