Title
Understanding the Mechanisms of Stress Related Pathways in Rett Syