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Deregulated Gene Expression in the Brain of Rett Syndrome Patients and *Mecp2*-null Mice

by

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A DISSERTATION

Presented to the Department of Cell and Developmental Biology and

the Oregon Health & Science University School of Medicine

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

May 2005

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ACKNOWLEDGEMENTS

I love science. Following my passion for science, I crossed the Pacific Ocean from Hong Kong and came to OHSU to pursue my Ph.D. training. Over the past six and a half years of my graduate career here at OHSU, there have been many ups and downs, challenges as well as unexpected turning points. Throughout all of these, there have been many people who have been uplifting and have contributed to both my academic and personal growth in their own unique ways. I feel blessed and grateful for having these wonderful people and experiences in this chapter of my life.

My first thanks goes to my advisor, Dr. Sergio Ojeda, for giving me the great opportunity to work on this project and for supporting me through these years of work on this project. I am deeply indebted to every member on my thesis advisory committee (TAC), Drs. Richard Maurer, Charles Roberts and Klaus Freuh, for their valuable scientific advice, productive discussion, time, effort and cooperation, especially when commuting between the two OHSU campuses to every TAC meeting was challenging. Without their support, I couldn't have gone this far to get where I am standing now. I'd also like to extend my special thanks to our collaborator, Dr. Matthew Frerking, for his sage advice, inputs, and stimulating discussion, and also for the tremendous amount of time and effort as well as the excellent expertise that he contributed to the electrophysiological study of this project. I'd like to thank Dr. Sudge Budden for bringing this

wonderful project onto the lab bench and for sharing her passion for research on Rett syndrome. I'd also like to thank Northwest Rett Syndrome Foundation for allowing me the chance to attend their annual meetings and to interact with many families and girls affected with RTT. It was those experiences that ultimately evoked strong passion and drive within me that gave this project a meaning much deeper than that of a Ph.D. degree. I'm grateful to have worked with every member of the Ojeda lab and thank you for your friendship and support over these past five years.

My never-ending appreciation goes to my parents, who, as compassionate physicians, imparted to me and fostered my strong interest in life science and human diseases during my early childhood; and whose unconditional love nurtured a deep sense of security within me that has always empowered me to explore and to pursue my dreams. Thank you, my dear mother and father, for your endless love, support, understanding and sacrifice! I'd also like to thank my dearest older sister for her love, patience, courage and sharing of perspectives in every aspect of life, even though we are apart from each other for all these years.

Finally I'd like to thank all those people who have left special and beautiful footprints in my life while I was working on this Ph.D. project. Thank you, Bin and Dennis, for sharing those special experiences that have deepened my perception and eventually let me grow stronger as a whole person. Thank you, all my dear friends-- See-yan and Luk, Jasper, Wei, Jenny, Matthew and many others, for

your love and encouragement, for your precious friendship, and for your continuing support. Thank you, Anuji, my fellow graduate student and dear friend, for your valuable inputs, critiques as well as kind help and support, especially during the final phase of the dissertation preparation on those winter days when the weather was relentlessly cold and snowy and you had to walk home on foot. Once again, I thank everyone who has impacted and helped shape my life greatly. Without you, I could not have become who I am now.

ABSTRACT

Found almost exclusively in young females, Rett syndrome (RTT) is a progressive X chromosome-linked neurodevelopmental disorder characterized by loss of acquired psychomotor abilities, decelerated head growth, mental retardation, stereotyped hand movements, autism, and seizure. Girls affected with RTT usually present a dynamic age-dependent clinical course with an abrupt onset of symptomatic manifestation after 6-18 months of seemingly normal development.

Mutations in the *MECP2* gene, encoding methyl-CpG-binding protein 2 (MeCP2), are responsible for a majority of RTT cases. MeCP2 acts as a transcriptional repressor that interprets DNA methylation signals in the mammalian genome by binding to methylated DNA, and subsequently induces gene silencing via chromatin remodeling by recruitment of Sin3A-histone deacetylases (HDAC1/2) corepressor complex. Gene silencing, mediated by DNA methylation, represents an important epigenetic mechanism and is essential for many biological processes during normal development in vertebrates.

The MeCP2 protein is most abundantly expressed in mature neurons of the mammalian central nervous system (CNS) and is thought to play a role in maintenance and modulation of neuronal maturity and plasticity. The precise

function of MeCP2 in the brain and the mechanism by which MeCP2 mutations cause RTT currently remain elusive. Given the molecular nature of MeCP2, it is predicted that loss of MeCP2 function may lead to widespread aberrant gene expression in the CNS. Identification of the neuronal targets of MeCP2 will not only advance our current knowledge about the role of MeCP2 in neuronal functioning, but will also shed light on the neuropathology of RTT. In this thesis study, using Affymetrix GeneChip oligonucleotide arrays, we compared gene expression profiles of postmortem brain tissues from RTT patients with their age-matched control subjects. We found that the gene expression level of *FXYD1* was abnormally elevated in the frontal cortex of RTT patients. We further detected that the *FXYD1* gene was also selectively overexpressed in the frontal cortex of *Mecp2*-null mice, a mouse model of RTT.

The *FXYD1* gene encodes a small, single-span membrane protein, namely FXYD domain-containing ion transport regulator 1 *(*FXYD1*)* or phospholemman (PLM), which regulates cell excitability via modulation of tissue-specific Na^+,K^+-ATPase activity. Here, we demonstrated that the *FXDY1* gene harbored multiple methylated CpG sites in its promoter regions, and that MeCP2 was physically associated with the native FXYD1 promoters in both human cell culture and mouse brain tissues. These results suggested that the *FXYD1* gene was subjected to DNA methylation-dependent epigenetic transcription repression mediated by MeCP2. We further evaluated functional consequences of FXYD1 overexpression by electrophysiological studies. The data revealed that Na^+,K^+-

ATPase-dependent currents were reduced in cortical neurons of *Mecp2*-null mice, possibly attributable to FXYD1 overexpression.

Taken together, results presented in this thesis have suggested for the first time that FXYD1 is a *bona fide* MeCP2-target gene, whose selective derepression in the frontal cortex due to MeCP2 deficiency may contribute to the neuropathogenesis of RTT by altering neuronal Na^+, K^+ ATPase activity. This finding will potentially provide a new line of investigation for designing therapeutic strategies and agents to treat this debilitating childhood disorder.

Keywords: Rett syndrome (RTT), DNA methylation, MeCP2, FXYD1, Gene expression, Transcription repression, Affymetrix GeneChip arrays, Frontal cortex, Na^+, K^+-ATPase, *Mecp2*-null mouse.

CHAPTER ONE

INTRODUCTION

Rett Syndrome and Epigenetic Gene Regulation Mediated by Methyl-CpG Binding Protein 2 (MeCP2)

Vivianne W. Deng

Overview of Rett Syndrome

Clinical Characteristics:

Rett syndrome (RTT, OMIM #312750) is a severe X-linked neurodevelopmental disorder that predominantly affects young females with a prevalence of about 1:10,000-15,000 worldwide [1-3]. It is characterized by an abrupt arrest and deterioration of brain development that becomes symptomatically evident after the first 6-18 months of seemingly normal postnatal growth [4]. The disease subsequently leads to a variety of neurological abnormalities, including "acquired" microcephaly, impaired language development, loss of voluntary movement, stereotypical hand wringing, mental retardation, irregular breathing, autism, hypotonia, ataxia, apraxia, and seizure [4, 5]. Born apparently normal, affected individuals soon lose their ability to stand, walk, talk and use their hands. Surpassed only by Down syndrome, RTT is the second leading cause of mental retardation in females [6]. The course of the disease is irreversible, and the disease is incurable at the present time. Although young girls with RTT can generally live into their adulthood, the mortality rate is 1.2% per year, with about 25% of these deaths reported as sudden and unexplained [7]. It has been speculated that breathing dysfunction and cardiac abnormalities may account for these incidents of sudden death [8].

Neuropathology:

Due to the neurological regressive features presented in RTT, the syndrome was initially thought to be a neurodegenerative disease. It is not until

recently that evidence from neuropathologic studies has supported RTT as being a disorder of arrested neuronal development [9]. Although the gross brain anatomy reveals only subtle and nonspecific abnormalities, the brain size of individual with RTT is about 30% smaller than normal at any given age [9-11]. Further studies using neuroimaging and postmortem brain weight have indicated that the caudate nucleus and frontal cortex display the largest reduction in volume [10, 12], suggesting a selective decrease in the size of certain brain areas [11]. Impaired neuronal development has also been implicated by the observation that the dendrites of pyramidal neurons in the frontal and motor cortex were markedly shortened and had much less complexity [13, 14]. In fact, the results from three-dimensional confocal microscopy studies confirmed these findings and further detected a reduction in the number of dendritic spines [15]. Finally, while neuronal migration in RTT brains seemed normal, neurons in the areas of cerebral cortex, basal ganglia, thalamus, hippocampus, amygdala, and substantia nigra showed smaller soma and were more densely packed than in control subjects [16]. The mechanisms underlying the neuropathology of RTT are currently unknown, however, the dendritic alterations demonstrated in the above studies suggest that a disrupted synaptic development is involved, and may be associated with the reduced levels of a number of molecules, such as microtubule-associated protein 2 (MAP2, an important cytoskeletal component of neuronal dendrites), substance P (a neuropeptide that stimulates neurite extension in cultured neuroblastoma cells), and prostaglandin endoperoxide H synthase-2 (PGHS-2, the rate-limiting enzyme in prostanoid biosynthesis that localizes to dendritic spines and marks

mature neurons) [17-19] . And yet, whether or not there is a causative relationship between changes in synaptic connectivity and the levels of these molecules remains to be determined by in-depth studies in the future.

MECP2 as a RTT-responsible Gene and Mecp2-null Mouse as a Rodent Model for RTT:

Even though the almost exclusive involvement of females is best explained by X-linked dominant inheritance with lethality in the hemizygous males [4], the molecular genetics underlying the pathology of RTT had been an enigma for several decades since the first description of the syndrome in1966 by Andreas Rett [20], a Viennese physician. RTT is mostly sporadic in nature with a familial recurrence of only approximately 1% of the total reported cases [21], making it difficult to map genetic loci that are responsible for the disease. In 1999, the identification of a disease-associated gene, namely methyl-CpG-binding protein 2 (*MECP2*), marked a significant breakthrough in understanding of molecular genetics of RTT [22]. The *MECP2* gene is localized on the X-chromosome, and it encodes methyl-CpG-binding protein 2 (MeCP2), a chromosomal protein that is involved in epigenetic regulation of gene silencing. The *MECP2* gene in both humans and mice is subject to X inactivation (XCI). Thus, it has been postulated that the pattern of XCI may influence the symptomatic presentations of MECP2 mutations in females. Conceivably, *MECP2* mutation types along with XCI patterns in females may generate a

surprisingly wide spectrum of clinical phenotypes with various degrees of severity [23].

The fact that mutations in MECP2 have been found in about 80% of classic RTT patients implies that the resultant deficiencies due to *MECP2* mutations are primarily, if not solely, responsible for the onset of the disease. This notion has been substantially supported by evidence derived from studies on transgenic lines of mice carrying a MeCP2 deficiency. Using Cre-*loxP* gene knockout technology, two independent research groups led by Bird and Jaenisch generated *Mecp2*-null mice and central nervous system (CNS)-specific *Mecp2*-deficient mice, respectively. In agreement with each other, both groups observed that mutant mice displayed Rett-like neurological abnormalities [24, 25]. Notably, although the MECP2 gene is ubiquitously expressed in all tissues, selective deletion of MECP2 in the mouse CNS generated an identical phenotype to that of *Mecp2*-null mutants, suggesting the Rett-like phenotype is due to MeCP2 deficiency in CNS rather than in peripheral tissues. Moreover, conditional deletion of MeCP2 in postnatal CNS neurons also resulted in similar neuronal phenotypes as seen in *Mecp2*-null mice, surprisingly implicated that MeCP2 might play an essential role in the maintenance of mature neuronal functions. Indeed, a recent report from Zoghbi H, et al. has substantiated this concept by showing that MeCP2 is abundantly expressed in neurons with a certain degree of maturity, and that the timing of MeCP2 expression in mice and humans correlates with the maturation of CNS [26]. Shahbazian and Zoghbi H., et al. also

generated a transgenic line of mice harboring a truncating mutation of MECP2 similar to those found in RTT patients [27]. Following six weeks of seemingly normal development, these mice exhibited progressive neurological abnormalities mimicking many features of RTT. The finding that all these aforementioned mutant mouse strains mirror virtually every clinical aspect of RTT patients, and particularly, recapitulates highly specialized hand-wringing movements, strongly suggests that the pathways originating from dysfunctional MeCP2 and leading to each of these features are conserved between mice and humans. Thus, these Mecp2 mutant mice provide a powerful model system for *in vivo* study of the physiological roles of MeCP2 in brain development and function as well as in the pathobiology of RTT.

DNA Methylation and MeCP2-mediated Epigenetic Gene Regulation

Transcription Regulation and Epigenetics:

In multicellular organisms, cells of different types share identical genetic makeup, but are morphologically and functionally heterogeneous due to distinct gene expression patterns. Tightly controlled and efficiently executed, the gene expression program is essential for normal development and proper function of the organism. Gene expression patterns are created and regulated mainly at the level of transcription, a highly synchronized activity that involves dynamic and intricate interplays of transcription factors with their cognate DNA sequences. While the initial research in transcription regulation focused on transcription activation, transcriptional repression has gained increasing appreciation and

understanding during the recent years. Studies have convincingly demonstrated that mammalian transcription regulation is mediated by active engagement of both transcription activator and repressor complexes, which selectively assemble on specific DNA sequences of a target gene in response to proper cellular signaling cascades. These regulators work in concert with chromatin modifiers and remodeling factors to reshape the local chromatin architecture by inducing changes in chromatin conformations, thus either sustaining a silencing state or promoting a transcriptionally active state. It is currently believed that lasting alterations in chromatin structure is a direct consequence of epigenetic phenomenon, which refers to a set of self-perpetuating, post-translational modifications of DNA and nuclear proteins that indirectly influences patterns of gene expression without involvement of changes in DNA sequences [28]. Epigenetic effects allow heritable and lasting alterations of gene expression in a short term, and are critical for control of gene transcription during mammalian development. The possibility that epigenetic mechanism may play an active role in synaptic plasticity and memory formation, thus in behavior, neurophysiology and neuropathology, has recently begun to emerge as a burgeoning theme in modern neurobiology [28].

DNA Methylation:

In vertebrates, DNA methylation and histone modification are two common epigenetic mechanisms utilized by cells in regulation of high-order chromatin architectures in the event of gene transcription [29]. DNA methylation is primarily

responsible for stable transcriptional repression of genes, and has a profound effect on gene expression in many biological processes, as illustrated in tissue-specific gene expression, cellular differentiation, X-chromosome inactivation, and imprinted gene silencing [30]. In the mammalian genome, DNA methylation occurs predominantly at the C-5 position of cytosines in 60-90% of CpG dinucleotide sites (5'-m^5CG-3'), which represents the most frequent form of post-replication modifications of DNA. Such a methylation is catalyzed by various DNA methyltransferases (DNMTs). DNA methylation patterns are initially established by *de novo* DNA methyltransferase 3a and 3b (DNMT3a and 3b) during early embryogenesis and subsequently maintained by DNA methyltransferase 1(DNMT1) in proliferating cells [31-33]. Faithful maintenance of appropriate methylation patterns is required for normal development in mice [33, 34]. Aberrant methylation patterns have been shown to be associated with human tumors and many developmental abnormalities [35, 36]. Studies of mice that are deficient in DNA methyltransferases have suggested a critical role for DNA methylation in normal development of the nervous system [33, 37, 38]. Increasing evidence has also demonstrated that DNA methylation explicitly influences regulation of neuronal gene expression [39-42]. Moreover, a recent study has shown that neuron-specific imprinting of the ubiquitin-protein ligase E3A gene (UBE3A) is mediated by DNA methylation. UBE3A is associated with majority of cases of Angelman syndrome, an imprinting neurodevelopmental disorder sharing a few overlapping features with RTT [43].

MeCP-mediated Epigenetic Regulation:

The cellular machinery responsible for interpreting the information coded by DNA methylation is the methyl-CpG-binding domain (MBD) protein family, including MeCP1-2 and MBD1-4 proteins. Most of MBD proteins contain a methyl-DNA-binding domain (MBD), which targets the protein to methylated CpG sites in genomic DNA. MeCP2 is the archetypical member of the MBD protein family and functions as a major mediator of downstream consequences of DNA methylation [44]. Although studies have shown that MeCP2 may mediate DNA methylation-dependent transcription repression either via or independent of chromatin remodeling, compelling evidence supports the prevailing model that MeCP2 exerts transcription repression by recruiting histone remodeling complexes. More specifically, MeCP2 can selectively bind to a single methylated CpG by its MBD and recruits the histone deacetylase (HDAC)-containing core complex to the promoter regions of target genes via the interaction with the Sin3A co-repressor at its transcriptional repressor domain (TRD), thus rendering the chromatin structure inaccessible to transcription machinery and stably silencing gene transcription of the loci [45, 46]. In such a manner, MeCP2 establishes an important molecular link in the three-way connection between DNA methylation, chromatin architecture and gene silencing. The meCpG-MeCP2-Sin3A-HDAC machinery converges two epigenetic mechanisms mediated by DNA methylation and histone modification, and is perceived as a global mechanism for stable transcriptional repression at the sites of DNA methylation. The fact that MeCP2 is highly expressed in the brain with apparently

ubiquitous presence in mature neurons, and that MeCP2 deficiency results in phenotypes restricted to the nervous system in both mice and humans, strongly suggests a pivotal role for MeCP2 and DNA methylation in brain development and function. In this sense, RTT represents a disease caused by missteps in MeCP2-mediated epigenetic regulation, which in turn, offers a unique disease model system to appreciate and dissect the role of epigenetic transcription repression mediated by DNA methylaltion and MeCP2 in maintaining the integrity of neuronal functions.

Neuronal Activity-dependent Gene Epigenetic Regulation by MeCP2:

Noteworthily, an exciting breakthrough in understanding DNA methylation and MeCP2-mediated epigenetic regulation in neuronal functions comes from a recent investigation of gene expression of the neuronal gene encoding brain-derived neurotrophic brain function factor (BDNF), a key molecule involved in synaptic plasticity and cognition functions of the brain. In contrast with the long-standing belief that DNA methylation and MeCP2 associate with heritable, stable transcriptional repression, the finding that BDNF gene transcription is subjected to transcription repression mediated by DNA methylation and MeCP2 in an neuronal activity-dependent fashion [47, 48] indicates that dynamic regulation of the DNA methylation status and the association of MeCP2 with chromosome DNA can contribute to acute genomic regulation in response to excitatory stimuli transmitted through signaling networks from synapses to the nucleus. This observation demonstrates that DNA methylation, MeCP2-mediated chromatin

remodeling in activity-dependent neuronal gene regulation is essential for synaptic development and neural plasticity in the brain. As a matter of fact, DNA methylation is remarkably dynamic during mammalian development and in some tumor cells [49, 50]. Changes in the methylation status of the entire genome, certain chromosomes, and specific genes are present to respond to developmental stages and environmental cues, and can result in significant changes in gene expression [29, 49]. For instance, methylation patterns and the resultant gene expression in postmitotic neurons have been shown to be altered in both aging [51] and ischemia conditions [52].

Toward Understanding the Role of MeCP2 in the Brain and Neuropathology of RTT

Given that MeCP2 is ubiquitously expressed throughout the human body, it is perplexing that MeCP2 mutations give rise to a constellation of clinical manifestations in RTT that may only derive from altered CNS functions. Currently, the causal relationship between MeCP2 deficiency and the neuropathology of RTT still remains a central focus of the RTT research. A prerequisite for elucidation of the pathogenesis of RTT is knowledge of the MeCP2 function in the brain. While both recapitulation of RTT-like phenotypes by mice with a conditional *Mecp2*-deletion in CNS postnatal neurons and involvement of MeCP2-mediated transcription repression in BDNF gene regulation suggest that the MeCP2 function may be crucial for neuronal maintenance and plasticity in the mature CNS, at present, little is known about

the precise physiological role of MeCP2 in the brain. In the past several years, increasing attention and effort has been drawn to this area of study worldwide, and major progress has been made in understanding of: (1) the distribution of MeCP2 in the brain during development; and (2) downstream neuronal targets of MeCP2 that are subjected to MeCP2-mediated transcriptional regulation in the brain.

MeCP2 Expression Patterns in the Brain:

Extensive studies with mouse, rat, and non-human primate brains have revealed a regional, temporal, and cell type-specific expression pattern of the MeCP2 protein in the brain. First, MeCP2 is more abundantly expressed in the brain than in many other peripheral tissues [26, 53], with the highest expression in olfactory bulb and in frontal cortex [54]. Secondly, MeCP2 is selectively expressed in the majority of neurons but not in glia in the brain, and has an apparent nuclear localization [26, 53, 55]. Third, MeCP2 expression levels increase throughout development and correlate with neuronal maturation [26, 55, 56]. Specifically, the timing of MeCP2 expression is coincident with synaptogenesis [57]. Finally, MeCP2 is present in both excitatory glumatergic and inhibitory GABAergic neurons [55]. Together, these findings demonstrate that MeCP2 expression is developmentally regulated during neuronal maturation and synaptic formation, and that a regional and cellular heterogeneity in MeCP2 distribution is present in different brain structures. This expression pattern further supports the current hypothesis that

MeCP2 acts to maintain or modulate neuronal maturity and plasticity, and that these functions are spatiotemporally dynamic.

The Search for MeCP2-target Genes in RTT:

Further understanding of the roles of MeCP2 that are specific to mature neurons still relies on identification of MeCP2 neuronal targets and the resultant cascade of events following their deregulation due to MeCP2 deficiency.

The molecular function of MeCP2 as a global gene silencer has led to a conjecture that MeCP2 deficiency may disrupt the epigenetic program of gene silencing in normal brain development and result in abnormal gene activation in the brain. Deregulated expression of these genes may contribute to neurological abnormalities in Rett syndrome. However, independent studies of global gene expression profilings in both clonal cell culture from RTT patients and *Mecp2*-null mouse brains unexpectedly failed to detect obvious changes in gene expression on a whole-genome scale [58, 59]. Although the first genome-wide gene expression profiling analysis by Colantuoni et al. suggested a few differential gene expressions in postmortem RTT brain tissues [60], the result was not conclusive owning to a small sample size, the limitation of gene profiling techniques, as well as lack of substantial confirmation data from the RTT mouse model. Moreover, the findings that MeCP2 expression is development, region and cell-specific and that MeCP2 may dynamically regulate its neuronal target genes in an activity-dependent manner might also provide explanation for the subtle changes in gene

expression of a small set of genes detected by these gene profiling studies. Nevertheless, the studies on neuronal activity-dependent gene regulation and imprinting gene regulation using *Mecp2*-null mice have recently discovered two genes as MeCP2 neuronal targets: the aforementioned BDNF gene and a DNA-binding homeobox protein, DLX5, encoding the enzyme that synthesizes gamma-aminobutyric acid (GABA). Evidence from neuropathology and brain imaging studies of RTT has suggested that the disease is associated altered neurotransmission [61]. BDNF enhances glutamatergic neurotransmission at excitatory synapses, whereas Dlx5 stimulates the synthesis of GABA in most GABAergic neurons. The finding of BDNF and DLX5 as MeCP2 target genes in neurons further supports the hypothesis that disruption in synaptic formation and neuronal circuits may underlie the neuropathology of RTT [61, 62].

On the whole, the current knowledge about RTT and MeCP2 leads to a general concept that the methylation-dependent gene silencing mediated by MeCP2 may represent an essential component in regulation and maintenance of proper neuronal functions in mammalian CNS development. MECP2 mutations may release the MeCP2-mediated transcriptional repression, causing aberrant activation of genes that should normally remain silenced during postnatal development, and eventually contributing to perturbation and disruption in normal functioning of the maturing CNS. Identification of additional neuronal target genes of MeCP2 in the brain will provide insights into the regulatory elements and pathways required for normal brain development, shed light on the molecular

mechanisms responsible for neuropathology of RTT, and help identify molecular markers and candidate genes as potential targets for future diagnostic agents and therapeutic intervention of the disease.

The overall objective of this dissertation project is to identify abnormal gene expression in the brain affected by MeCP2 deficiency in RTT, and to functionally characterize the deregulated expression of these target genes related to MeCP2 dysfunction as well as the RTT neuropathology. During the planning phase of this dissertation project, because nothing was known about MeCP2 expression patterns and MeCP2-mediated gene regulation in mammalian brains, and also because of limited availability of brain tissues affected with RTT, it was decided to take advantage of Affymetrix GeneChip technology to conduct a genome-wide analysis of gene expression profiling in postmortem brain tissues from RTT patients and their age-matched normal controls. Affymetrix GeneChip technology applies combinatorial chemistry and photolithographic technology to enable the manufacture of high-density oligonucleotide microarrays of high quality, reproducibility, and consistency. The unique advantage of Affymetrix GeneChip arrays over other microarray platforms is that each array provides multiple, independent measurements for each gene transcript of interest, generating a complete expression data set with accurate, reliable, reproducible results from every experiment. Using Affymetrix GeneChip Human U95A arrays followed by subsequent clustering analysis, I found that the brains of RTT patients expressed abnormally high levels of a novel gene, termed

FXYD domain-containing ion transport regulator 1 (FXYD1) or phospholemman (PLM).

FXDY1 and the *FXYD* Gene Family

The *FXYD* gene family encodes a novel family of small, single-span membrane proteins that are characterized by a signature sequence of 35 amino acids containing an FXYD motif, a single transmembrane span, and other invariant and highly conserved amino acids [63] (Figure 1.1). The mammalian FXYD protein family consists of seven members, namely FXYD1 through FXYD7[64]. Based on structural features and the observation that several FXYD proteins induce ion-specific conductance when overexpressed in *Xenopus* oocytes [65-68], it has been thought that the FXYD proteins may function as channels or regulators of ion channels [64]. Except for FXYD2 being known as a regulatory subunit of Na, K-ATPase [69], the molecular and physiological functions of FXYD proteins were largely unknown until recently. Although FXYD proteins appear to be functionally versatile, current studies have just started to unravel the common role of FXYD proteins as isoform-, tissue-specific regulators of Na,K-ATPase [63, 70-74]. The FXYD1, 2, 4 & 7 proteins exhibit tissue-specific distribution patterns, with FXYD1 in heart and skeletal muscle [75, 76], FXYD2 (the γ-subunit of Na,K-ATPase) [69] and FXYD4 (corticosteroid hormone-induced factor or CHIF) in the kidney [77], and FXYD7 in the brain [73]. These FXYD proteins have been shown to associate, regulate and fine tune the transport activity of Na,K-ATPase in a tissue-specific manner and in such a way as to fulfill functional demands of

distinct tissues on Na^+ and K^+ handling by Na,K-ATPase in various biological events, including Na^+-reabsorption, muscle contraction, and neuronal excitability [63, 70-73]. In fact, interaction of FXYD proteins with Na,K-ATPase has become a common theme for modulation of the Na,K-ATPase function.

The *FXYD1* gene is co-localized in a gene cluster with other three *FXYD* genes, *FXYD3, 5 & 7*, on chromosome 19 [75]. The gene expresses two transcript variants possibly from alternative transcription start sites, *FXYD1a* and *FXYD1b*, encoding an identical isoform but with different 5' UTR sequences. *FXYD1* is mainly expressed in electrically excitable tissues, highly in skeletal muscle and heart, and moderately in brain. Just recently, the FXYD1 protein has been found abundantly present in distinct structures in the CNS, in both neurons and glia, with the highest expression in cerebellum where it was detected in the molecular layer, Purkinje neurons, and axons traversing the granule cell layer[78]. FXYD1 is also expressed in choroid plexus and certain tanycytes or ependymal cells of the ventricle wall [78].

FXYD1 was first discovered as a phosphorylated protein in sarcolemma from cardiac and skeletal muscles in 1977 [79] and has been most extensively studied since then, although mostly in cardiac and skeletal muscle tissues. The human FXYD1 protein contains 20-amino acid cleavable signal sequence and the mature form is a 72 amino acid membrane-span protein [80], with the N-terminus on the extracellular and the C-terminus on the cytoplasmic side of the membrane.

Besides its well-recognized role as a heart- and skeletal muscle-specific Na,K-ATPase modulator in regulation of muscle contractility, FXYD1 appears to play diverse molecular and physiological roles in other experimental systems: (1) FXYD1 induces a hyperpolarization-activated chloride current when expressed in Xenopus oocytes [65]; (2) FXYD1 selectively induces ion and zwitterionic taurine permeability in oocytes and lipid bilayers [81, 82], and mediate taurine efflux in response to cell swelling , implying a role in cell volume regulation[83, 84]; (3) In response to insulin and adrenergic stimulation, FXYD1 is a major target in heart for multiple protein kinases, including PKA, PKC, myotonic dystrophy kinase and NIMA (Never in mitosis gene A) kinase [85-88] , suggesting it may serve as a cellular integrator in these signaling pathways, although the physiological role of FXYD1 phosphorylation is poorly understood; (4) FXYD1 modulates myocyte contractility in cardiac muscle partly by inhibiting Na+/Ca2+ exchanger 1 (NCX1), a nine-transmembrane protein that plays a critical role in maintaining intracellular Ca2+ and Na+ homeostasis [90].

The very recent finding demonstrating that FXYD1 has a unique distribution in the cerebellum and choroid plexus of the CNS where it may act as an accessory protein of Na^+,K^+-ATPase to regulate its transport activities, clearly suggests that FXYD1 plays important yet undefined physiological roles in the CNS [78]. Because choroid plexus is responsible for CSF secretion in the ventricles, enrichment of FXYD1 in choroid plexus also implies that it may be involved in regulation of CSF secretion. The sodium and potassium ion gradient

established by the Na^+,K^+-ATPase is crucial in countless membrane transport processes, particularly in calcium transport and neurotransmitter reuptake, and is responsible for maintaining ion homeostasis and proper excitability in neurons. Regulation of Na^+,K^+-ATPase by FXYD1 is, therefore, of great importance in the brain physiology and pathophysiology. However, the research in this area is currently much neglected.

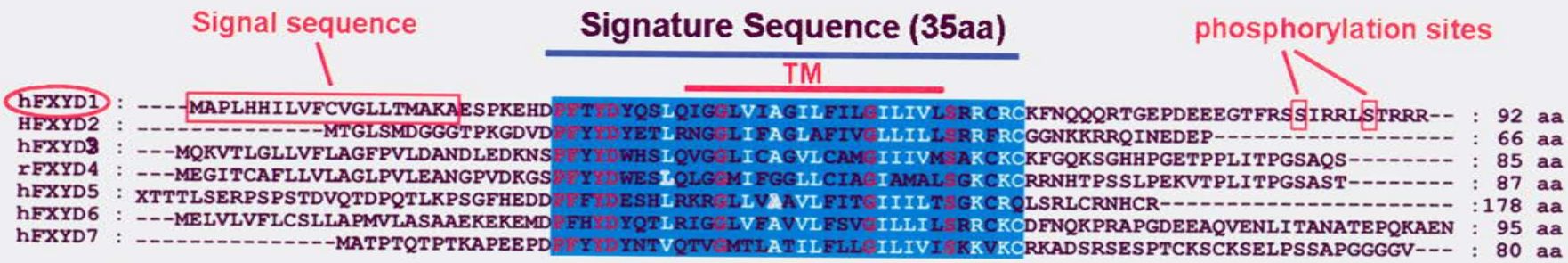

Figure 1.1. The FXYD protein family. Alignment (CLUSTAL X software) of human FXYD1-3, rat FXYD4, and human FXYD 5-7. The extended N-terminus of FXYD5 has been omitted (indicated by X). Amino acids in red are identical in all FXYD proteins, with the exception of the proline (P) residue preceding the FXYD motif, which is a lysine in *Xenopus* FXYD2. Amino acids in white are similar in at least 80% of mammalian FXYD proteins. The signal sequence and known phosphorylation sites of FXYD1 are shown in red boxes. (Adapted from Crambert G, Geering K., Sci STKE. 2003 Jan 21;2003(166) [63].

General Hypothesis and Specific Aims:

It appears that MeCP2 is essential for regulating expression of a subset of genes functioning in postnatal neurons. My initial finding of the *FXYD1* gene overexpression in postmortem brain tissues affected with RTT leads to a speculation that the *FXYD1* may be one of these genes whose transcriptional silencing mediated by MeCP2 is critical in neuronal functions during postnatal brain development. Hence, it can be hypothesized that the *FXYD1* gene expression in the brain is regulated by DNA methylation and MeCP2-mediated transcriptional repression. As formerly mentioned, both MeCP2 and FXYD1 exhibit brain region, cell-specific distribution patterns, thus it is possible that such a regulation is also brain region-, or even cell-specific. The *FXYD1* gene may represent a class of genes that, if not silenced shortly after birth, may disrupt subsequent brain development by persistently altering neural cell activity, thereby contributing to the neurological abnormalities of Rett syndrome.

Because FXYD1 has been found to associate with and act as a Na+,K+-ATPase regulator in the brain [78], it can also be hypothesized that *FXYD1* overexpression in Mecp2-null mice may result in predictable effects on Na+,K+-ATPase activity. As a result, deregulated *FXYD1* gene expression may cause alteration in Na+, K+-ATPase activity, which in turn, may lead to perturbation of neuronal excitability and ion homeostasis, thus contribute to some aspects of the RTT neuropathology.

To test these hypotheses, I propose the following specific aims and Chapter Two in this thesis will describe studies that address these specific aims:

Aim1: To validate the initial Affymetrix GeneChip array results and verify the upregulated gene expression of *FXYD1* in additional RTT brains and their age-matched normal controls. The FXYD1 mRNA level will be determined by quantitative real-time PCR.

Aim2: To determine whether the *FXYD1* gene and protein expression are abnormally elevated in *Mecp2*-null mice, and to determine whether this overexpression is brain region-specific, using a combination approach of quantitative real-time PCR, in situ hybridization, immunohistochemistry and western blotting analyses.

Aim3: To determine whether the *FXYD1* gene is subjected to transcriptional silencing mediated by MeCP2 in a DNA methylation-dependent manner. To this end, whether or not DNA methylation plays a role in *FXYD1* gene regulation will be determined by demethylation analysis using a demethylation reagent (5-azacytidine), and the methylation status of the *FXYD1* promoter regions will be mapped using bisulfite genomic DNA sequencing. *In vitro* methylation and a promoter-reporter system will also be applied to assess whether *in vitro* methylation would repress the FXYD1 promoter activity in the presence of MeCP2. Finally, whether MeCP2 is physically associated with the native FXYD1

promoters *in vivo* will be determined by chromatin Immunoprecipitation (ChIP) assays with both MeCP2-transfected human cells and mouse brain tissues.

Aim4: To determine whether *FXYD1* overexpression affect the Na+, K+-ATPase activity in the brain of *Mecp2*-null mice using electrophysiology techniques. Given that FXYD1 is recognized as a Na+, K+-ATPase regulator, an altered Na+,K+-ATPase activity in *Mecp2*-null mice would be at least one of the functional consequences that can be predicted from *FXYD1* overexpression in these mice. For this purpose, whole-cell voltage clamp recordings will be performed in Layer II/III pyramidal cells from the frontal cortex slices of *Mecp2*-null mice and their gender-matched wild-type littermate controls. The Na+,K+-ATPase activity will be assessed by ouabain-sensitive currents.

In addition, given that *FXYD1* is localized in a close proximity to *FXYD7*, a newly identified *FXYD* gene family member, encoding a brain-specific Na+,K+-ATPase regulator, it would also be very interesting to test whether these two genes share a common regulatory mechanism mediated by MeCP2, and whether they play relevant functional roles in the brain. To this end, ontogeny of gene expression and cellular expression sites of *FXYD1* and *FXYD7* will be examined. The information derived from this study would further the understanding of physiological functions of FXYD1 in the brain, and hopefully would also provide insights into the neuropathology of RTT implicated by *FXYD1* overexpression.

The preliminary results of this facet of study will be summarized in Appendix Chapter One of this thesis.

Besides the *FXYD1* gene, the initial GeneChip data analysis also revealed the presence of a group of genes involved in neurogenesis that showed differential expression in the brain tissues affected with RTT. Among these genes, two genes, termed SRY (sex determining region Y)-box 2 (*SOX2*) and Wnt inhibitory factor-1 (*WIF1*), respectively, are of particular interest. The human *SOX2* gene (NM_003106) is an intronless gene encoding a member of the SRY-related HMG-box (SOX) family of transcription factors involved in the regulation of embryonic development and in the determination of cell fate [91]. The SOX2 protein inhibits neuronal differentiation and maintains neural progenitor identity [92, 93]. The *WIF1* gene encodes Wnt inhibitory factor-1 (WIF1), a secreted antagonist of Wnt signaling that functions by directly binding to WNT proteins in the extracellular space and inhibiting their activities [94]. The WNT proteins belong to a large family of secreted, cysteine-rich proteins that play key roles as intercellular signaling molecules in diverse developmental processes, including segmentation, CNS patterning, and control of asymmetric cell divisions [95, 96]. It has been found that the methylation status of the CpG island within the *WIF1* promoter region is an important factor in control of Wnt activation in human cancers [97]. Therefore, further in-depth analysis of the expression of *SOX2* and *WIF1* genes in the brains of RTT patients as well as in *Mecp2*-null mice will provide valuable new information that may shed light on the neuropathogenesis of RTT. With this in

mind, both *SOX2* and *WIF1* gene expression in postmortem brain tissues of RTT patients and *Mecp2*-null mice will be accessed by quantitative real-time PCR. Appendix Chapter One and Two of this dissertation will present the preliminary data from these analyses.

CHAPTER TWO

FXYD1 is a MeCP2-target Gene Overexpressed in the Brain of Rett Syndrome Patients and Mecp2-null Mice

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Running Title: *FXYD1* overexpression in Rett syndrome

Key Words: gene overexpression, neuronal excitability, MeCP2-target genes, FXYD1, phospholemann, Na^+, K^+- ATPase

Submitted, in part, to Nature Neuroscience, the manuscript has been reviewed, and now in revision.

Abstract

Methyl-CpG-binding protein 2 (MeCP2) binds to methylated DNA and induces gene silencing via chromatin remodeling. Mutations in the *MECP2* gene are associated with most cases of Rett syndrome (RTT). The neuronal genes derepressed in RTT due to MeCP2 deficiency are just beginning to be uncovered. Here we report that expression of the *FXYD1* gene is selectively overexpressed in the frontal cortex of both RTT patients and *Mecp2*-null mice. *FXYD1* encodes a small, single-span membrane protein, termed FXYD1 or phospholemman, which regulates cell excitability via modulation of tissue-specific Na^+, K^+-ATPase activity. Here we show that the *FXDY1* gene was subjected to DNA methylation-dependent transcription repression mediated by MeCP2 and that Na^+, K^+-ATPase -dependent currents were reduced in cortical neurons of *Mecp2*-null mice. Our findings have identified *FXYD1* as a MeCP2-target gene, whose selective derepression in the frontal cortex affected with RTT may contribute to the neuropathogenesis of RTT through altering neuronal Na^+, K^+-ATPase activity and excitability.

Introduction

Mutation of the methyl-CpG-binding protein 2 (MECP2) gene is a molecular hallmark of Rett syndrome (RTT), an X-linked neurodevelopmental disorder that affects mainly females [6]. Most cases of RTT are associated with mutations of the methyl CpG-binding protein 2 (MeCP2) [98, 22, 99], indicating that loss-of-function of the MeCP2 protein is the primary cause responsible for the pathology of RTT. This notion has been supported by the finding that deletion of the *Mecp2 gene* in mice results in neurological abnormalities strikingly similar to those of RTT [24, 25]. Yet, the mechanisms by which MeCP2 mutations give rise to RTT are largely unknown.

MeCP2 was originally recognized by virtue of its ability to bind specifically to a single symmetrically methylated cytosine in CpG dinucleotides[100], and to promote gene silencing by recruiting a Sin3A-HDAC1/2 transcription co-repressor complex to the surrounding chromatin [101, 45]. Because of these characteristics, MeCP2 has been considered as a global gene silencer. Surprisingly, genome-wide transcriptional profiling of *Mecp2-null* mice reveals that MeCP2 deficiency does not appear to result in widespread deregulation of gene expression [58], but instead, might affect a discrete subset of genes required for neuronal functions [58,102]. Although MeCP2-mediated transcriptional silencing has been shown to regulate the expression of specific methylated genes, such as multidrug resistance gene 1 (MDR1 or ABCB1) and thrombospondins 1 (THBS1) in cancer cells, fragile X mental retardation 1 (FMR1) in fragile X syndrome [103],

and the BDNF gene in primary neurons [47], the neuronal targets that are derepressed in RTT as a result of MeCP2 deficiency are just beginning to be appreciated. Most recently, the imprinted gene distal-less homeobox 5 (*Dlx5*) has been identified as a direct MeCP2 target gene [104]. Given that *Dlx5* regulates the expression of enzymes involved in the production of γ-aminobutyric acid (GABA), the most abundant inhibitory neurotransmitter in the central nervous system (CNS), the loss of maternal-specific imprinting of *Dlx5* in RTT patients and the increased *Dlx5* expression observed in the brain of *Mecp2*-null mice indicate that deregulated Dlx5 gene expression may be one of the mechanisms contributing to the pathogenesis of RTT [104]. The identification of additional downstream neuronal targets of MeCP2 remains an important goal in the RTT research, because it may not only help unravel biochemical pathways that are perturbed in RTT and devise therapeutic strategies to treat this devastating disease, but also further our understanding of epigenetic mechanisms and regulatory elements required for normal development and functioning of the brain.

The high-throughput microarray technology allows one to monitor gene expression on a genome-wide scale simultaneously. In this study, we took the advantage of Affymetrix oligonucleotide microarrays to compare the mRNA abundance of the frontal cortex between RTT patients and their age, brain region-matched normal controls. We found that the expression of a gene, namely *FXYD1*, was persistently elevated in the frontal cortex of RTT patients. *FXYD1* belongs to a gene family encoding small, single-span membrane proteins thought

to control ion transport across the cell membrane, and to regulate cell excitability via modulation of Na^+,K^+-ATPase activity. We further detected that both FXYD1 mRNA and FXYD1 protein levels were selectively elevated in the frontal cortex of *Mecp2*-null mice. Demethylation treatment of genomic DNA by 5'-azacytidine increased FXYD1 mRNA levels in human cells lack of detectable *FXYD1* gene expression. Bisulfite genomic DNA sequencing mapped multiple methyl-CpG dinucleotides in the human *FXYD1* promoter region, and chromatin immunoprecipitation (ChIP) assays demonstrated that the endogenous MeCP2 protein physically associated with the native *FXYD1* promoters *in vivo*. Electrophysiological study using whole-cell patch clamp on mouse cortical neurons showed that Na^+, K^+ ATPase-dependent currents in cortical neurons were reduced in *Mecp2*-null mice, indicating that Na^+, K^+-ATPase activity was inhibited in neurons of these mutant mice. Taken together, these findings strongly suggest that the *FXYD1* gene is subjected to MeCP2-mediated, methylation-dependent transcriptional repression in both human and rodent brains, and that loss of the MeCP2 function leads to FXYD1 overexpression. Such an overexpression may result in an inhibition of neuronal Na^+, K^+-ATPase activity, which in turn may alter neuronal excitability and ultimately contribute to some aspects of neurological abnormalities in RTT. The results presented here demonstrate the first example of *FXYD1* gene regulation that is controlled by an epigenetic mechanism mediated through DNA methylation and MeCP2 in a tissue- and brain region-specific manner. More significantly, these findings imply, for the first time, a novel link between MeCP2-mediated epigenetic gene

regulation and neuronal excitability homeostasis maintained by a Na+, K+ - ATPase function. Missteps in these regulatory processes due to MeCP2 deficiency may modify neural activity and underlie the neuropathogenesis of RTT.

In summary, Chapter Two of this thesis reports that the *FXYD1* gene, encoding a protein involved in the regulation of cell excitability via modulation of Na^+,K^+-ATPase activity [70], is a direct MeCP2-target that becomes selectively overexpressed in the absence of functional MeCP2 in the frontal cortex of both RTT patients and Mecp2-null mice.

Materials and Methods

Tissue Acquisition and RNA Isolation. Human brain tissue from the frontal lobe of RTT patients and age-matched normal controls were acquired from two sources: the Kennedy Krieger Research Institute and the Harvard Brain Tissue Resource Center (**Table 2.1**). The *Mecp2*-null mice and their littermate wild-type controls were obtained by breeding heterozygous MeCP2-deficient female mice (JAX: strain B6.129P2©-Mecp2$^{tm1.1Bird}$) with wild-type C57BL/6 males. Cerebral cortex tissue from 40 days-old wild-type and *Mecp2*-null mice was dissected in cold PBS, submerged immediately in RNALater (Ambion, Austin, TX) and stored at –20°C until use. Total RNA from both human and mouse tissues was isolated and purified using RNeasy Mini Columns and DNase On-Column digestion (Qiagen, Valencia, CA) according to the manufacturer's specifications.

Affymetrix GeneChip Oligonucleotide Array Analysis. Total RNA from five Rett syndrome patients was pooled to generate samples representing three age groups (2-4, 5-6, and 8-10 year of age) and these samples were compared to their respective age-matched normal controls. Total RNA was labeled and hybridized to six Affymetrix GeneChip oligonucleotide arrays at the Gene Microarray Shared Resource (GMSR), OHSU. To ensure the quality of hybridization labeled RNA samples were first hybridized to a test array containing 345 control probe sets in total, including 37 human probe sets. Quality-tested samples were then hybridized to the HU_U95Av2 array containing 12,625 human probes. Results were processed with Affymetrix Microarray Suite 4.0 (MAS 4.0). Comparative analysis was performed for each paired sample (i.e., RTT patients

and age-matched normal controls) and the difference in hybridization intensity of each pair of samples was calculated and expressed as a fold-change (RTT/ normal control). Genes showing an absence call on all 6 arrays and genes consistently showing less than 2-fold change in either direction between RTT and normal controls in any age group were removed from the analysis. Genes sharing a similar expression pattern in all age groups were clustered and visualized via a K-means clustering algorithm using J-Express2.0 software.

Quantitative RT Real-time PCR. To verify and expand the results of the DNA arrays we subjected RNA samples from RTT and normal brains to Real-time PCR analysis. The procedure used (**Supplementary Note 1**) has been described in detail elsewhere [121].

Western Blots. FXYD1 and MeCP2 were detected in immunoblots using previously characterized polyclonal antibodies [25,78], and following the procedure described in **Supplementary Note 2.**

5' Aza-Cytidine Treatment and RT-PCR. Human IMR32 neuroblastoma and embryonic kidney 293 cells were seeded into 6-well culture plates at low density (100,000 cells/well) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Twenty-four h after plating, the cells were treated with 5-aza-cytidine (Sigma) (5 or 50 μM) or were mock-treated with the same volume of phosphate-buffered saline for 48h changing the medium every 24 h. Cells were rinsed once with PBS and scraped

into cold PBS. Total RNA was isolated using TRI Reagent (Molecular Research Center, Inc, Cincinnati, OH), and reverse transcribed (1μg) using the Omniscript® RT kit (Qiagen) and random hexamers primers. Thereafter, 1μl of cDNA was PCR-amplified using Hot Star Taq polymerase (Qiagen) and primers specific to the human FXYD1 coding region. PCR conditions were 15 min at 94 °C, then 30 cycles of 45 sec at 95 °C, 1 min at 55 °C, 1 min at 72 °C, and a final extension period of 5 min at 72 °C. The products were resolved on a 1.5% agarose gel. Each of the PCR assays was run in duplicate.

Bisulfite PCR Sequencing of Human Genomic DNA. Genomic DNA from adult human brain and heart were purchased from BioChain, Inc (Hayward, CA). The presence of 5'-methylcytosines was determined either by bisulfite PCR or bisulfite genomic sequencing. Bisulfite conversion of DNA was performed as described [122], with slight modifications (see **Supplementary Note 3**). After bisulfite conversion, 2μl of the treated DNA were used for PCR amplification in a 50μl volume, using a HotStarTaq DNA polymerase (Qiagen). PCR conditions were 15 min at 95 °C, then 35 cycles of 1 min at 95 °C, 1 min at 60°C, 1 min at 72 °C, and finally 5 min at 72 °C. The primers used for bisulfite-PCR are listed in **Table 2.2**. The primers were designed to span 1.86 kb of the 5' flanking region upstream of exon 1b and 1a of the human FXYD1 gene according to the GenBank sequence (NT_011109.15). The bisulfite-PCR products were resolved on a 1.5% agarose gel, gel-purified using the Qiaquick™ Gel Extraction kit (Qiagen) and cloned into pGEM®-T Easy vector (Promega, Madison, WI). Plasmid DNA from at least 10 clones was purified using the Qiaprep® Spin

Miniprep kit and subjected to automated DNA sequencing from both directions using M13 forward and M13 reverse sequencing primers (Applied Biosystems, Foster City, CA).

***In vitro* Methylation and Luciferase Reporter Assay.** A 1518bp fragment from the 5' franking region of the human FXYD1 gene encompassing the two predicted FXYD1 alternative promoter was cloned into pGL3-Basic luciferase expression vector. The pGL3-FXYD1 promoter construct and the control pGL3-Basic vector were methylated with M SssI CpG methylase or left intact. One μg of the methylated or unmethylated plasmid was transiently transfected into 293T cells grown to 50% of confluence in 6-well plates, with or without an expression plasmid carrying the MECP2 gene (HA-MeCP2). Luciferase activity was measured 48h later according to the manufacture's recommendations (Promega).

Plasmid Constructs and Transient Transfections. The pCMV-HA-MeCP2) construct and the MeCP2 mutant construct (pcDNA-MeCP2-TRD) were the generous gift from A. Bird (University of Edinburgh, UK) and Dr. Robert A. Drewell (California Institute of Technology, Pasadena, CA), respectively. Human embryonic kidney 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. When reaching 70% confluence, the cells were transfected with 1μg of pCMV-HA-MeCP2 or pcDNA-MeCP2-TRD using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to

the manufacturer's protocol. After 48h, the cells were subjected to chromatin immunoprecipitation assay.

Chromatin Immunoprecipitation Assay (ChIP). Chromatin immunoprecipitation assays were carried out using a Chromatin Immunoprecipitation Assay Kit (Upstate, Lake Placid, NY) as described in **Supplementary Note 4**. To amplify DNA fragments containing *FXYD1* 1a and 1b promoter regions, PCR analysis was carried out using HotStar Taq polymerase (15 min at 95 °C, then 32-34 cycles of 30 sec at 94 °C, 30 sec at 60 °C, and 45 sec at 72 °C. The PCR primer pairs used for amplification are listed in **Table 2.2**.

Immunohistochemistry and *in situ* hybridization. To localize FXYD-1 mRNA in the mouse brain we utilized a 274nt cRNA probe transcribed from a cDNA template generated by PCR amplification of mouse cerebral cortex RNA. The procedure used for tissue preparation and hybridization has been previously reported [123] and it is briefly described in **Supplementary Note 5**. To detect FXYD1 immunoreactive protein we used a procedure previously described [43] (see also **Supplementary Note 5**), and FXYD1 antibodies produced in rabbits (generously provided by Dr. Randall Moorman, University of Virginia, Charlottesville, Virginia).

Electrophysiology. Slice preparation and recording techniques were similar to those described previously [125]. Slices of frontal cortex (400 μm thick) were prepared from 40-45 day old mice using a Vibratome. After >1 hour of recovery

time, slices were transferred to a recording chamber perfused at room temperature with a solution consisting of (in mM): 119 NaCl, 26 $NaHCO_3$, 10 glucose, 2.5 KCl, 4 $CaCl_2$, 4 $MgSO_4$, and 1.0 Na_2HPO_4, bubbled with 95% O_2/5% CO_2. Layer II/III Pyramidal cells were visualized by infrared differential interference contrast microscopy, and recorded with patch electrodes (1-5 MΩ) using whole-cell voltage clamp.

Na, K-ATPase activity was assessed by the change in holding current induced by the specific Na, K-ATPase inhibitor, ouabain. Ouabain elicits membrane currents due to cessation of electrogenic pump activity [126], but also due to other subsequent effects of pump blockade, including extracellular potassium accumulation [127,128] and depolarization-evoked transmitter release [129], which eventually lead to spreading depression and cell damage. To enhance the relative contribution of the pump current to our recordings, we added 100 μM $CdCl_2$ to the external solution to prevent depolarization-induced calcium influx. We also used an internal solution that was loaded with sodium to increase the electrogenic pump current, as reported [126]. The internal solution contained (in mM): 25 Na gluconate, 115 K gluconate, 5 HEPES, 2 $MgCl_2$, 0.2 EGTA, 4 MgATP, 0.3 Na_3GTP, adjusted to pH 7.2, 270-290 mOsm.

Cells were held in voltage-clamp at –60 mV. Whole-cell currents were filtered at 2 kHz and digitized at 2.5-5 kHz. Data were analyzed online with Igor Pro and offline with Sigma Plot. All experiments and analysis were performed

blind with respect to animal genotype. Wild type and *Mecp2*-null mice were taken from the same colony, and 6 animals were used in each group.

Results

FXYD1 gene expression is abnormally increased in the frontal lobe of RTT patients

To identify genes that might be overexpressed in the brain of RTT patients due to MeCP2 mutations, we utilized Affymetrix oligonucleotide arrays to compare the gene expression profile of postmortem brains affected by RTT to that of age-matched normal brains (Table 2.1). Because previous studies have shown that the prefrontal cortex is most evidently affected by the disease [105], we selected the superior frontal gyrus as a representative brain region in our study. Total RNA extracted from five RTT and three normal controls of ages spanning the first 10 years of postnatal life was hybridized to a total of six Affymetrix Human U95v2.0 arrays. Data analysis was carried out using Affymetrix MAS4.0 software and a data filtering strategy was applied according to the criteria described in Material and Methods. A total of 1232 genes fulfilled these criteria and showed fold changes of two-fold or greater between RTT and normal controls in at least one age group. The genes with similar expression patterns across all three age groups were clustered and visualized using a K-means clustering algorithm provided by the J-Express software. The data output revealed the presence of a cluster of 26 genes (Fig. 2.1-a) whose expression was elevated in all age groups (between 2 and 10 years of age). Within this cluster, the gene most consistently overexpressed was *FXYD1* (Fig. 2.1-a, red profile). The *FXYD1* gene encodes the FXYD1 protein or phospholemman (PLM), a small, single-spanned membrane protein shown to control excitability and contractility in heart and

skeletal muscles by modulating Na^+, K^+-ATPase activity [15]. Most recently, FXYD1 has been found in the CNS with prominent sites of expression in cerebellum and choroid plexus, where it interacts with the Na^+, K^+-ATPase alpha subunit isoform [78].

To validate the array results, FXYD1 mRNA was measured by quantitative real-time PCR in the RNA samples used for array hybridization. The results showed that FXYD1 mRNA abundance was, indeed, increased in the RTT brains (Fig. 2.1-b). To determine whether this overexpression is also evident in other brain samples derived from RTT patients, we performed quantitative real-time PCR analysis of reverse-transcribed RNA samples generated from three additional RTT patients and three normal controls at older ages. As shown in Figure 2.1-c, FXYD1 mRNA levels were also increased in these patients in comparison with their normal controls.

The *FXYD1* gene is selectively overexpressed in the prefrontal cortex of *Mecp2*-null mice

We then determined whether the relative abundance of *FXYD1* mRNA and FXYD1 protein would also increase in *Mecp2*-null mice, a rodent model that mimics the symptoms observed in human RTT patients [24, 25]. We dissected brain tissues from *Mecp2*$^{-/y}$ male mice of 40-45 day old, the time when the mutant mice become evidently symptomatic. Various anatomical structures that can be reproducibly dissected were chosen to minimize the experimental noise. The

FXYD1 mRNA level assessed by quantitative real-time PCR was found to increase about 2-fold in the cerebral cortex of *Mecp2*$^{-/y}$ male mice as compared with wild-type littermate controls (Fig. 2.2-a). This change in FXYD1 mRNA level was accompanied by a relatively smaller but significant increase in FXYD1 protein abundance, detected by Western blot analysis (Fig. 2.2-b). To confirm the loss of MeCP2 protein in the cerebral cortex of *Mecp2* null mice, we performed immunohistochemistry and found MeCP2 immunoreactive material to be absent in neuronal nuclei (Fig. 2.2-c and -d), as previously reported in the brain of *Mecp2-null* mice using the same antibodies [24]. To define the sites of *FXYD1* mRNA and protein expression in the cerebral cortex of *Mecp2* mutants and wild-type mice, we performed *in situ* hybridization and immunohistochemistry experiments. Hybridization histochemistry using a ^{35}S-UTP-labeled mouse *FXYD1* cRNA probe demonstrated an overall increase (~ 2-fold) in *FXYD1* mRNA levels in the cerebral cortex of *Mecp2*-null mice (Fig. 2.2-e and -f) consistent with the increase detected by real-time PCR (Fig. 2.2-a). No specific signal was observed in sections hybridized to a sense RNA probe (Fig. 2.2-g). Immunohistochemical detection of the FXYD1 protein demonstrated an apparent increase in the number of neurons with detectable immunoreactivity in the brain of *Mecp2-null* mice as compared with their wild-type littermate controls (Fig. 2.2-h-l). As previously reported [5], the neuronal cell bodies in *Mecp2*-deficient mice appeared smaller and more densely packed than in controls (Fig. 2.2-k and -l). Omission of the FXYD1 antibodies in adjacent sections resulted in no detectable

staining (Fig. 2.2-j). Thus, as observed in RTT patients, the *FXYD1* gene was overexpressed in the brain of *Mecp2*-null mice.

Our studies to this point focused on the frontal cortex, as this region is one of the most affected areas by RTT. To determine whether the regulation of FXYD1 expression is similarly region-specific, we compared *FXYD1* mRNA expression in different brain regions of wild-type and *Mecp2*-null mice. We found that FXYD1 was overexpressed in the brain of *Mecp2*- null mice in a region-specific manner, with the highest amount of overexpression in frontal cortex, less in premotor cortex and no significant change in any of several other brain regions examined (Fig. 2.3). This region-specific increase in *FXYD1* gene expression was, in general, inversely related to the pattern of *FXYD1* mRNA distribution detected in the brain of wild-type mice, i.e. the *FXYD1* mRNA level in wild-type mice was significantly lower in the frontal cortex than in all other brain regions studied, including the hippocampus, hypothalamus, cerebellum and brain stem (Fig. 2.3, inset). Consistent with a recent report on FXYD1 protein distribution in the rat brain[78], our data also showed that the *FXYD1* mRNA appeared to be expressed at the highest level in the cerebellum. Intriguingly, the brain region-specific overexpression of *FXYD1* seemed coincident with the regional and cellular heterogeneity in MeCP2 protein distribution in rat and non-human primate brains with the highest level in the frontal cortex [54, 55].

FXYD1 gene expression is subjected to transcriptional repression mediated by DNA methylation

Methylation of cytosines in CpG dinucleotides, the most frequent epigenetic modification in mammalian genomes, represents a common epigenetic mechanism for transcriptional inactivation of genes [106]. Tissue-specific genes are often methylated in non-expressing tissues. MeCP2 has been shown to mediate transcriptional silencing by selectively binding to methylated cytosines in CpG dinucleotides and recruiting a transcription repressor complex [46]. Given that the *FXYD1* gene is overexpressed in the cerebral cortex of both RTT patients and *Mecp2*-null mice, we hypothesize that the *FXYD1* gene is subjected to MeCP2-mediated transcriptional repression in a DNA methylation-dependent fashion. To test this idea, we challenged the human embryonic kidney 293T and neuroblastoma IMR32 cells with the demethylating agent 5'-aza-cytidine. We found that *FXYD1* mRNA levels, barely detectable in these cells, increase markedly in response to demethylation treatment (Fig. 2.4-a), indicating that FXYD1 gene expression is regulated in a methylation-dependent fashion in these cells.

We then sought to determine the methylation status of the native human FXYD1 promoter. With this purpose on mind, we carried out bisulfite genomic DNA sequencing using genomic DNA from human frontal lobe and heart. *FXYD1* expression has shown to be low in the former, but abundant in the latter. The human *FXYD1* gene is localized to chromosome 19q13.1 and two transcript variants (*FXYD1a* and *FXYD1b*) encoding the same coding region with different 5' UTR sequences have been described [75]. Based on our *in silico* analysis using

the Genomatix promoter database (http://www.Genomatix.de), *FXYD1a* and *1b* appear to be transcribed from alternative transcription sites using different promoters. Two sets of PCR primers were designed to amplify either the *FXYD1a* or the *FXYD1b* promoter (Fig. 2.4-b) after bisulfite conversion of genomic DNA. The PCR products were subsequently cloned into the pGEM-T plasmid and at least 10 independent clones were sequenced from each promoter fragment. Our results showed that the two *FXYD1* promoters contained methylated cytosines in both the frontal lobe and heart, but that they – and in particular the *FXYD1a* promoter - were much more methylated in the frontal lobe than in the heart (Fig. 2.4-b). Notably, a predicted CpG island in the *FXYD1a* promoter contains the putative binding sites of transcription factors, such as SP1 and other basal transcription factors (TBP and TFIID), identified by the TESS program (http://cbil.upenn.edu/tess). These results strongly suggest that DNA methylation plays an important role in the tissue-specific regulation of *FXYD1* gene expression, and that methylation of the *FXYD1* promoter may contribute to the transcriptional repression of *FXYD1* gene expression in the frontal cortex of humans and mice. In fact, such a repression is also consistent with the lowest level of *FXYD1* gene expression in the cerebral cortex of both humans and rodents in comparison with other brain regions ([78] and Fig. 2.3).

To determine the effects of methylation on *FXYD1* promoter activity *in vitro*, we cloned a 1510 bp fragment of the *FXYD1* 5' flanking region encompassing the putative *FXYD1b* and *FXYD1a* promoters into a luciferase

reporter vector (pGL3-Basic), and subjected this construct to *in vitro* methylation catalyzed by M. SssI CpG methylase. The methylated and non-methylated empty vector and promoter construct were then transfected into 293T cells .The promoter activity was analyzed 48 h after transfection by measuring luciferase activity [21]. Although basal luciferase activity of the methylated pGL3-Basic vector alone was much lower than that of the non-methylated vector, the activity of the methylated *FXYD1* promoter construct was much more strikingly reduced (25-fold) relative to the unmethylated promoter (Fig. 2.4-c). Next we determined whether MeCP2 could cause further reduction in the transcriptional activity of the methylated *FXYD1* promoter construct. To do so, 293T cells were cotransfected with the reporter constructs along with either a pCMV HA-tagged MeCP2 expression vector [25] or the empty pCMV plasmid. As shown in Figure 2.4-c, indeed, expression of the MeCP2 protein further repressed the transcriptional activity of the methylated *FXYD1* promoter construct, but had no effect on that of the methylated empty vector (Fig. 2.4-c). Therefore, MeCP2 may specifically interact with and repress the *FXYD1* promoter activity.

The FXYD1 promoter is an endogenous MeCP2-target *in vivo*

MeCP2 selectively binds to a single methylated CpG dinucleotide site regardless of the sequence context via its methyl CpG binding domain (MBD). It then recruits a histone deacetylase (HDAC)-containing complex to the methyl-CpG region by interacting with the co-repressor Sin3A via its transcriptional repression domain (TRD). Formation of this complex leads to chromatin

condensation and thus promotes gene silencing [46]. Thus far, brain-derived neurotrophin factor (*BDNF*) is the only gene known to be transcriptionally repressed by MeCP2 in an activity-dependent fashion in the mammalian brain [47, 48]. To determine whether *FXYD1* is a MeCP2-target *in vivo*, we performed ChIP assays using a rabbit polyclonal MeCP2 antibody. The ChIP assays, using five sets of *hFXYD1* promoter-specific primers (Fig. 2.5-a), revealed that MeCP2 bound to the native FXYD1 promoter region in human 293T cells transiently transfected with a pCMV-HA-MeCP2 expressing vector (Fig. 2.5-b). No FXYD1 promoter fragments were detected in cells transfected with a mutant MeCP2 construct lacking the transcription repression domain (TRD) (Fig. 2.5-b), perhaps due to the failure of MeCP2 antibody to recognize the mutant proteins. Western blot analysis of protein extract from the transfected cells confirmed that the MeCP2 protein was indeed expressed after transfection, as a molecular specie identical in size to the MeCP2 protein detected in the cerebral cortex of wild-type mice (Fig. 2.5-c). To further determine whether the native FXYD1 promoter is an endogenous MeCP2 target in the mouse cerebral cortex, and whether the association of MeCP2 with the FXYD1 promoter is abrogated in *Mecp2*-null mice, we conducted ChIP assays using cortical tissues from wild-type and *Mecp2*-null mice. The results showed that MeCP2 was indeed recruited to the *FXYD1* promoter region in the cerebral cortex of wild-type, but not *Mecp2*-null mice (Fig. 2.5-f), thus demonstrating that endogenous MeCP2 physically associates with the native *FXYD1* promoter in a physiological context in the mouse brain. In additional ChIP assays, and using a polyclonal antibody against HDAC1, we also

detected endogenous HDAC1 associated with the *FXYD1* promoter region in mouse cerebral cortex, suggesting that the HDAC1-containing co-repressor complex is also recruited to the *FXYD1* promoter region (Fig. 2.5-d).

Na^+, K^+-ATPase function is altered in the cerebral cortex of *Mecp2*-null mice

The aforementioned results do not address the issue as to whether overexpression of FXYD1 has a functional consequence. FXYD1 is known to associate with the Na^+, K^+-ATPase [reviewed in [63]], and coexpression of FXYD1 with the Na^+, K^+ -ATPase inhibits Na^+, K^+-ATPase function by reducing the affinity of the ATPase for Na^+ and K^+ [70]. A FXYD1 homologue has also been found to inhibit Na^+, K^+-ATPase in sharks [74]. Very recently, FXYD1 overexpression has shown to inhibit Na^+, K^+-ATPase activity in postmyocardial infarction myocytes of rats [135]. These results suggest that the increase in FXYD1 expression seen in *Mecp2*-null mice might inhibit Na^+, K^+-ATPase activity in the cerebral cortex of these mice.

To test this possibility, we made whole-cell voltage clamp recordings from pyramidal neurons in slices of cerebral cortex from *Mecp2*-null and wild-type mice. The Na^+, K^+-ATPase is tonically active, and this activity can be assessed by monitoring the effects of ATPase inhibition. We found that the Na^+, K^+-ATPase inhibitor ouabain (1 mM) induced a change in the holding current of pyramidal neurons of both *Mecp2*-null and wild-type mice (Fig. 2.6-a). When quantitatively compared between genotypes, this ouabain-induced current was reduced in cells

from *Mecp2*-null mice (n=8) when compared to those from wild type mice (n=8; Fig. 2.6-b and -c). Neither the holding current (Fig. 2.6-d) nor the input resistance (Fig. 2.6-e) of pyramidal cells differed between wild type and *Mecp2*-null mice, indicating that the basal electrical properties of these cells are not genotype-dependent. These results suggest that Na^+, K^+-ATPase activity is altered in *Mecp2-null* mice, consistent with the expected consequences of FXYD1 overexpression in these animals.

Discussion

Following the demonstration that mutations of the *Mecp2* gene are responsible for most cases of RTT [22], much endeavor has been devoted to unravel the mechanisms by which MeCP2 dysfunction causes RTT. The key is to identify MeCP2 downstream neuronal targets, as knowing which genes are derepressed in *Mecp2*-deficient individuals will not only improve our understanding of the etiology of RTT, but also help the design of viable therapeutical strategies. Although MeCP2 has been implicated in the activity-dependent regulation of neuronal *BDNF* transcription [47], to our knowledge only one gene has been shown to be both directly targeted by MeCP2 and deregulated in RTT and Mecp2-null mice [104]. *DLX5*, an imprinted gene expressed in GABAergic neurons and required for the synthesis of glutamic acid decarboxylase enzymes involved in GABA synthesis, loses imprinting in RTT patients and is overexpressed in the brain of *Mecp2*-null mice [104]. We have now identified *FXYD1* as another *bona fide* MeCP2-target gene. We show that the *FXYD1* gene was selectively overexpressed in the frontal cortex of both humans affected by RTT and *Mecp2*-null mice, and further demonstrate that expression of the *FXYD1* gene was subjected to MeCP2-mediated, DNA methylation-dependent transcriptional repression in the frontal cortex of both species. FXYD1, also known as phospholemman (PLM), belongs to a family of small, membrane-spanning proteins involved in the tissue-specific homeostatic regulation of the Na^+, K^+-ATPase [63].

Epigenetic transcription regulation of *FXYD1* by DNA methylation and MeCP2

FXYD1 was originally described as being highly expressed in the heart and skeletal muscle [75]; more recently, it was also shown to be present in the brain where it is physically associated to the alpha subunits of the Na^+, K^+-ATPase [78]. Notably, FXYD1 immunoreactivity has a brain region-specific distribution, with highest levels seen in the cerebellum and choroid plexus [78]. In agreement with these findings, our results show that *FXYD1* mRNA content varies widely in different regions of the mouse brain, with the highest value in the cerebellum and lowest in the frontal cortex.

It has been recently postulated that MeCP2 may not act as a global transcriptional repressor in mammalian as previously thought [58, 108], and instead, MeCP2 plays a specific role in the CNS. This notion is well supported by the developmentally heterogeneous, and region-, cell-specific expression patterns of MeCP2 in both rodent and non-human primate brains[26, 54, 55]. Possibly, MeCP2 may only control gene expression in selected subsets of neuronal genes, as suggested by recent discoveries of its regulatory effect on *DLX5* imprinting expression [104] and on activity-dependent changes in *BDNF* gene transcription [47, 48]. While MeCP2 expression in the frontal cortex is the highest [55], the *FXYD1* expression, as shown here, is the lowest in the mouse brain. This feature and the selective deregulation of *FXYD1* expression observed in the absence of functional MeCP2 suggest that MeCP2-mediated gene silencing is critical in regulation of *FXYD1* gene expression in the frontal cortex. Because no such

deregulation is observed in other brain areas, the control of *FXYD1* expression by MeCP2 appears to be region-specific. Interestingly, the frontal cortex is the brain region most severely affected in RTT [105]. One possible explanation for aforementioned region-specificity is that it is caused by local differences in MeCP2 expression; an alternative explanation is that methylation of the *FXYD1* promoter is less pronounced in other brain regions than the frontal cortex. Indeed, our results from bisulfite methyaltion mapping demonstrated the *FXYD1* promoter regions were differentially methylated in the human frontal lobe, further supporting the concept that DNA methylation-dependent, MeCP2 mediated epigenetic effects may contribute to brain region-, and possibly cell-specific, transcriptional regulation of *FXYD1* gene expression in the brain.

Functional consequences of *FXYD1* overexpression in *Mecp2*-null mice

FXYD1 regulates Na^+, K^+-ATPase activity by lowering the affinity of the enzyme's subunits for both the external K^+ and the internal Na^+ ions [70]. Therefore, we reasoned that Na^+, K^+-ATPase activity might be decreased in the FXYD1-overexpressing frontal cortex of *Mecp2*-null mice. In consistence with this prediction, our results show that blocking the pump with ouabain in cortical pyramidal neurons resulted in a much smaller current in *Mecp2* null mice than in their wild-type counterparts. Thus, a reduced neuronal Na^+, K^+-ATPase activity is a distinct abnormality of cerebro-cortical neurons in *Mecp2*-null mice. A relative inability of the neurons to maintain adequate Na^+ and K^+ gradients across the cell membrane under resting conditions, and/or to restore these gradients during

neuronal activity, might result in abnormal neuronal excitability. In fact, a decrease in Na^+, K^+-ATPase activity causes neuronal depolarization [109, 110] and increases neuronal excitability [111]. Because of this effect, inhibition of Na^+, K^+-ATPase activity has been implicated as a mechanism underlying epileptogenic seizures [109, 111, 112], a feature commonly observed in RTT patients and *Mecp2*-null mice.

Another potential mechanism linking an excess of FXYD1 to defects in neuronal excitability resides in the relationship between FXYD1 and Na^+/Ca^{2+} exchanger 1 (NCX1). Driven by the sodium electrochemical gradient generated by Na^+, K^+-ATPase, NCX1 efficiently extrudes Ca^{2+} in exchange for Na^+, thus maintaining a low resting level of intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$)[132]. In cardiac cells, FXYD1 has shown to downregulate the activity of NCX1 [113]. The fact that NCX1 is also highly expressed in cerebro-cortical neurons [114, 115], coupled to the finding that loss of Na^+,K^+-ATPase activity increases Ca^{2+} conductance [111], implicates that FXYD1 overexpression may also increase neuronal excitability by inhibiting NCX1 activity, directly or indirectly via modulation of the Na^+,K^+-ATPase activity.

Implications for the RTT neuropathology

Our results indicate that *FXYD1* gene expression is deregulated in RTT patients. Intriguingly, RTT does not appear to be the only brain disorder in which *FXYD1* gene expression is dysregulated. Recent studies have shown that *FXYD1* expression is strikingly down-regulated in schizophrenia [116] and up-

regulated in the aging brain [117]. Thus, FXYD1 appears to represent an important, but hitherto underappreciated, molecule required for the homeostatic normalcy of neuronal function.

In this context, it is also noteworthy that mutations in Na^+, K^+-ATPase subunits, such as α2 subunit (ATP1A2), α3 subunit (ATP1A3), and γ-subunit (also known as *FXYD2*) have also been linked to several genetic disorders that share clinical features and neuronal abnormalities with RTT. These disorders include familial hemiplegic migraine and benign familiar infantile convulsions [118], rapid –onset dystonia parkinsonism [119], and akinesia in mouse [120]. Like in RTT, individuals affected by these syndromes exhibit various degrees of motor dysfunction, mental retardation, respiratory irregularity, and seizures, implying that some overlapping mechanisms of neuropathy may involve dysfunction of ion transportation mediated by Na^+, K^+-ATPase. Therefore, it is conceivable that inhibition of brain Na(+), K(+)-ATPase activity due to *FXYD1* gene deregulation may account, at least in part, for neuronal dysfunction characteristic of RTT patients.

Acknowledgement

These studies were supported by the Northwest Rett Syndrome Foundation in addition to NIH grants HD25123, U-54 HD18185, and RR00163 (SRO). Dr. Matthew Frerking is supported by NIH grant NS045101. I especially thank Dr. Matthew Frerking for conducting the excellent electrophysiology experiments and for productive scientific inputs and discussions. I thank Dr. Sarojini Budden for her support and her share of passion for the RTT study. I would also like to thank Drs. Mary Blue (Kennedy Krieger Institute) and Jonathan Pevsner (Johns Hopkins) for providing valuable RTT brain samples.

Figures and Tables

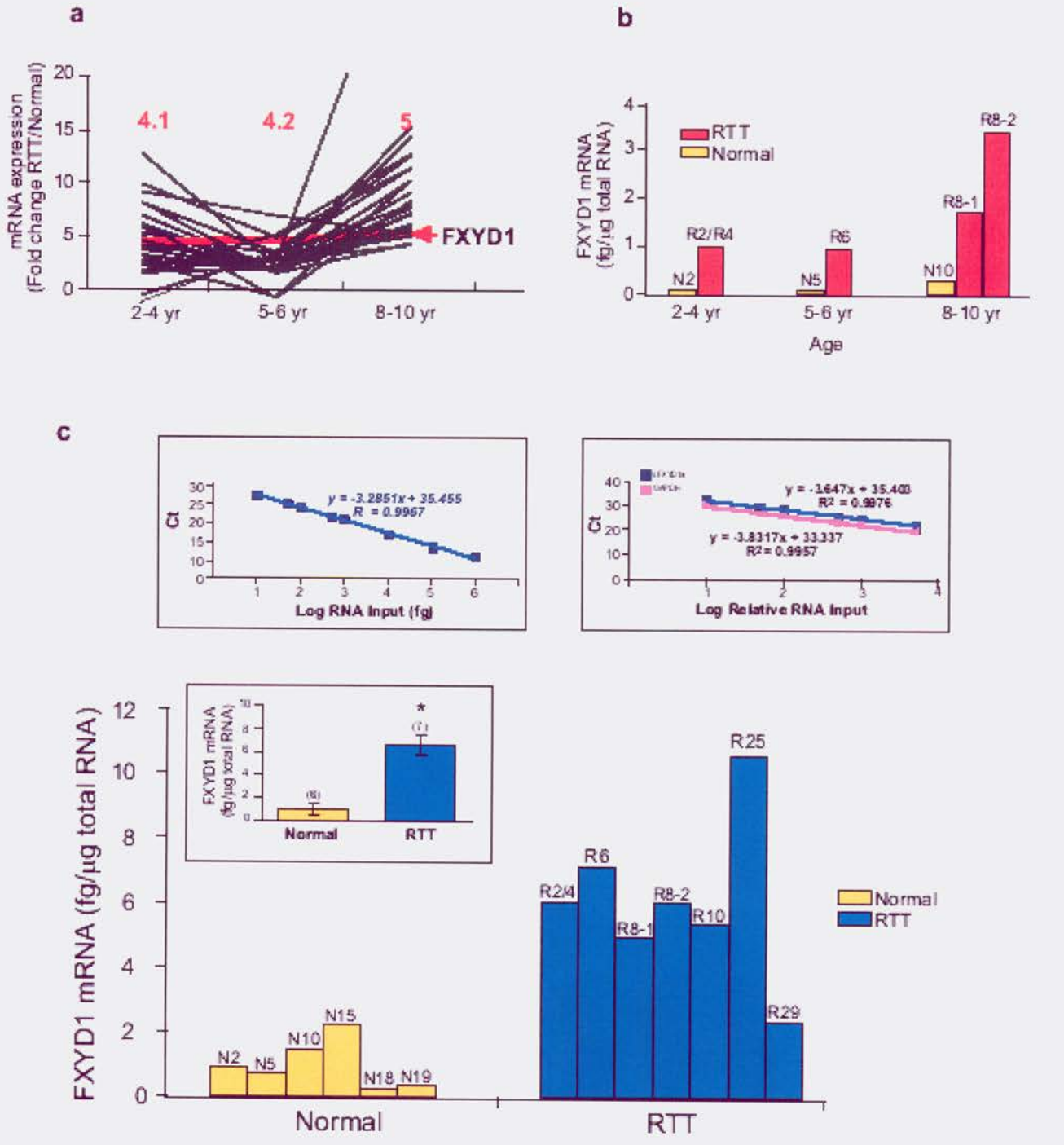

Figure 2.1. FXYD1 gene expression is consistently upregulated in RTT brains as detected by Affymetrix GeneChip arrays and quantitative real-time PCR.

(a) Total RNA extracted from the frontal lobe of five RTT patients and three normal controls with ages spanning the first 10 years of postnatal life were hybridized to six Affymetrix U95v2.0 arrays. The cluster (J-Express) shown here represents a group of 26 genes with increased expression in RTT brain as compared to age-matched normal controls across all age groups. Each line in the cluster represents the expression pattern of a single gene. The red line represents the *FXYD1* gene. The numbers in red indicate the fold-increase in *FXYD1* gene expression detected in RTT brains. (b) Quantitative real-time PCR verification of the change in *FXYD1* gene expression detected by DNA arrays in RTT patients (R2, R4, R6, R8-1 and R8-2) as compared to normal, age-matched controls (N2, N5, and N10). R2/R4 denotes a pooled RNA sample with equal contribution from R2 and R4. The same RNA samples used for the arrays were reverse-transcribed with random hexamer primers and used for real-time PCR amplification. 18S ribosomal RNA was used as an internal control for normalization purposes. For other details see Supplemental Note 1. (c) Quantitative real-time PCR analysis of FXYD1 mRNA expression in the frontal cortex from three additional RTT patients (R10, R25 and R29) and three normal controls (N15, N18 and N19) plus a re-measurement of the samples previously assayed (shown in Fig.1b). The *upper left panel* depicts a representative standard curve constructed with different concentrations (1 fg to 1ng) of FXYD1

mRNA transcribed from a FXYD1 cDNA isolated from adult human brain and cloned into the vector pGEM-T Easy. The *upper right panel* shows a representative relative standard curve constructed by serially diluting and PCR amplifying RNA from an experimental sample (R8-1). GAPDH was used as internal control. GAPDH mRNA content of each sample was estimated by referring each individual value to the relative standard curve. The *bottom panel* shows FXYD1 mRNA levels detected in all RTT and normal samples. The real-time PCR reactions were carried out as described in **Supplemental Note 1.**

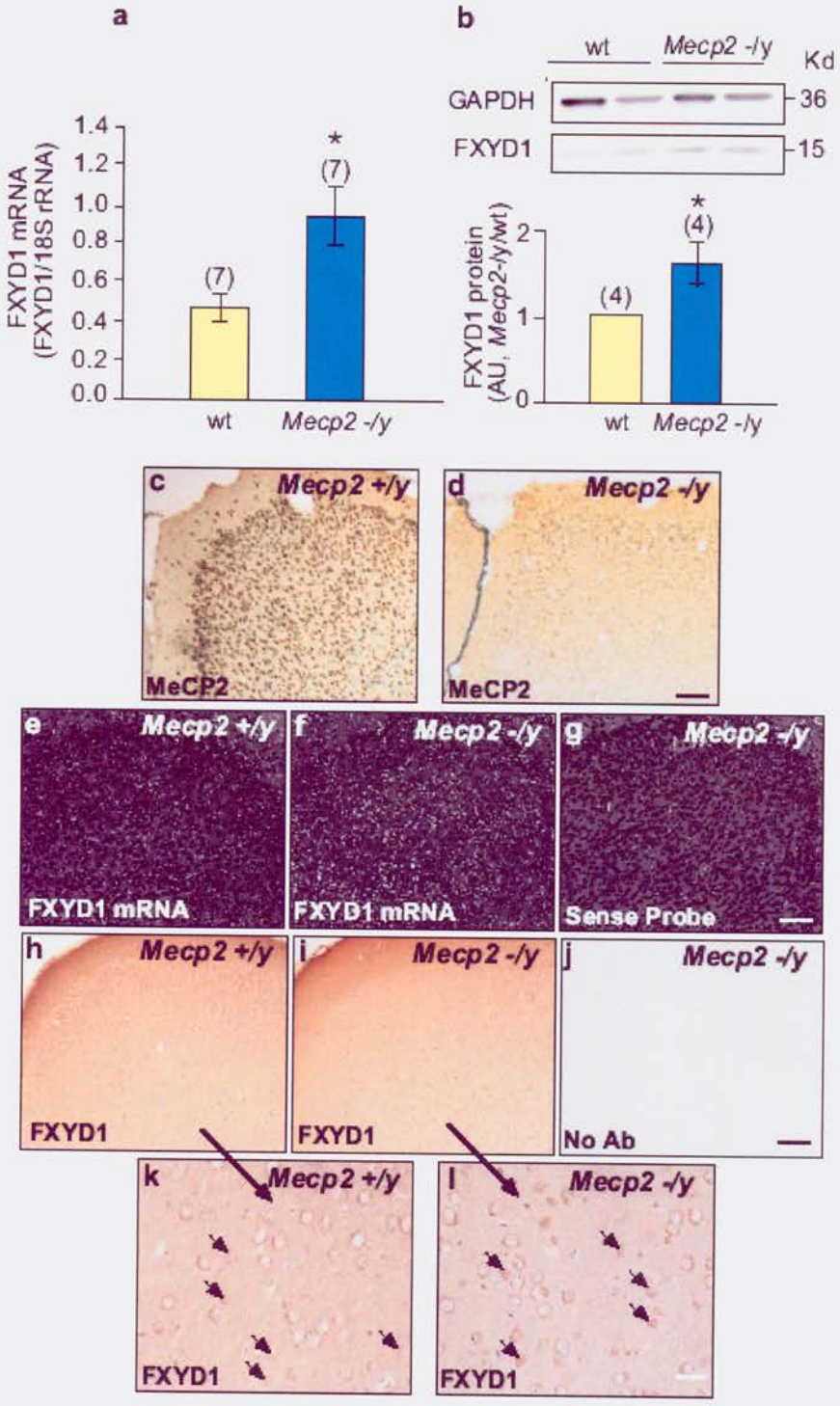
a
b
wt
Mecp2 -/y
Kd
GAPDH
36
FXYD1
15
FXYD1 mRNA (FXYD1/18S rRNA)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
(7)
(7)
*
wt
Mecp2 -/y
FXYD1 protein (AU, Mecp2-/y/wt)
2
1
0
(4)
(4)
*
wt
Mecp2 -/y
c Mecp2 +/y
MeCP2
d Mecp2 -/y
MeCP2
e Mecp2 +/y
FXYD1 mRNA
f Mecp2 -/y
FXYD1 mRNA
g Mecp2 -/y
Sense Probe
h Mecp2 +/y
FXYD1
i Mecp2 -/y
FXYD1
j Mecp2 -/y
No Ab
k Mecp2 +/y
FXYD1
l Mecp2 -/y
FXYD1

Figure 2.2. FXYD1 mRNA and protein content are increased in the cerebral cortex of *Mecp2*-null mice (a) FXYD1 mRNA abundance is significantly increased in the cerebral cortex of *Mecp2*-null mice as detected by real-time PCR. FXYD1 mRNA levels are expressed as the mean ratio of each FXYD1 value over its respective 18S rRNA value. (b) FXYD1 protein content is also elevated in the cerebral cortex of *Mecp2*-null mice as determined by Western blotting. GAPDH was used as a loading control. The values shown were calculated by first determining the FXYD1: GADPH ratio in each sample, and then expressing this ratio for each *Mecp2*-deficient mice as fold-change with regard to the value observed in its respective littermate control. In both a and b, bars represent group means, vertical lines are S.E.M., and number in parenthesis above bar are number of animals per group. *: $p<0.05$, significantly different from wild-type controls. (c-l) The increase in FXYD1 mRNA and protein abundance seen in cerebral cortex of Mecp2-null mice can also be detected at the cellular level. MeCP2 immunoreactivity detected in cerebrocortical cells of wild-type mice (c) is absent in *Mecp2-null* mice (d). The cerebral cortex of wild-type mice express low levels of FXYD1 mRNA distributed diffusely throughout the different layer of neurons (e); FXYD1 mRNA abundance is moderately increased in MeCP2-deficient mice (f), whereas no hybridization signal is detected in a adjacent section hybridized to a sense FXYD1 RNA probe (g). FXYD1 immunoreactive cells are detected through the cerebral cortex of both wild-type (h) and MeCP2-deficient mice (i), but they appear to be more numerous

in the latter, probably reflecting the detection of cells with increased FXYD1 content. No FXDY1 immunoreactivity is observed in sections incubated in the absence of FXYD1 antibodies (j). Higher magnifications views of the cerebral cortex of both control (k) and MeCP2-deficient mice (l) show that the increase in FXYD1 protein occurs in individual cells of apparently the same morphological phenotype (arrows). Bars in c-j = 100 μm; in k and l = 20 μm.

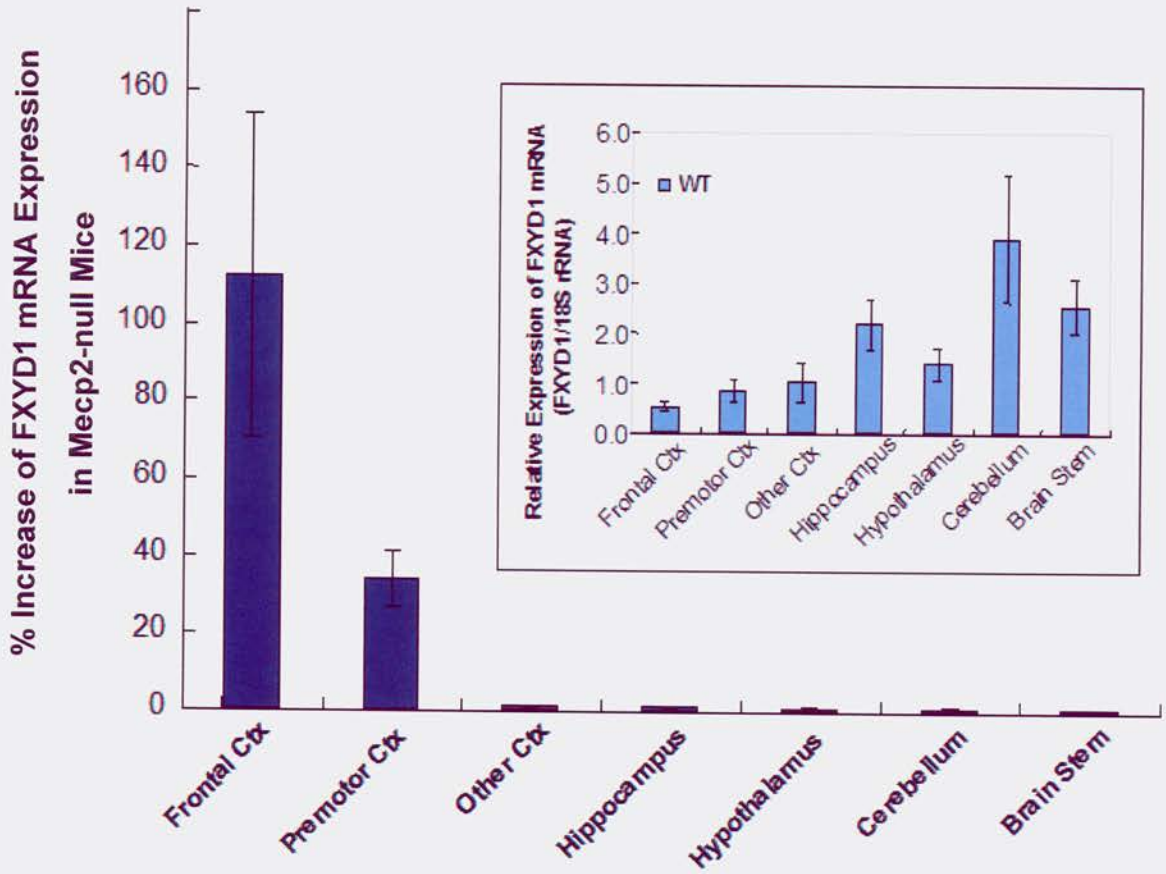

Figure 2.3. The FXYD1 gene is selectively overexpressed in the frontal cortex of MeCP2-null mice. Different brain regions derived from a total of five pairs of wild-type and *Mecp2*-null littermates were quickly dissected and submerged in RNALater for 24h at 4°C. Total RNA was then extracted and the FXYD1 mRNA content was measured by quantitative real-time PCR. Each FXYD1 mRNA value (FXYD1 mRNA/18s rRNA) derived from *Mecp2*-null tissues is expressed as a percent of the value detected in the respective littermate wild-type controls. *Inset:* FXYD1 mRNA content in different brain regions of wild-type mice. The changes in FXYD1 mRNA content observed in *Mecp2*-null mice (and depicted in the main panel) were calculated using these values as a reference.

Bars represent means and vertical lines are S.E.M. (n=5 mice per group).

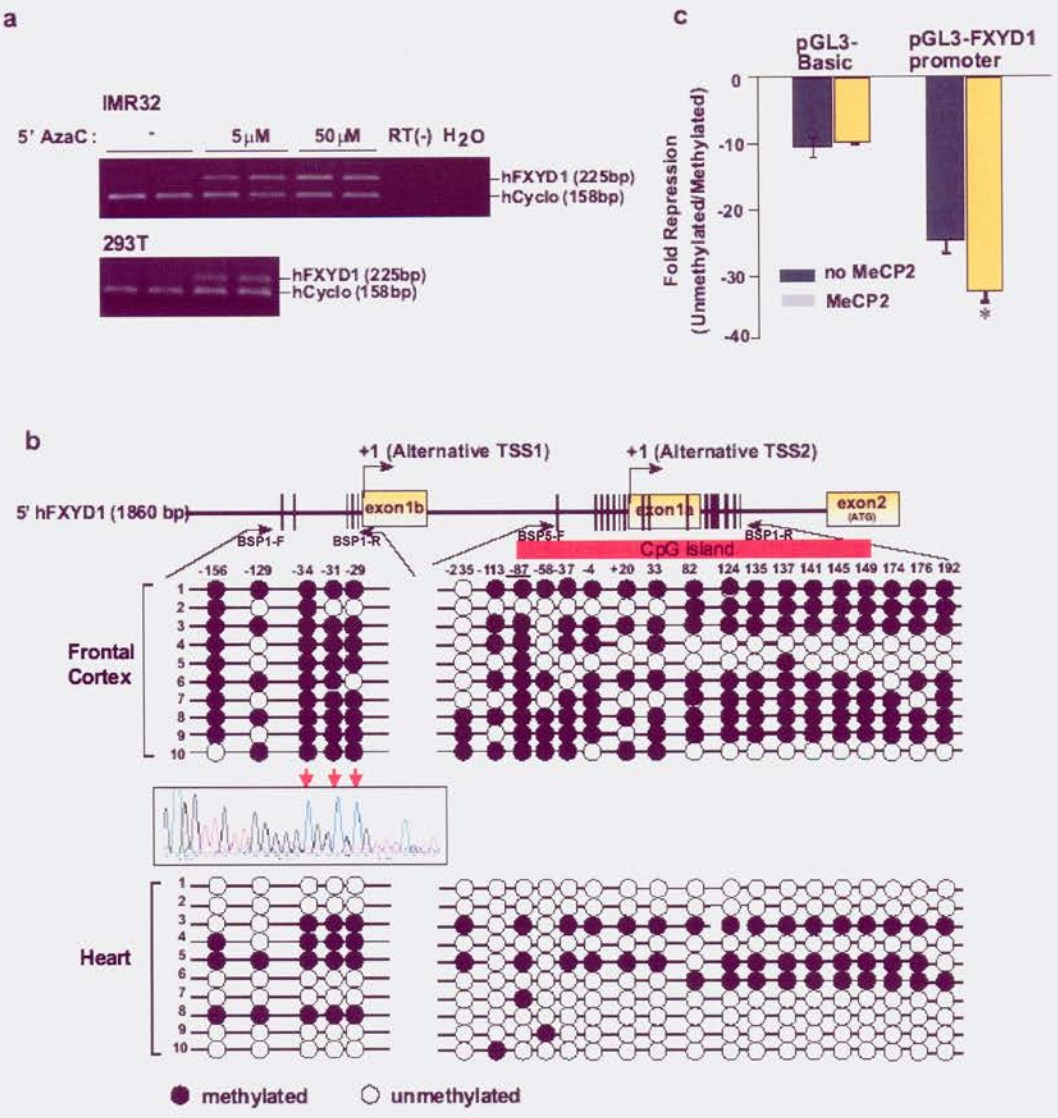

Figure 2.4. FXYD1 gene expression is subjected to methylation-dependent transcriptional regulation. (a) Activation of *FXYD1* gene expression by 5' azaC-induced demethylation. Human neuroblastoma IMR32 and human embryonic kidney 293T cells were plated in DMEM medium containing 10% FCS and then treated with 5 or 50 μM 5' azacytidine (5' AzaC) for 48h changing medium at 24h.

Total RNA was isolated and subjected to RT-PCR analysis using primers that amplify a 225bp DNA segment within the FXYD1 coding region (nt 148-372, GenBank accession No. NM_005031.3). A 158 bp fragment of human cyclophilin mRNA (nt 432-589, GenBank Accession No. M80235) was amplified as a control for procedural losses (b) Mapping the methylation status of the human *FXYD1* promoter region by bisulfite genomic DNA sequencing. Genomic DNA isolated from human frontal lobes and human heart was subjected to bisulfite treatment. The bisulfite-converted DNA was PCR amplified with a set of primers targeting overlapping DNA segments of 400-500 bp in length in the putative *FXYD1* promoter region, favoring bisulfite-converted DNA. The PCR products were cloned into pGEM-T EASY vector and the plasmid DNA from at least ten individual colonies was purified for subsequent DNA sequencing. The top diagram depicts the 5' flanking region of the FXYD1 gene with its two alternative transcription start sites (TSS1 and TSS2) used for transcripts NM_021902 and NM_005031, respectively. Arrows show location of primers used for PCR amplification of bisulfite-converted genomic DNA. The positions of CpG sites are numbered relative to the annotated FXYD1b or FXYD1a mRNA start site (+1). Positions underlined identify methyl-CpG sites located near or within transcription factor binding sites predicted using the TESS program (http://cbil.upenn.edu/tess). Each row represents individually sequenced PCR products. Methylated-CpG sites are shown as closed circles and unmethylated CpG sites as open circles. For CpG sites at –34, -31 and -29, automatic sequencing was also performed directly from PCR products amplified from

bisulfite converted genomic DNA from human frontal lobe to determine their methylation status. The sequencing profile is shown. The red arrows point to methyl-cytosines at these CpG sites, which are protected from bisulfite conversion from C to T. (c) Methylation reduces *in vitro* FXYD1 promoter activity and MeCP-2 accentuates this reduction. A 1,518 bp fragment from the 5' flanking region of the human *FXYD1* gene encompassing the predicted *FXYD1a* and *FXYD1b* promoters was cloned into pGL3-Basic luciferase expression vector. The pGL3-FXYD1 promoter construct and the control pGL3-Basic vector were then methylated by M. SssI CpG methylase or left unmethylated, and were transiently transfected into 293T alone or with either a MeCP2-expressing plasmid (pCMV-HA-MeCP2) or a pCMV control plasmid. Luciferase activity was measured 48 h later. The degree of promoter repression was calculated by dividing the values obtained with each methylated plasmid by the values obtained from their respective unmethylated counterparts. A decrease in luciferase activity, shown as a negative value, indicates fold repression of luciferase activity. Notice that MeCP2 enhances the reduction in methylated FYXD1 promoter activity, but not that of the plasmid alone. *: $p<0.05$, student t-test.

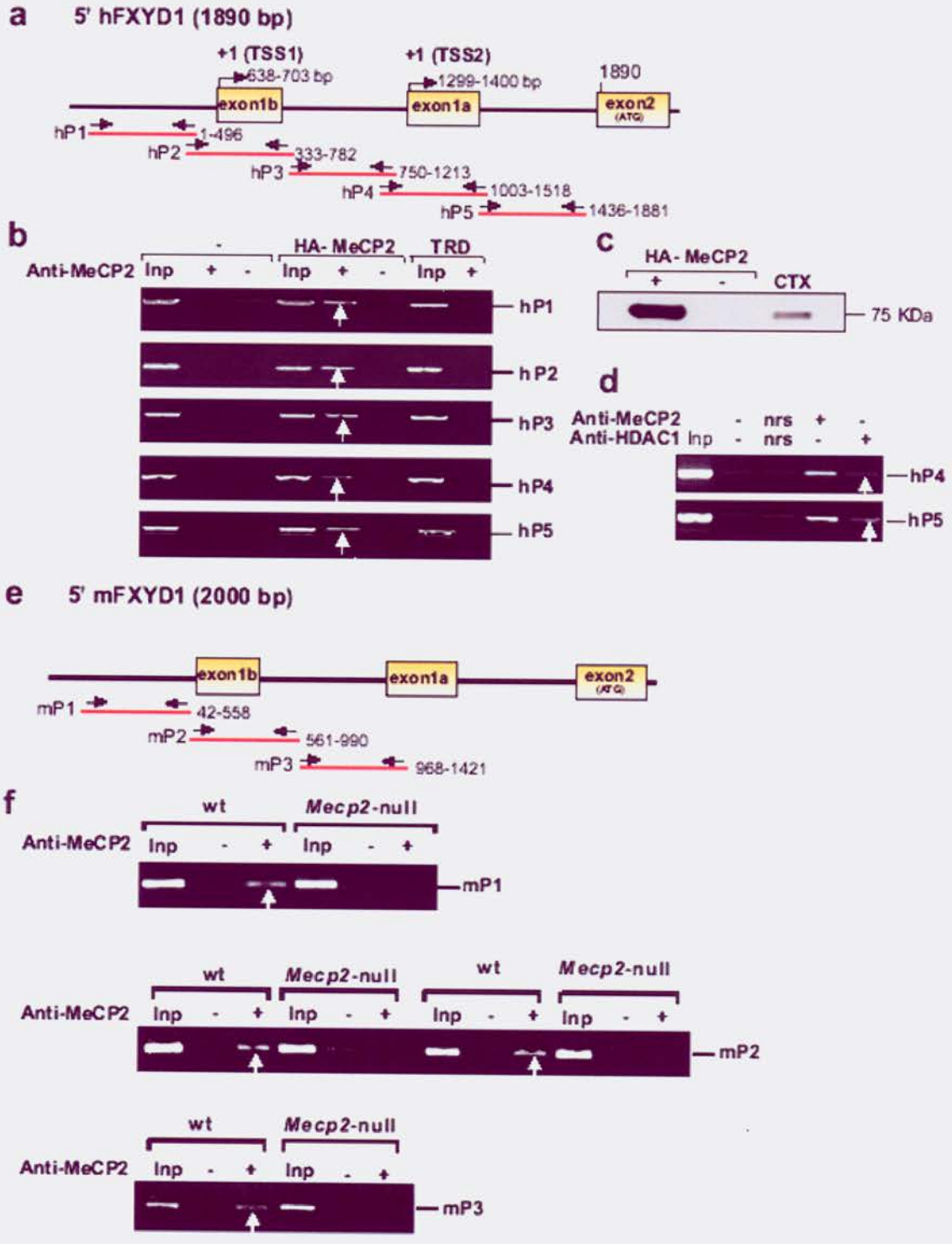
a
5' hFXYD1 (1890 bp)
+1 (TSS1)
638-703 bp
+1 (TSS2)
1299-1400 bp
1890
exon1b
exon1a
exon2
(ATG)
hP1
1-496
hP2
333-782
hP3
750-1213
hP4
1003-1518
hP5
1436-1881
b
HA- MeCP2
TRD
Anti-MeCP2
Inp
hP1
hP2
hP3
hP4
hP5
c
HA- MeCP2
CTX
75 KDa
d
Anti-MeCP2
Anti-HDAC1
nrs
hP4
hP5
e
5' mFXYD1 (2000 bp)
exon1b
exon1a
exon2
(ATG)
mP1
42-558
mP2
561-990
mP3
968-1421
f
wt
Mecp2-null
Anti-MeCP2
Inp
mP1
mP2
mP3

Figure 2.5. MeCP2 physically associates with the human FXYD1 promoter in MeCP2-expressing 293T cells (a) and with the mouse FXYD1 promoter in the frontal cortex of wild-type mice, but not *Mecp2*-null mice (f). Protein–DNA complexes derived from human 293T cells transfected with HA-MeCP2 or with TRD-MeCP2, a mutated form of MeCP2 lacking the transcriptional repression domain [49], or from untreated mouse cerebro-cortical tissue were cross-linked by incubation with 1% formaldehyde at 37°C. The chromatin was ultrasonically sheared to a length ranging from 500 to 1000 bp. Chromatin immunoprecipitation (ChIP) assays were performed using MeCP2 or HDAC1 polyclonal antibodies, using normal rabbit serum as a negative control (-). **a,** diagram depicting the 5 sets of primer pairs (hP1-hP5) used to PCR-amplify segments of 400-500bp from the 5' flanking region of the human FXYD1 gene, **b,** each set of primers amplifies DNA fragments from the FXYD1 promoter immunoprecipitated by the MeCP2 antibodies. The antibodies failed to immunoprecipitate any of these DNA segments from cells transfected with TRD-MeCP2, **c,** Western blot showing that the cells transfected with HA-MeCP2 express the MeCP2 protein, detected as a band identical in size to MeCP2 present in mouse cerebral cortex (CTX), **d,** endogenous HDAC1 also associates to the human FXYD1 promoter region in cells transfected with HA-MeCP2 as HDAC1 antibodies immunoprecipitate protein-DNA complexes containing DNA segments (hP4 and hP5) from the FXYD1 promoter, **e,** diagram showing the 3 sets of primers (mP1, mP2, mP3) used to PCR-amplify specific DNA segments from the mouse FXYD1 promoter, **f,** Protein-DNA complexes immunoprecipitated

with MeCP2 antibodies from the cerebral cortex of normal mice contain each of the DNA segments from the FXYD1 promoter targeted for PCR amplification, indicating that endogenous MeCP2 is associated with the native FXYD1 promoter under physiological conditions. This association was not present in the cerebral cortex of *Mecp2*-null mice. Arrows show FXYD1 promoter fragments amplified from chromatin immunoprecipitated by anti-MeCP2 or anti-HDAC1 antibodies. "Inp" = nonimmunoprecipitated input DNA ($1/100^{th}$ of the total sheared DNA). "nrs" = normal rabbit serum.

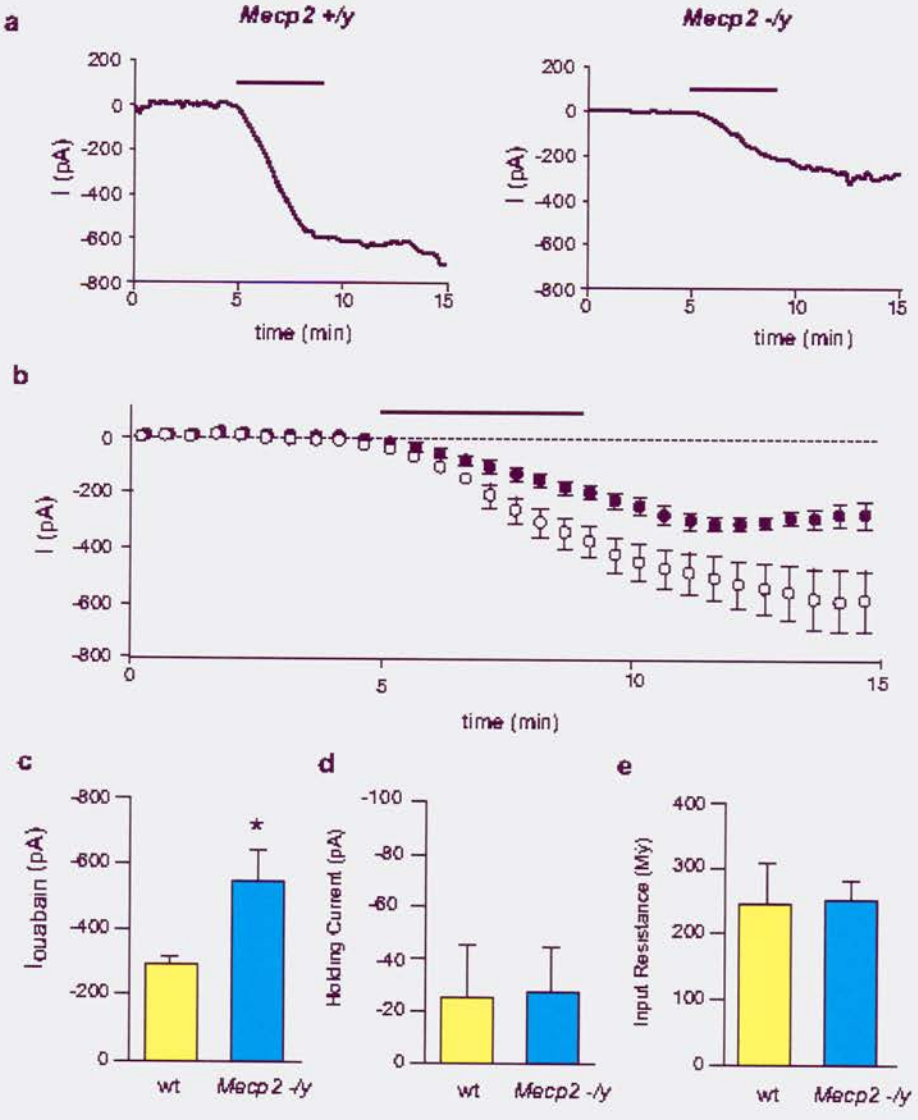

Figure 2.6. Na^+, K^+-ATPase activity is altered in the cerebral cortex of *Mecp2*-null mice. a, Representative experiments showing the effects of ouabain (1 mM) on the holding current of a pyramidal cell from a wildtype (*Mecp2+/y*) or a *Mecp2 -/y* mouse. Bar indicates time of ouabain application. In roughly half of the recorded cells, the effects of ouabain slowly reverted over a span of 15-20 minutes upon washout; however, in the remaining cells, ouabain induced a stable change in current that was followed during the wash by a sharp 1-2 nA inward current,

similar to the spreading depression-like depolarization seen during ouabain application in hippocampus (eg. [46,50] and data not shown). The induction of this large inward current was not obviously affected by mouse genotype and was not analyzed further. b, A summary graph showing the ouabain-induced current in wild-type (open circles) and *Mecp2* null mice (filled circles). c, The ouabain-induced current is significantly smaller in cells from the MeCP2-deficient mice (P=0.02). d, The holding current, measured immediately after obtaining the whole-cell configuration, was not genotype-dependent (P=0.94). e, Likewise, the input resistance of cells was not genotype-dependent (P=0.95).

Table 2.1. Postmortem brain tissues used in this thesis study

Condition	Age	Sex	Case#	MeCP2 Mutation	PMI	Affymetrix GeneChip Assay	Real-time PCR	Brain Region	Source
RTT (n=9)	2 Year	F	# 448	R168X	20	Yes	Yes	Superior Frontal Gyrus	KK
	4 Year	F	# 2665	n/a	n/a				
	7 Year	F	# 2400	n/a	n/a	-	Yes		
	10Year	F	# 1814	R270X	5	-	Yes		
	6 Year	F	# 2963	n/a	31.5	Yes	Yes	Area8/9	Harvard
	8 Year	F	# 2684	R106W	12			Area8/9	
	8 Year	F	# 4687	R225X	2.9			Area8/9	
	25 Year	F	#2575	R294X	11			Superior Frontal Gyrus	
	29 Year	F	#2854	No coding region mutation detected	35				
Normal (n=7)	2 Year	F	# 103	-	11	Yes	Yes	Superior Frontal Gyrus	KK
	4 Year	F	# 449	-	n/a	-	-		
	5 year	F	#269	-	36	Yes	Yes		
	10 Year	F	# 24	-	n/a	Yes	Yes		
	15 Year	F	# 104	-	16	-	Yes		
	18 Year	F	#72	-	29				
	18 Year	F	#26	-	16				
	19 Year	F	#55	-	17				

KK: Kennedy Krieger Research Institute

Harvard: Harvard Brain tissue Resource Center

PMI: Postmortem Interval in hours

Table 2.2. Primer pairs for bisulfite genomic PCR/sequencing and ChIP/PCR analysis.

Primers are shown in 5' to 3' orientation. **F,** forward primer; **R,** reverse primer; **m,** *Mus musculus*; **h,** *Homo sapiens*.

Bisulfite genomic PCR or sequencing primers:

The positions of the 5' end of each primer in the encompassed genomic DNA sequence are indicated in parentheses.

The following primer pairs are designed for PCR amplifications targeting fragments of 400-500bp within a genomic sequence of 1860bp encompassing the 5' flanking region, exons 1a and 1b of the human *FXYD1* gene:

TTGTTGTTTTTTTTGAATTGAGAGA, hFX1b-BSP1-F (419-443)

ACTAAACACTTTAAACCCCCTAACC, hFX1b-BSP1-R (641-617)

GGGAGAGGTTGTTGTTTATGTTTA, hFX1a-BSP5-F(1008-1031)

CCCAAATCCCACCTACCTACATAC, hFX1a-BSP5-R(1488-1465)

ChIP primers:

For the human *FXYD1* gene, five sets of primer pairs are designed for five overlapping PCR amplifications with each targeting a DNA fragment of 400-500bp within a genomic sequence of 1892bp encompassing the 5' flanking region, exons1a and 1b, and part of exon2 of the gene (including the "ATG" translation start site):

CCGGGTCTTTGGGTTTCTCTATCA, hFXYD1 ChIP Primer1-F

GAC TGC CCT GGC CCG CTT GGT TA, hFXYD1 ChIP Primer1-R

GCAGCCGCCTCAGCCCAAAAG, hFXYD1 ChIP Primer2-F

GGCCACTCCTGCTCCCAAAATAG, hFXYD1 ChIP Primer2-R

GCTGGGCCGCCTATTTTG, hFXYD1 ChIP Primer3-F

CCGCCTCTGGGTGACATCTT, hFXYD1 ChIP Primer3-R

TACACCCCACGTCCTGCTCCAACC, hFXYD1 ChIP Primer4-F

CGCCCAAGTCCCACCTGCCTACAT, hFXYD1 ChIP Primer4-R

GAGCGGGCGGGCGGAGAG, hFXYD1 ChIP Primer5-F

GGGGGACACCACGGATTAGG, hFXYD1 ChIP Primer5-R

For the mouse *FXYD1*, three sets of PCR amplification primers are designed for there overlapping PCR amplifications with each targeting a DNA fragment of 400-500bp within a genomic sequence of 1943bp encompassing the 5' flanking region, exons 1a and 1b of the gene (including the "ATG" translation start site):

CTTCTGGTTCCCGAGTCTTGTTAC, mFXYD1 ChIP Primer1-F

ATTGCGTGTGGACTGGACTATGAG, mFXYD1 ChIP Primer1-R

CATGCTGCTCCTGGGACTGTGCT, mFXYD1 ChIP Primer2-F

TCTCTAACTCTCCCGCCCCTGTGC, mFXYD1 ChIP Primer2-R

CACAGGGGCGGGAGAGTTAGAGAC, mFXYD1 ChIP Primer3-F

GCCCAGCCAGGAGCCACATC, mFXYD1 ChIP Primer3-R

CHAPTER THREE

CONCLUSIONS AND FUTURE DIRECTIONS

Vivianne W. Deng

Conclusions

The *FXYD1* gene is abnormally overexpressed, at both mRNA and protein levels, in the frontal cortex of human RTT patients and *Mecp2*-null mice

Since Amir et al. mapped *MECP2* as a gene responsible for RTT by mutation analysis in 1999 [22], the RTT research has emphasized on identification of target genes of MeCP2. The establishment of MeCP2 as a global transcriptional repressor that is widely expressed in the mammalian brain has prompted the search for MeCP2 neuronal target genes, and this undertaking still remains a major challenge in the RTT research field. While it can obviously be reasoned that loss of MeCP2 function may result in widespread aberrant gene expression, which in turn may interrupt normal brain development and function, previous global transcriptional profiling studies using either postmortem brain tissues of RTT patients or brain tissues from *Mecp2*-null mice have only generated divergent results. It is possible that the various sample sources and disparate technology platforms used in these studies contributed to the observed discrepancy, making it difficult to identify genuine MeCP2-target genes. In any event, the thesis work presented here reports the discovery of *FXYD1* as an overexpressed gene in the brain of RTT patients and *Mecp2*-null mice, specifically in the frontal cortex, an area that has been reported to be affected most severely in RTT. By employing Affymetrix GeneChip HumanU95A oligonucleotide arrays, followed by clustering data analysis and subsequent quantitative real-time PCR verification, the *FXYD1* mRNA expression was identified as being abnormally overexpressed in the postmortem tissues from the

frontal cortex of RTT patients. Further investigation utilizing a combination of cellular and molecular approaches into *FXYD1* gene expression in *Mecp2*-null mice revealed that levels of both *FXYD1* mRNA and FXYD1 protein were significantly elevated in the frontal cortex of *Mecp2*-null mice. Interestingly, such overexpression of the *FXYD1* gene occurred selectively to the frontal cortex of mice, suggesting the *FXYD1* gene deregulation caused by MeCP2 deficiency is brain-region specific. One unique feature in this thesis work is that we combined study on human RTT patients with *Mecp2*-null mice, and were able to demonstrate that results derived from these two different model systems converged to the same conclusion, i.e. the *FXYD1* gene is significantly overexpressed in the frontal cortex of both species, although the level of overexpresion appeared more dramatic in human RTT samples. Therefore, this observation suggests that the two species may share a conservative pathway involving FXYD1 in brain function. It should also be pointed out that differential sensitivity to MeCP2 dysfunction may exist between humans and mice, leading to different degrees of changes in gene expression. This may provide further explanation for inconsistent results obtained by several other gene expression profiling studies. However, to date, the *FXYD1* gene is the first example of deregulated gene expression found in both humans and mice due to MeCP2 deficiency.

The *FXYD1* gene expression in the brain is subjected to epigenetic transcription repression mediated by DNA methylation and MeCP2

Based on the above observations, I hypothesized that the *FXYD1* gene regulation in the frontal cortex of mammalian brains might involve an epigenetic regulation mechanism mediated by MeCP2. A major focus of this dissertation was to test this hypothesis. It is well known that DNA methylation represents a common form of epigenetic modification contributing to transcription repression of gene activity primarily through a mechanism mediated by MeCP2. Therefore, I first examined whether DNA methylation plays a role in controlling *FXYD1* gene expression. To this end, an array of experiments using demethylation treatment, bisulfite genomic DNA sequencing, and *in vitro* methylation in conjunction with promoter-reporter transfection assays was carried out. I was able to obtain the following results: (1) Demethylation induced by 5'azacytidine activated *FXYD1* gene expression in cells that don't normally express *FXYD1*, suggesting that FXYD1 gene expression is regulated in a methylation-dependent manner; (2) Bisulfite genomic sequencing analysis of genomic DNA from human frontal cortex, in parallel with that from human heart, revealed that differential methylation of cytosine occurred in multiple CpG dinucleotides, particularly those located at potential transcription factor binding sites predicted *in silico*. These methylated CpGs may provide potential binding sites for MeCP2; (3) *In vitro* methylation of *FXYD1* promoter repressed the *FXYD1* promoter transcription activity in 293T cells, and this repression was further enhanced by exogenous expression of the MeCP2 protein. Together, these results demonstrate that the *FXYD1* gene is subjected to DNA methylation-dependent transcriptional repression in a tissue-specific manner and is possibly mediated by MeCP2. To

further determine whether the *FXYD1* gene is a direct MeCP2 target *in vivo*, chromatin immunoprecipitation analysis was performed with cell lysates from MeCP2-expressing 293T cells as well as from the frontal cortex of wild-type and *Mecp2*-null mice. The results showed that MeCP2 and endogenous HDAC1 were recruited to the *FXYD1* promoter region in MeCP2-expressing cells, and an endogenous MeCP2 was associated with native *FXYD1* promoters only in the frontal cortex of wild-type but not *Mecp2*-null mice. Thus, for the first time, this data provides compelling evidence to support the innovative notion that the *FXYD1* gene is a MeCP2-target gene subjected to DNA methylation-dependent gene silencing in the frontal cortex of mouse brains. This finding provides us with insight into the molecular mechanism by which the *FXYD1* gene is selectively silenced, possibly in a cell-specific fashion, in the frontal cortex of mouse brains. Such selective gene silencing may be critical for normal functioning of mature neurons. Given that multiple CpG sites in the *FXYD1* promoters region were also demonstrated to be methylated in human frontal cortex, which may serve as potential MeCP2 binding sites, these conclusions may also apply to human brains.

The functional consequences from the abnormal overexpression of *FXYD1* in mouse frontal cortex may include a reduction of Na^+, K^+-ATPase activity

Finally, one important aspect of this thesis study is that we were able to detect one of the functional consequences potentially conferred by *FXYD1* overexpression in a physiological context. Our electrophysiological study using

whole-cell patch clamp recordings from layer II/III pyramidal cells in the frontal cortex of wild-type and *Mecp2*-null littermates revealed that the Na^+, K^+-ATPase activity, measured as ouabain-sensitive currents, was significantly reduced in *Mecp2*-null mice. This observation coincides with a possible outcome that one would predict from the selective overexpression of *FXYD1* in mouse frontal cortex. As mentioned in the previous chapter, because alterations of Na^+, K^+-ATPase activity have been implicated in numerous neurological disorders with clinical symptoms reminiscent of RTT, it is likely that the inhibited Na,K-ATPase activity due to *FXYD1* deregulation may also underlie at least some of these common neurological abnormalities in RTT. Although we were unable to provide more direct evidence to establish a causative and functional relationship between *FXYD1* overexpression and altered Na^+, K^+-ATPase activity, the work presented here implicate a novel concept – that MeCP2-mediated epigenetic gene silencing may play a role in maintaining and modulating ion homeostasis and cell excitability in mature neurons in the brain, through regulation of neuronal Na^+, K^+-ATPase activity by controlling *FXYD1* gene expression. This notion is in agreement with the current hypothesis that RTT is a disorder caused by synaptic defects. At the present time, the physiological significance of Na^+, K^+-ATPase in neuronal activity, of defects in this ATPase in human pathophysiology, is somewhat underappreciated. It is possible that FXYD1/ Na^+, K^+-ATPase system is a potential contributing factor to the RTT neuropathology, and may perhaps be a foothold toward therapeutic targets for disease treatment. In addition, the work presented here represents the first example of a study in the field where

deregulated gene expression in RTT was investigated from a molecular and cellular level up to a physiological level to understand the neuropathology of RTT.

Summary of Findings

Overall, the work described in this dissertation leads to several findings as listed below.

First, an abnormal up-regulation of *FXYD1* gene expression was identified in the frontal cortex of RS patient brains by using Affymetrix GeneChip microarray analysis followed by quantitative real-time PCR verification.

Second, the *FXYD1* gene was also found to be selectively overexpressed in the frontal cortex of *Mecp2*-null mice, a rodent model of RTT, not only at the mRNA level but also in the FXYD1 protein content.

Third, *in vitro* methylation of the *FXYD1* promoter region repressed transcriptional activity of *FXYD1* promoters *in vitro,* and the presence of MeCP2 protein further enhanced such a repressive effect; demethylation induced by 5'-azacytidine activated *FXYD1* gene expression in cells that don't normally express *FXYD1* mRNA, implying that *FXYD1* gene transcription is regulated in a methylation-dependent manner.

Fourth, bisulfite genomic DNA sequencing analysis revealed that the *FXYD1* promoter region harbored more densely methylated cytosine in multiple CpG dinucleotides in human frontal cortex than in heart, particularly in CpGs

located at predicted binding sites of transcription factors, suggesting that *FXYD1* promoters are differentially methylated in a tissue-specific fashion.

Fifth, chromatin immunoprecipitation (ChIP) assays further demonstrated endogenous MeCP2 and HDAC1 were recruited to the native *FXYD1* promoter regions in both MeCP2-expressing human cells and mouse frontal cortex tissues, strongly suggesting that *FXYD1* is a MeCP2-target gene subjected to MeCP2-mediated, DNA methylation-dependent transcription repression.

Finally, electrophysiology study using whole-cell patch clamp recordings from layer II/III pyramidal in mouse frontal cortex showed that Na^+/K^+-ATPase activity was significantly reduced in *Mecp2*-null mouse, possibly as a result of *FXYD1* overexpression. Inhibition of Na^+/K^+-ATPase activity may have an profound impact on neuronal excitability in the brain.

Future Directions

The primary goal of this thesis was to uncover deregulated gene expression caused by loss of MeCP2 function in the brain of human RTT and Mecp2-null mice, as this may help understand molecular mechanisms underlying the neuropathology of RTT. The work presented here specifically describes that the *FXYD1* gene is overexpressed in the frontal cortex of both RTT patients and *Mecp2*-null mice. More importantly, our further investigation into FXYD1 gene regulation strongly indicates that *FXYD1* gene expression is subjected to DNA methylation-dependent transcriptional repression mediated by MeCP2, and this negative transcriptional regulation is selectively compromised in the frontal cortex of both human RTT patients and *Mecp2*-null mice where MeCP2 function is impaired. The evidence provided here and the recent finding that FXYD1 has the lowest expression in the frontal cortex imply that this region-, or even cell-specific gene silencing of FXYD1, regulated by a MeCP2-mediated epigenetic mechanism may be critical for proper functioning of cortical neurons in frontal cortex.

1. Epigenetic regulation of FXYD1 gene expression in CNS mediated by MeCP2

Conclusions drawn from this thesis work lay a solid ground work for additional studies aimed to further understand the mechanisms by which the FXYD1 gene is regulated by MeCP2 in the CNS. Future work can be directed to

determine: (1) whether MeCP2-mediated *FXYD1* gene repression is brain region- and/or cell type-specific in order to adapt to varying functions of different brain structures; (2) whether such a regulation works in a neuronal activity-dependent manner in response to hormonal stimulation, just like the case of BDNF, given that FXYD1 is the substrate of multiple kinases, including PKA and PKC, and hormonal regulation of NKA function is a critical mechanism for cells to maintain homeostasis; (3) whether MeCP2 mediates *FXYD1* gene silencing in a HDAC-dependent manner; (4)whether the *FXYD1* gene promoter regions is differentially methylated in discrete neuronal types and brain structures, and whether the methylation pattern is subject to dynamic changes that contributes to differential gene expression of *FXYD1*. For instance, the *FXYD1* promoter may be hypomethylated in choroid plexus and cerebellum where *FXYD1* is highly expressed.

2. Implication of selective overexpression of FXYD1 in the RTT neuropathology

Many important questions also remain unanswered regarding the functional relevance of *FXYD1* overexpression in RTT, for example: (1) Is the *FXYD1* overexpression one of the primary causes of RTT or the secondary outcomes due to confounding effects in RTT? (2) How does *FXYD1* overexpression affect the brain functions? (3) Is the *FXYD1* overexpression sufficient to give rise to any of the disease phenotypes presented in RTT?

Obviously, future studies should focus on elucidating neuronal functions and physiological roles of FXYD1 in the CNS.

To this end, perhaps transgenic animals with controlled *FXYD1* overexpression or deficiency would offer valuable model systems for this line of study. It should be pointed out that the results of this type of analysis may be difficult to interpret due to confounding effects of loss of function or overexpression of the gene. For this purpose, cortical culture ex vivo can be used to test experimental conditions and an inducible expression vector (or a viral vector) may be applied to titrate the levels of protein expressed to mimic endogenous FXYD1 levels. If these efforts prove fruitful, generating knock-in mouse lines, which would be useful for dissecting the physiological role of FXYD1, could also be utilized for electrophysiological studies to explore topics described in the next paragraph.

3. The physiological roles of FXYD1 and modulation of Na^+, K^+-ATPase by FXYD1 in the CNS

In this thesis, I have also described that the Na^+, K^+-ATPase (NKA) activity was reduced in cortical neurons of Mecp2-null mice. It is tempting to assume the observed effect was caused by the FXYD1 overexpression. Yet, we haven't been able to establish the direct link between the two. Future studies should be designed to address whether the FXYD1 overexpression is responsible for altered NKA activity in Mecp2-null mice. If this is true, it would be important to

investigate how this deregulation contributes to the neuropathology of RTT. Better understanding of the regulatory mechanisms by which FXYD1 modulates and fine-tunes functional properties of neuronal NKA , and of the mechanisms by which the FXYD1/NKA system contributes to the adaptation and maintenance of proper neuronal excitability and function in the brain, will provide vital information to answer these questions and will be a challenging subject for future investigation.

Another important note with regard to FXYD1's function in the brain is the relationship between FXYD1 and Na, Ca-exchangers (NCXs). Working in parallel with Na^+,K^+-ATPase, NCXs handles calcium extrusion. NCXs are enriched in neurons and it plays a housekeeping role in maintaining a low intracellular Ca2+ concentration, which is critical to ensure appropriate excitability of neurons [33, 34, 132]. Since FXYD1 has shown to act as an endogenous inhibitor of NCX1 in cardiac tissues [133], it would not be surprising if FXYD1 is found to play a role in regulation of Ca^{++} homeostasis through modulating Na, Ca-exchanger activity in the nervous system. Therefore, it would be very interesting to explore the relationship between FXYD1 and NCX1 in the brain. Future experiments to determine co-localization, specific protein-protein interaction of FXYD1 and NCX1, and functional consequences of the interaction on neuronal excitability should be warranted.

Altered *FXYD1* expression has been implicated in several human pathological conditions, including schizophrenia, aging, brain injury and ischemia [116, 117]. It is possible that FXYD1 plays any other important, yet underscored roles required for the homeostatic normalcy of neuronal function. Exploration of these aspects would open up an exciting new research field for many future studies. Discovering any additional roles for FXYD1 will also help clarify the functional relevance of the aforementioned *FXYD1* overexpression in the neuropathology of RTT.

Alteration of Na^+, K^+-ATPase activity has also been suggested to be involved in depression. It would be interesting to examine behavioral aspects of *Mecp2*-null mice and to determine whether there is a correlation between mood-related behavior and alteration in the FXYD1-Na+, K+-ATPase system [134]. Given that synaptosomal NKA plays an important role in synaptic transmission and that my preliminary data also showed that FXYD1 was detected in synaptosomal preparation, it is possible that FXYD1 may modulate ionic transport at synapses via regulating synaptosomal membrane activity.

4. Identification of other MeCP2 targets in the CNS and understanding the cascade of events that follows their deregulation

My initial clustering analysis of Affymetrix microarray results revealed that some other genes in the *FXYD1* gene cluster also showed altered gene expression in brain samples affected with RTT when compared to the age-

matched control subjects. These genes are potential MeCP2-regualted genes, and it would be worthwhile to investigate them further. Future validation of these additional deregulated gene expression in the RTT samples and in *Mecp2*-null mice by quantitative real-time PCR will yield new information to help delve into the roles of MeCP2 in the brain as well as the neuropathology of RTT.

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APPENDICES

Appendix Chapter I

Gene Expression of FXYD1 and FXYD7 in the Cortex and Hypothalamus in Rodents

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Materials and Methods

Tissue Acquisition and RNA Isolation. Brain tissues from rats of different postnatal ages were dissected in cold PBS, submerged immediately in RNALater (Ambion, Austin, TX) and stored at –20°C until use. Total RNA was isolated and purified using RNeasy Mini Columns and DNase On-Column digestion (Qiagen, Valencia, CA) according to the manufacturer's specifications.

Quantitative RT Real-time PCR. The procedure in **Supplementary Note 1**, has been described in detail elsewhere [40].

In situ hybridization. The brains were fixed by intracardiac perfusion of 4% paraformaldehyde-borate buffer, pH 9.5. Both solutions were made with DEPC treated water (0.1% diethyl pyrocarbonate) to minimize RNase contamination. After dissection of the brain, tissue blocks were postfixed overnight in the same fixative containing 20% sucrose, then blocked and frozen on dry ice, and stored at –80°C until sectioning, as reported [2,3]. The sections (30μm) were cut on a sliding microtome, mounted on Superfrost Plus slides (Fisher Scientific, Santa Clara, CA), dehydrated under vacuum overnight and then frozen at –80°C until processing for hybridization. The hybridization procedure was that recommended by Simmons et al. [4], as described earlier by us [3,5], using a ^{35}S-UTP-labeled FXYD1 cRNA probe. Following an overnight hybridization at 45°C, the slides were washed and processed for cRNA detection. After dehydration, the slides

were dipped in NTB-2 emulsion, and were exposed to the emulsion for three weeks at 4°C. At this time the slides were developed, quickly dehydrated, dried, and cover-slipped for microscopic examination.

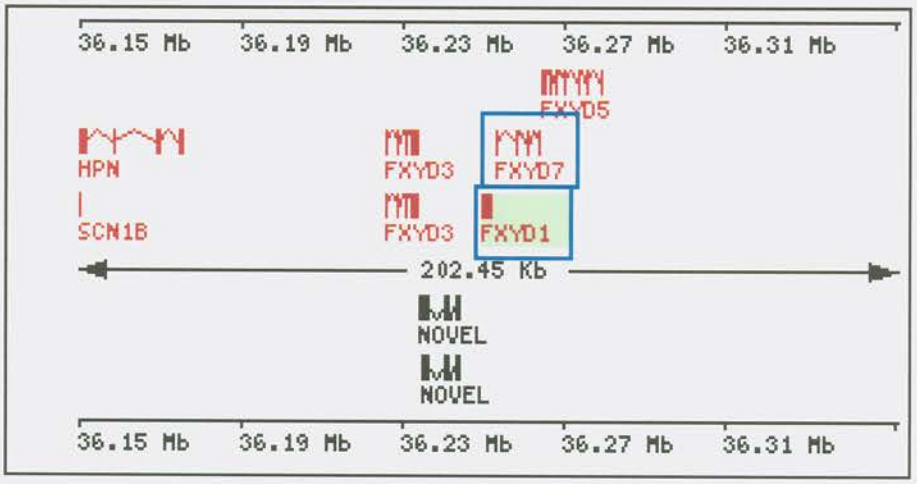
Results

In addition to FXYD1 to 6, the *FXYD7* gene has recently been discovered as a newcomer in the FXYD gene family [73]. *FXYD7* is expressed specifically in the brain and encodes for a brain-specific Na^+, K^+-ATPase regulator [73]. The gene is located within a gene cluster on human chromosome 19 that contains FXYD1, 3, and 5 (Appendix Figure 1.1a). Very intriguing, *FXYD1* and *FXYD7* lie unusually close – within 200 bp –to each other in this gene cluster (Appendix Figure 1.1b). It has been recognized that neighboring genes within eukaryotic genomes often times show similar expression patterns via a co-regulatory mechanisms. To explore the possibility that gene expression of *FXYD7* is co-regulated with that of *FXYD1*, thus also being relevant to studies on RTT-related genes, gene expression of both *FXYD7* and *FXYD1* was first examined in the cortex and hypothalamus tissues of mouse brains, and in the GT-7 cells originally derived from mouse hypothalamic tumors. For both genes, RT-PCR did reveal expression of mRNA in the brain regions examined, albeit *FXYD7* showing apparently much more robust expression in all samples (Appendix Figure 1.2). To further determine whether or not these two genes were concordantly expressed in the brain during development, the ontogeny of expression of both *FXYD1* (Appendix Figure 1.3) and *FXYD7* (Appendix Figure 1.4) in the hypothalamus and frontal cortex of rats was then investigated by quantitative real-time RT-PCR. Surprisingly, expression levels of *FXYD1* and *FXYD7* were not concordant, although each of them appeared to be developmentally regulated in the brain regions examined. In fact, temporal

expression patterns of these two genes showed almost no correlation, indicating that *FXYD1* and *FXYD7* gene expression are independently regulated during development and in distinct brain areas. Next, the distribution patterns of mRNA expression of *FXYD1* and *FXYD7* in rat hypothalamus and frontal cortex were determined by *in situ* hybridization. The result here demonstrated that both *FXYD1* and *FXYD7* were selectively expressed in specific brain structures, and the brain structure-specific expression of these two genes was not correlated by all means. As expected, *FXYD1* exhibited a high level of expression in choroid plexus and the ventricle wall (most likely tanycytes or ependymal cells), while *FXYD7* was highly expressed in cerebral cortex and hippocampus (Appendix Figure 1.5). Thus, even though *FXYD1* and *FXYD7* are localized in a close proximity on the same chromosome, these two genes showed completely different patterns of temporal and regional expression in the brain, implying that their gene expression may be regulated by different mechanisms and their functional roles may diverge to meet different demands in accordance to distinct anatomic and functional structures in the brain. Because the FXYD1 and FXYD7 proteins have been reported to modulate Na^+, K^+-ATPase activity [73, 78], further possible inference from the data presented here is that such a discrete spatiotemporal expression patterns of *FXYD1 and FXYD7* may serve to achieve brain region- and cell-specific regulation and fine tuning of neuronal Na^+, K^+-ATPase activity to ensure proper neuronal excitability in distinct brain structures.

Result Figures

a

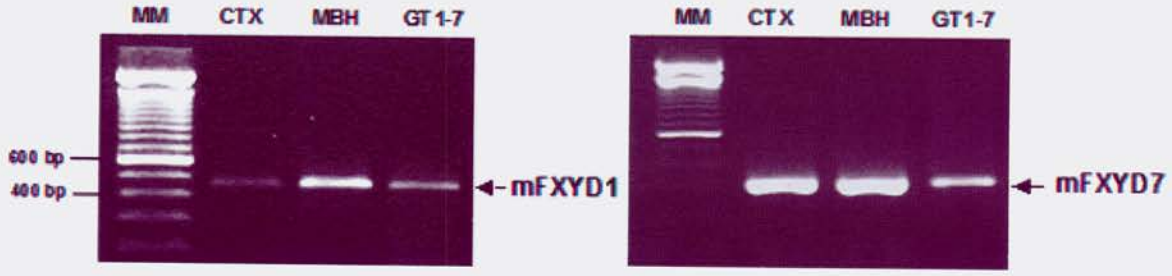


b

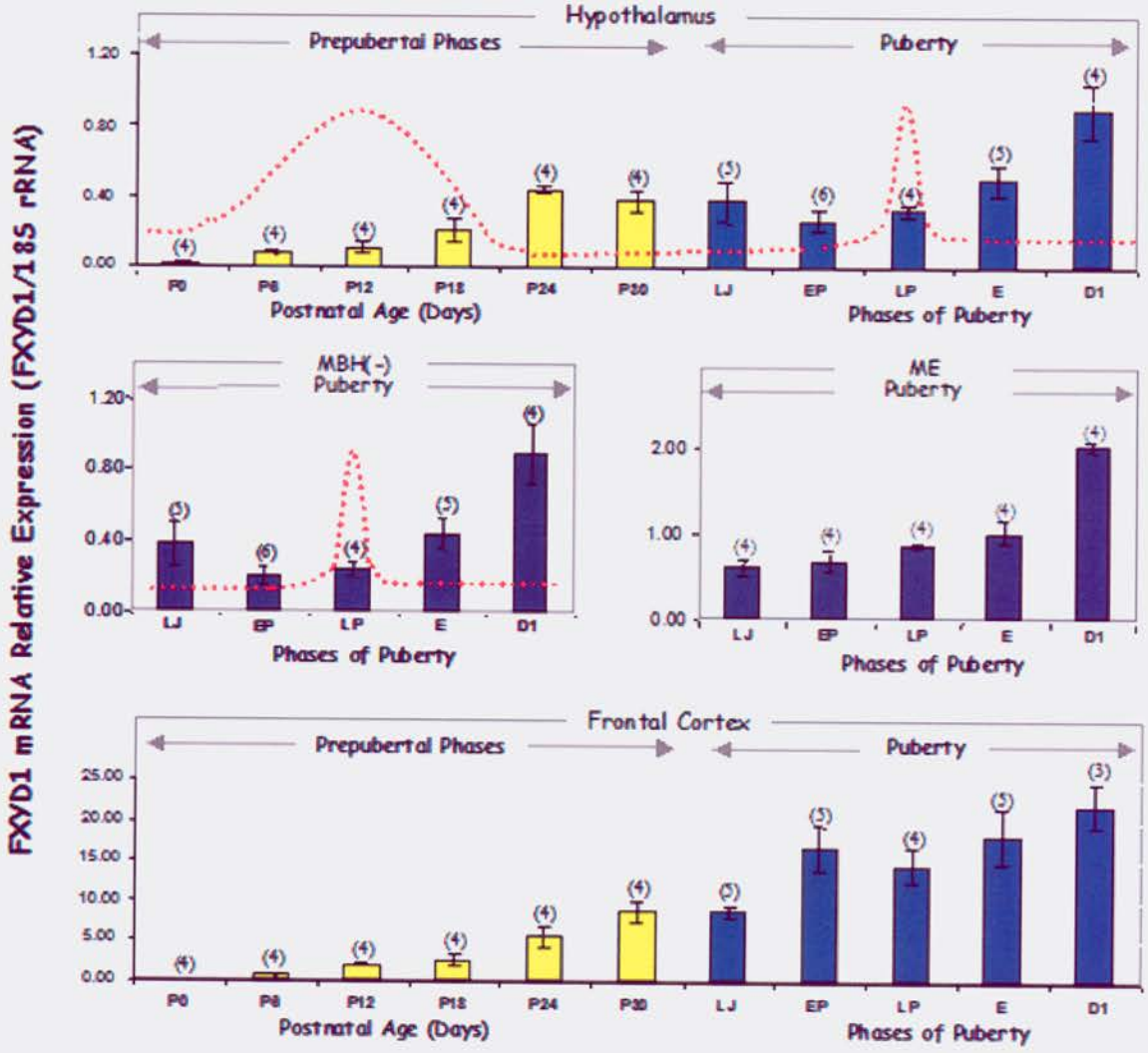
Appendix Figure 1.1. The FXYD1 & FXYD7 genes are localized in an unusually close proximity on human Chromosome 19.

(a) Transcript neighborhood of FXYD1 and FXYD7 on human chromosome 19. Four members of FXYD gene family, FXYD1, 3, 5 and 7, are clustered on human chromosome 19.

(b) The FXYD1 and FXYD7 genes are separated by a short nucleotide fragment of 199 base pairs. The FXYD1 (Accession number: NM_005031 & NM_021902) and FXYD7 (Accession number: BC018619.1) genes were mapped to human chromosome 19q. The sequence (NT_011109.15) was retrieved from NCBI Sequence Map Viewer v2.0.



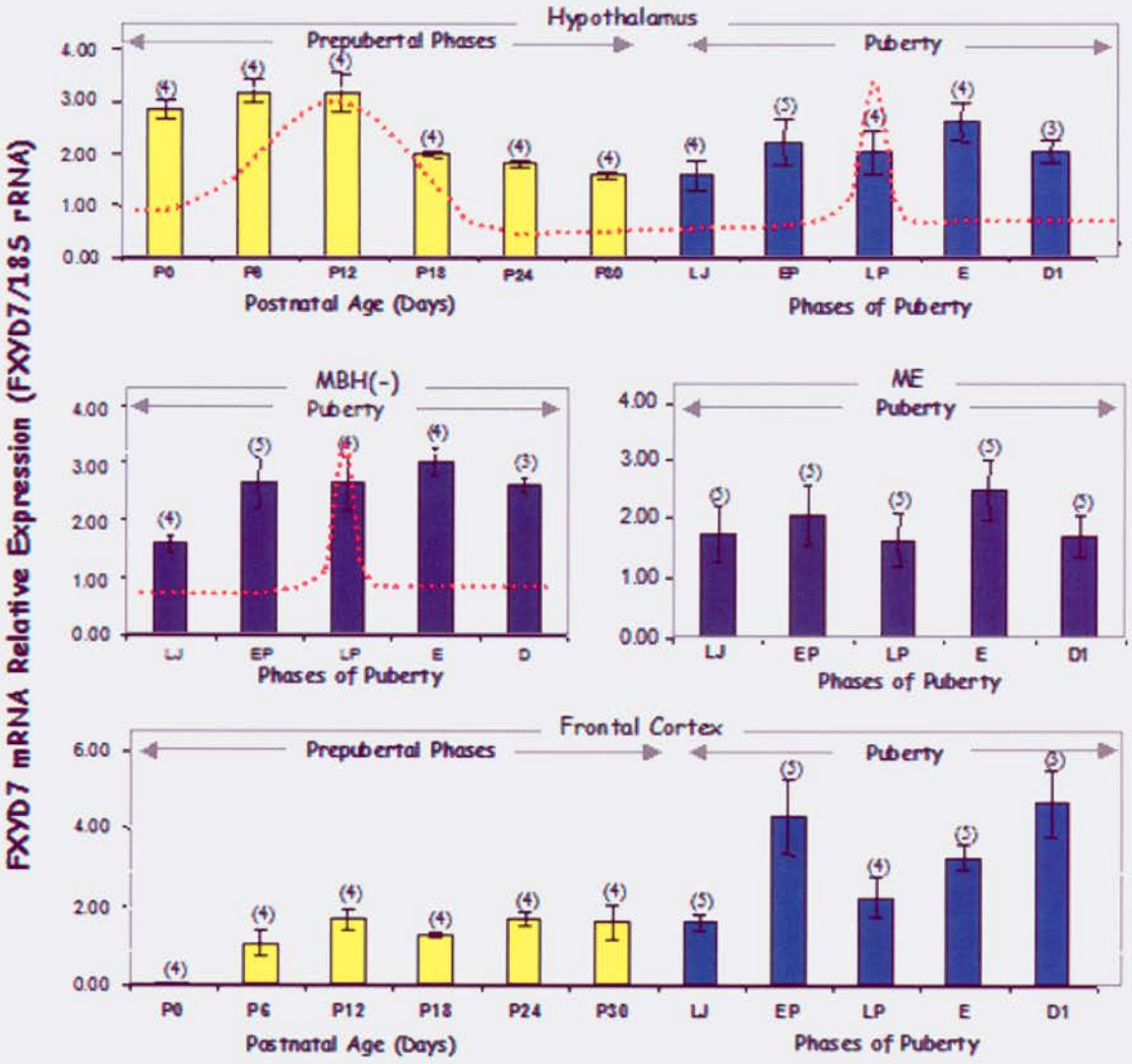
Appendix Figure 1.2. The FXYD1 & FXYD7 gene expression detected by RT-PCR in adult mouse brains and GT1-7 cells. Total RNA was extracted either from cerebral cortex and basal-medial hypothalamus tissues freshly dissected from adult wild-type mice, or from cultured GT1-7 cells by Tri-reagent RNA isolation solution. The RNA was subjected to RT-PCR analysis using primers specific for fragments residing within coding regions of human FXYD1 and FXYD7 genes.



Appendix Figure 1.3. The ontogeny of FXYD1 gene expression in the hypothalamus and frontal cortex of rats during development.

The FXYD1 gene is developmentally regulated in the hypothalamus (top panel), hypothalamus with medial basal hypothalamus removed (MBH(-), middle panel, left), median eminence (ME, middle panel, right), and frontal cortex (bottom panel) through the prepubertal and pubertal phases. Tissues were dissected into RNALater from total hypothalamus, MBH(-), ME, and frontal cortex of rats at indicated postnatal ages. Total RNA was isolated and subjected to quantitative

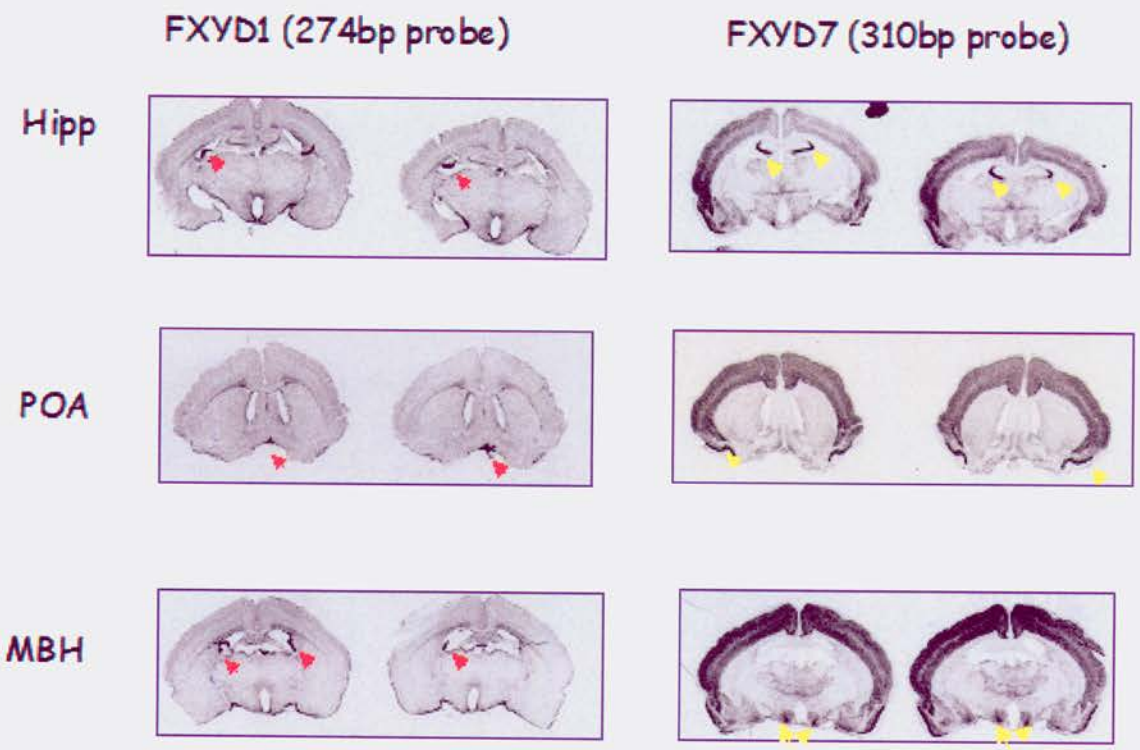
RT-PCR analysis with a primer set and Taqman real-time PCR probe specifically targeting a 96bp segment within the rat FXYD1 coding region. The red line represents the pulsatile secretion of luteinizing hormone (LH) from anterior pituitary in females.



Appendix Figure 1.4. The ontogeny of FXYD7 gene expression in the hypothalamus and frontal cortex of rats during development.

The FXYD7 gene is developmentally regulated in the hypothalamus (top panel), hypothalamus with medial basal hypothalamus removed (MBH(-), middle panel, left), median eminence (ME, middle panel, right), and frontal cortex (bottom panel) through the prepubertal and pubertal phases. Tissues were dissected into RNALater from total hypothalamus, MBH(-), ME, and frontal cortex of rats at

indicated postnatal ages. Total RNA was isolated and subjected to quantitative RT-PCR analysis with a primer set and Tagman real-time PCR probe specifically targeting a 96bp segment within the rat FXYD1 coding region. The red line represents the pulsatile secretion of luteinizing hormone (LH) from anterior pituitary in females.



Appendix Figure 1.5. FXYD1 and FXYD7 mRNA expression patterns differ in sections of rat hypothalamus and cortex as detected by in situ hybridization. To localize FXYD1 and FXYD7 mRNA in the rat brain we utilized a 274nt cRNA probe transcribed from a cDNA template generated by PCR amplification of mouse cerebral cortex RNA and 310nt cRNA from rat cortex RNA. The procedure used for tissue preparation and hybridization is briefly described in **Supplementary Note 5**.

APPENDIX CHAPTER II

Abnormal Expression of Genes Involved in Neurogenesis (SOX2 and WIF1) in the Brain of Rett Syndrome Patients and *Mecp2*-null Mice

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Materials and Methods

Tissue Acquisition and RNA Isolation. The same procedure as described in "Materials and Methods" in CHAPTER TWO of this thesis.

Quantitative RT Real-time PCR. The procedure in **Supplementary Note 1**, has been described in detail elsewhere [40].

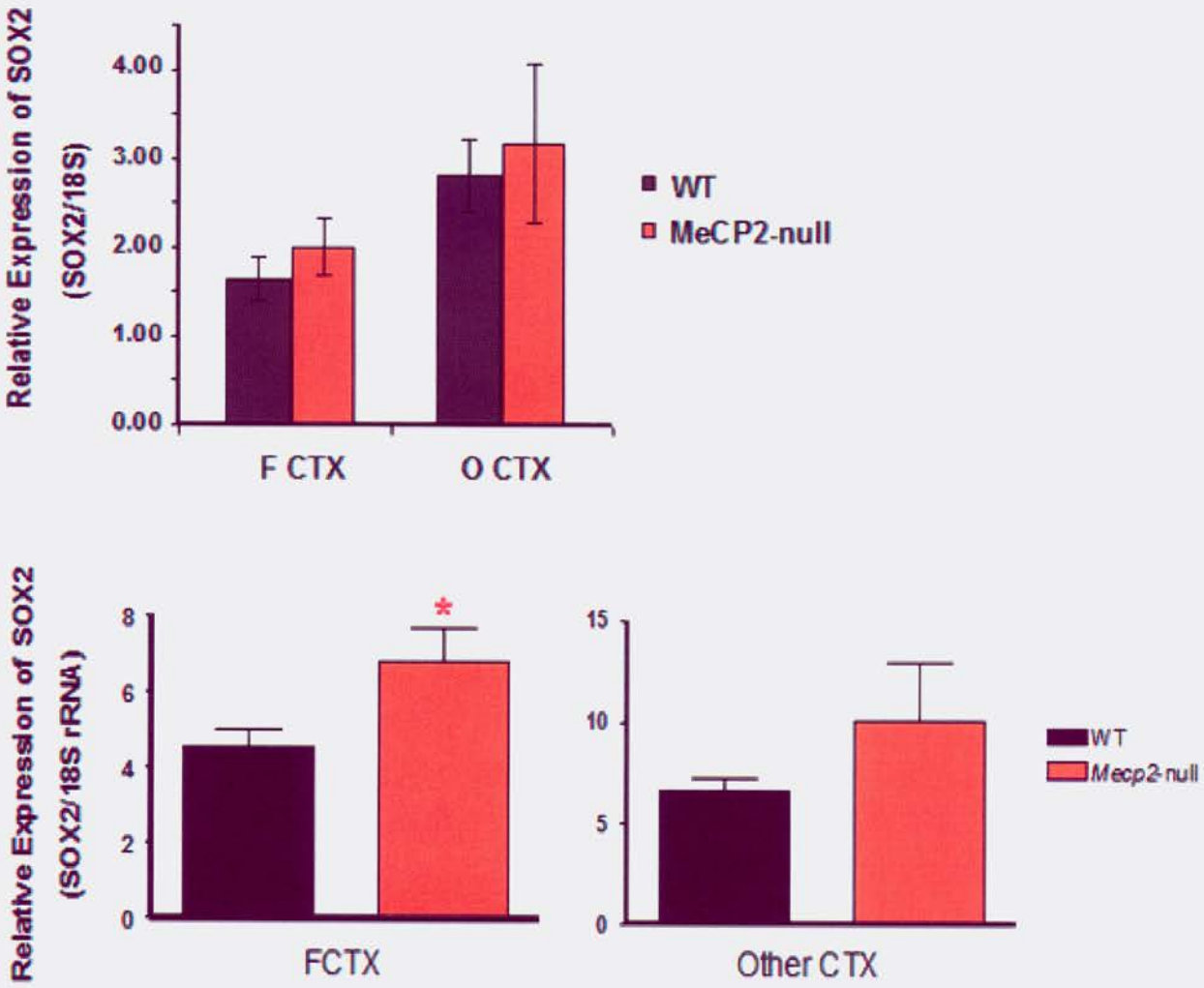
Results

In addition to FXYD1, described in Chapter Two of this dissertation, the initial gene profiling analysis from Affymetrix GeneChip experiments also revealed that two other genes that play important roles in neurogenesis, namely *SOX2* and *WIF1*, were overexpressed in the postmortem brain samples affected with RTT. This appendix chapter describes preliminary follow-up on those results.

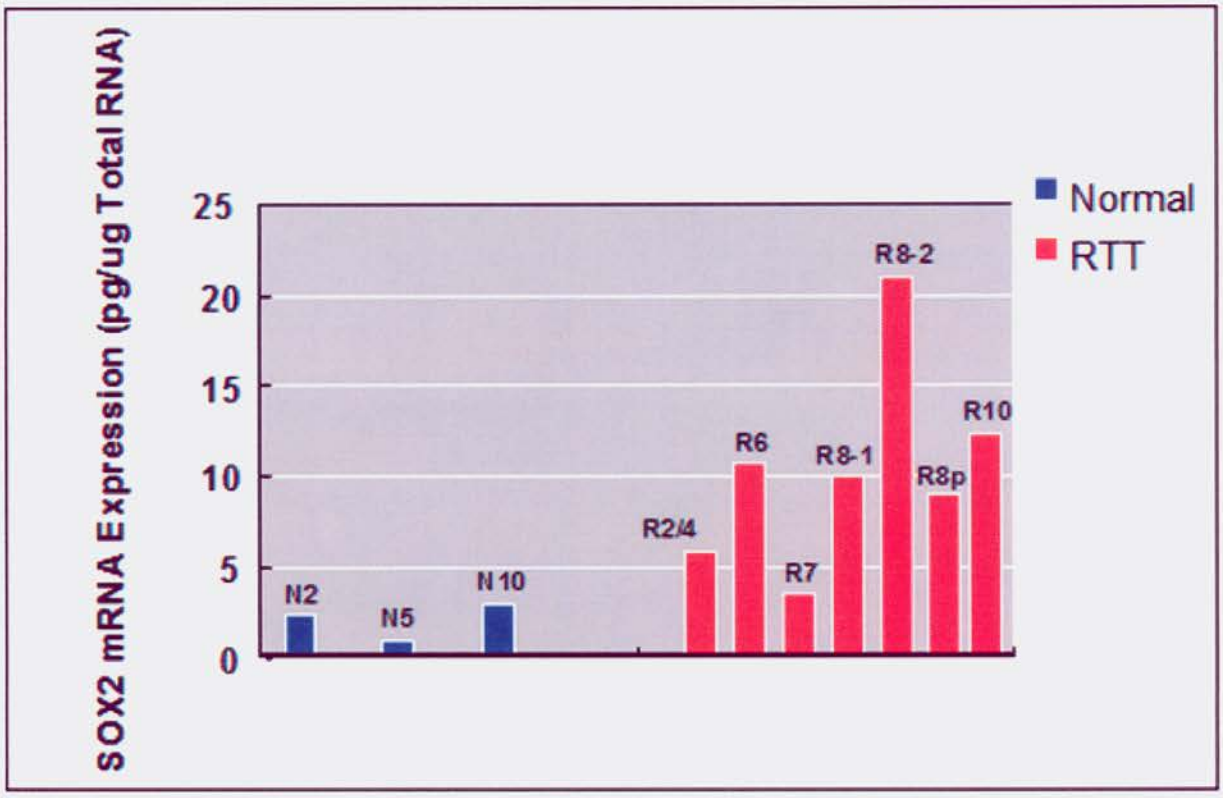
One of these genes, *SOX2*, is an intronless gene of the SRY-related HMG-box (SOX) family of transcription factors involved in developmental and cell-fate regulation in the embryo [91]. SOX2 has been shown to inhibit neuronal differentiation [92]. Therefore, given the neuronal and CNS phenotypes of RTT, it would be interesting to investigate whether deregulation of SOX2 is RTT-relevant. Verification of mRNA expression levels of *SOX2* in wild type and *Mecp2*-null mouse brain (Appendix Figure 2.1) and in RTT patient frontal cortex (Appendix Figure 2.2) was conducted by quantitative real-time PCR assays. Indeed, significantly higher levels of *SOX2* mRNA expression, as compared to age-matched control subjects, were observed in the frontal cortex of both species, although in *Mecp2*-null mice, the overexpression was found only at a younger age (P6). Further studies of this gene might, therefore, be illuminating.

The second gene found to be dysregulated in RTT patients in the Affymetrix GeneChip assay was *WIF1*. The WIF1 protein, which is secreted, binds and inhibits members of the WNT protein family [94]. Since the WNT proteins are often involved in CNS patterning during development [96], the expression level of the *WIF1* mRNA in brain tissues from MeCP2-null mice and RTT patients might reveal an important aspect of the RTT pathophysiology. *WIF1* mRNA expression levels were higher in *Mecp2*-null mouse brain and in RTT patient brains, but, unlike the case for *SOX2* gene expression, the regions of the brain that showed overexpression were not in agreement between the species. Specifically, *Mecp2*-null mice showed no significant changes in the frontal cortex (Appendix Figure 2.3) while the RTT samples were found remarked overexpressed in the frontal cortex (Appendix Figure 2.4). This divergence between human and mouse makes should be further examined in mice at different developmental ages.

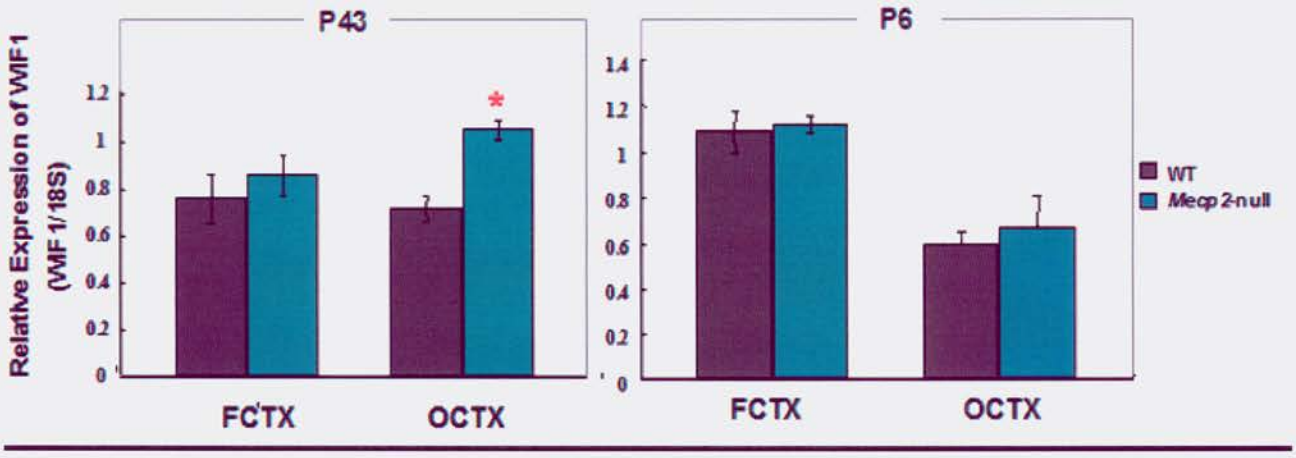
Result Figures



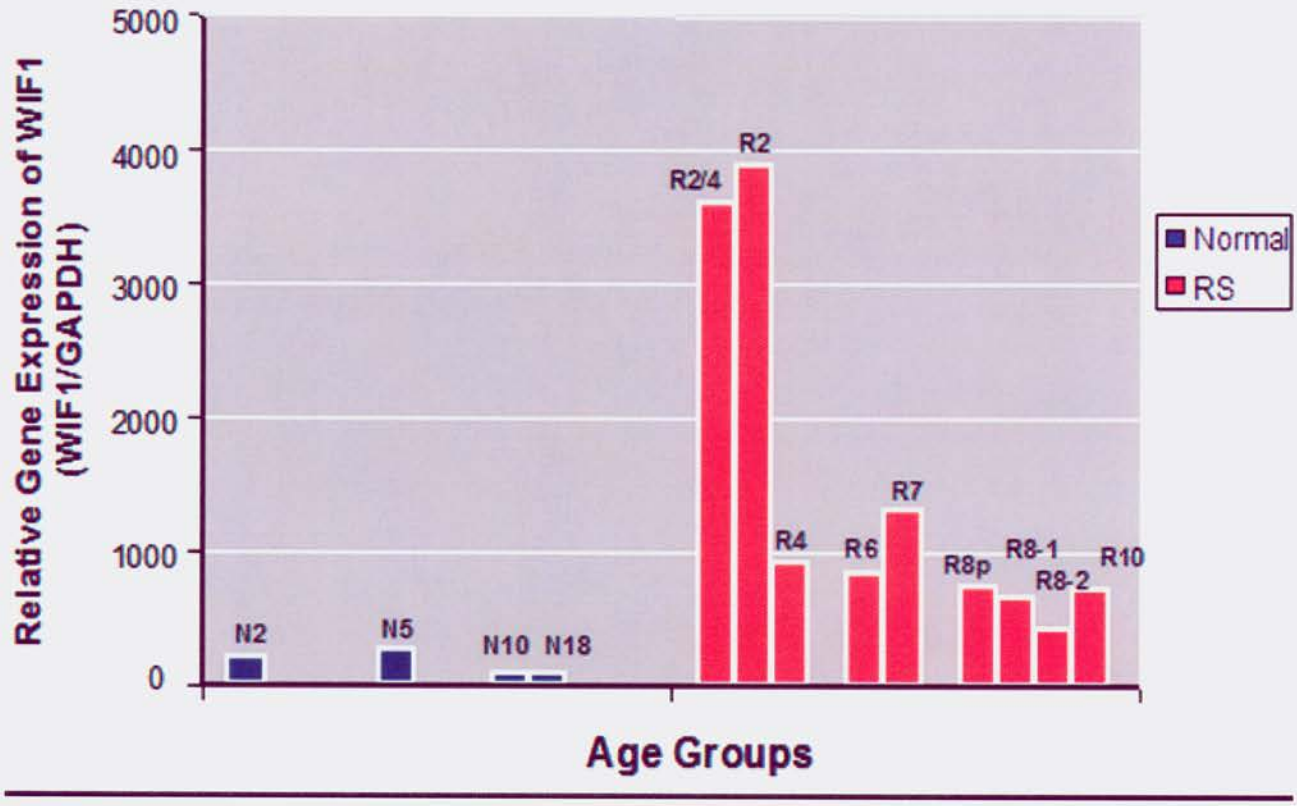
Appendix Figure 2.1. Quantitative RT real-time PCR analysis of SOX2 gene expression in the frontal cortex (FCTX) and other cerebral cortex (OCTX) of wild type and *Mecp2*-null mice at the postnatal ages of 45-day old (*upper panel*) and 6-day old (*lower panel*). SOX2 overexpression was only detected in the frontal cortex of mecp2-null mice at the age of 6 day old. *: P<0.05, paired t-test.



Appendix Figure 2.2. SOX2 gene expression was abnormally increased in the frontal cortex of RTT patient as detected by quantitative RT real-time PCR analysis.



Appendix Figure 2.3. Quantitative RT real-time PCR analysis of WIF1 gene expression in the frontal cortex (FCTX) and other cerebral cortex (OCTX) of wild type and *Mecp2*-null mice at the postnatal ages of 45-day old (*left panel*) and 6-day old (*right panel*). Significant overexpression of WIF1 was only detected in the other cortical region of mecp2-null mice at the age of 43 day old. *: P<0.05, paired t-test.



Appendix Figure 2.4. WIF1 gene expression was abnormally increased in the frontal cortex of RTT patient as detected by quantitative RT real-time PCR analysis. The RNA abundance of WIF1 was normalized by the level of human GAPDH and was represented as relative expression to GAPDH.

Supplementary Information:

Affymetrix raw image files for the six chip hybridization along with the absolute and comparison analysis results are available at:

http://murdock3.ohsu.edu:8893/amc/amcdb_welcome.html

Supplementary Note 1.

Reverse Transcription and Real-time PCR. RNA samples from the cerebral cortex of humans and mice were reverse transcribed using the Omniscript® RT kit (Qiagen) and random hexamer primer as suggested by the manufacturer. FXYD1 mRNA expression in human and mouse brain was quantitated by Real-time PCR, as previously described [1]. Briefly, 1ug of total RNA was reverse transcribed in a 20 μl volume, and 2 μl of the RT reaction were diluted 1:100 (human samples) or 1:500 (mouse samples) before Real-time PCR. Each sample was run in triplicates along with a relative and an absolute standard curve. Relative standard curves were generated by measuring FXYD1 mRNA and 18S ribosome RNA (18S rRNA) content in a serial of dilution of one sample. The content of 18S rRNA in each sample was then calculated using as a reference these relative standard curves and the values obtained were used to normalize the content of FXYD1 mRNA in each sample. The primer and probe used to detect 18S rRNA RNA were purchased as a kit (TaqMan Ribosomal RNA Control Reagents Kit, Perkin Elmer Applied Biosystems, Foster City, CA). Absolute standard curves were constructed from serial dilutions of FXYD1 mRNA

(1fg-100ng) transcribed *in vitro* using T7 RNA polymerase [1] from a hFXYD1 cDNA template cloned from human brain tissue into the pGEM-T plasmid. The threshold cycle number (C_T) from each sample was referred to the absolute curve to estimate the corresponding RNA content/sample, and each RNA value was then normalized for procedural losses using their respective 18S rRNA values estimated from the relative standard curve. C_T was the fractional cycle number at which the fluorescence accumulated to a level 10 times greater than 1 SD from basal values. The human and mouse FXYD1 primer were designed to amplify fragments with a length of 80-100bp within the FXYD1 mRNA coding region. Both primer and fluorescent probes were designed to target a DNA fragment of 101bp within the FXYD1 coding region using Primer Express 2.0 software (Perkin Elmer Applied Biosystems). The forward primer were 5' AGGGACAATGGCGTCTCTTG 3' (hFXYD1a-97F, Homo sapiens FXYD1 coding region, GenBank Accession number NM_005031) and 5' TCCATGCCAGTGCAGAA 3' (Mus-FXYD1-46F, Mus FXYD1 coding region, GenBank Accession number NM_019503); the reverse primers were 5' CGTAAGTGAACGGGTCGTGTT 3' (hFXYD1-197R) and 5' ATGAAGAGGATCCCAGCGATA 3 '(Mus-FXYD1-146R). The internal fluorescent oligodeoxynucleotide probe sequences 5' TGTGTGGGTCTCCTCACCATGGCC 3' (hFXYD1-134T) and 5' ACGATTACCACACCCTGCGATCG 3' (mus-FXYD1-92T) were covalently linked to the fluorescent dye, FAM, at the 5'-end, and to the quencher dye, TAMRA, at the 3'-end (Perkin Elmer Applied Biosystems). Real-time PCR reactions were

performed in a total volume of 10 µl, each reaction containing 2 µl of diluted cDNA or 2 µl of standard, 5 µl TaqMan Universal PCR Master Mix (PE Applied Biosystems), 250 nM of each gene-specific and ribosomal fluorescent probes, 300 nM of each gene specific primer, and 10 nM of each ribosomal primer. The real-time PCR program used consisted of an initial annealing period of 2 min at 50°C, followed by 10 min of denaturing at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C.

Supplementary Note 2.

Western blots. Tissue from the cerebral cortex of Mecp2-null mice and their littermate wild-type controls was dissected in cold PBS and snap frozen on dry ice. The tissue was then homogenized and extracted in lysis buffer (25 mM Tris pH 7.4, 50 mM glycerophosphate, 1% Triton X-100, 1.5 mM EGTA, 0.5 mM EDTA, 1mM sodium pyrophosphate, 1mM sodium orthovanadate, 10 µg/ml leupeptin and pepstatin A, 10 µg/ml aprotinin, and 1mM PMSF) using a PowerGen 700 (Fisher Scientific, 700 (Fisher Scientific, Hampton, NH) at a speed level 4 for 10 s. The protein extract was centrifuged for 15 min and the supernatant was resuspended in a sample buffer (62 mM Tris-HCl, pH 6.8, 1 mM EDTA, 10% glycerol, 5% SDS, and 50 mM dithiothreitol). Ten µg of total protein per lane were separated on a 4-20% pre-cast Tris-Glycine SDS–PAGE gel (Invitrogen, Carlsbad, CA) and transferred onto Immobilon-P membranes (Millipore, Billerica, MA) at 24V for 2 h using an electrophoretic transfer apparatus (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked in

freshly prepared TBST buffer containing 5% nonfat dry milk at room temperature for 1 hour and immunoblotted with anti-PLM rabbit polyclonal antibodies (generously provided by Dr. Randall Moorman, University of Virginia, Charlottesville, Virginia) at a dilution of 1:15,000 overnight with mild agitation at 4°C. The immunoreactions were detected by incubating with a anti-rabbit HRP conjugated IgG at room temperature for 1 hour and visualized using ECL reagents (Perkin-Elmer Life Sciences). When detecting MeCP2 in 293T cells transfected with pCMV-HA-MeCP2 we used rabbit polyclonal antibodies (Upstate Biotechnology, Lake Placid, NY) diluted 1:1,000.

Supplementary Note 3.

Bisulfite conversion of DNA. DNA (up to 2μg) was diluted into 50 μl with distilled H_2O and denatured by incubating with 5.5μl of freshly prepared 2M NaOH at 37°C for 30 minutes. Thereafter, 30μl of 10 mM hydroquinone (Sigma, St Louis, MO), freshly prepared by adding 55 mg of hydroquinone to 50μl of water, and 520μl of 3M Sodium bisulfite (Sigma S-8890), freshly prepared by adding 1.88 g of sodium bisulfite per 5 ml of H_2O, were mixed with the DNA and the pH was adjusted to 5.0 with NaOH. The mixture was incubated at 50°C for 16 hour. At this time, the DNA was desalted using the DNA Wizard Cleanup column kit (Promega A7280, Madison, WI) as instructed by the manufacturer. The DNA was eluted with 50μl distilled H_2O and was desulfonated in the presence of 3 M NaOH for 20 min at 37 °C. The DNA was then precipitated with 43 μl 7.5M NH_4Ac, 1 μl glycogen (20mg/ml stock, Boehringer Mannheim, Indianapolis IN)

and 3 volumes of 100% ethanol at -20°C overnight, washed with 70% ethanol, air dried and resuspended in 20 μl water.

Supplementary Note 4.

Chromatin Immunoprecipitation Assay. One million 293T cells were cross-linked by incubating with 1% formaldehyde in DMEM medium for 10 min at 37 °C, washed twice in ice-cold PBS containing a protease inhibitor cocktail (1mM phenylmethylsulfonyl fluoride (PMSF), 1μg/ml aprotinin and 1μg/ml pepstatin A) and then recovered by scraping and centrifugation at 2000 rpm for 4 min. Cell pellets were resuspended in 200 μl of chromatin lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1), incubated for 10 min at 4 °C, and then sonicated to yield chromatin fragments of 200-1000 bp using a micro-ultrasonic cell disrupter (Kontes, Vineland, NJ). The sonicated chromatin lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4 °C, and the supernatants were added to 10 ml of precipitation buffer (0.01% SDS, 1.1% Triton X-100, 167 mM NaCl, and 1.2 mM EDTA in 16.7 mM Tris-HCl, pH 8.1). After preclearing with 400 μl of salmon sperm DNA/protein A-agarose (Upstate Biotechnology) by incubation at 4 °C for 30 min with gentle agitation and then centrifugation at 1000 rpm for 1 min, nucleoprotein complexes were separated into 1 ml aliquots for immunoprecipitation using specific antibodies to MeCP2 (#07-013, Upstate Biotechnology). Ten μg of antibody were added to 1 ml nucleoprotein complexes. Antibody-nucleoprotein complex mixtures were incubated at 4 °C overnight with gentle agitation. Immunocomplexes were then collected by adding 60 μl salmon

sperm DNA/protein A-agarose (Upstate Biotechnology), incubated for 1 h at 4 °C with rotation, then centrifuged at 1000 rpm at 4 °C for 1 min. Pelleted immunocomplexes were washed sequentially with low salt wash buffer (0.1% SDS, 0.1% Triton X-100, 150 mM NaCl, and 2 mM EDTA in 20 mM Tris-HCl, pH 8.1), high salt wash buffer (0.1% SDS, 0.1% Triton X-100, 500 mM NaCl, and 2 mM EDTA in 20 mM Tris-HCl, pH 8.1), LiCl/Nonidet P-40/deoxycholate buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 1 mM EDTA in 10 mM Tris-HCl, pH 8.1), and with TE buffer (1 mM EDTA in 10 mM Tris-HCl, pH 8.0). The nucleoprotein complexes were eluted from the final washed immunoprecipitates into 250 µl of 1% SDS and 0.1 M $NaHCO_3$ by incubation at room temperature for 15 min. To reverse the cross-linking of protein to DNA, 20 µl of 5 M NaCl were added to the eluted immunoprecipitates and incubated at 65 °C overnight. Proteins were then digested by adding 2 µl of proteinase K (10 mg/ml), 10 µl of 0.5 M EDTA, and 20 µl of Tris-HCl, pH 6.5, and incubating the mixture for 1 h at 45 °C. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. To amplify DNA fragments containing the *FXYD1* promoter region, PCR was carried out using Hot Star Taq polymerase (95 °C for 15 min, then 32 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s.

Supplementary Note 5.

Immunohistochemistry and *in situ* hybridization. The brains were fixed by intracardiac perfusion of 4% paraformaldehyde-borate buffer, pH 9.5. Both solutions were made with DEPC treated water (0.1% diethyl pyrocarbonate) to

minimize RNase contamination. After dissection of the brain, tissue blocks were postfixed overnight in the same fixative containing 20% sucrose, then blocked and frozen on dry ice, and stored at –80°C until sectioning, as reported [2,3]. The sections (30μm) were cut on a sliding microtome, mounted on Superfrost Plus slides (Fisher Scientific, Santa Clara, CA), dehydrated under vacuum overnight and then frozen at –80°C until processing for hybridization. The hybridization procedure was that recommended by Simmons et al. [4], as described earlier by us [3,5], using a ^{35}S-UTP-labeled FXYD1 cRNA probe. Following an overnight hybridization at 45°C, the slides were washed and processed for cRNA detection. After dehydration, the slides were dipped in NTB-2 emulsion, and were exposed to the emulsion for three weeks at 4°C. At this time the slides were developed, quickly dehydrated, dried, and cover-slipped for microscopic examination.

To detect FXYD1 immunoreactive protein, tissue fragments containing the cerebral cortex of 40-day-old wild-type and MeCP2 null mice were fixed by immersion in Bouin's fixative for 4h at room temperature, embedded in paraffin and sectioned at 6μm. The sections were then subjected to an antigen retrieval protocol [6] before incubation with the primary antibodies. The FXYD1 antibodies were used at a 1:1,000 dilution; the immunohistochemical reaction was developed to a brown color using 3,3'-diaminobenzidine hydrochloride and an histochemical procedure previously reported [7,8].

Supplementary Reference

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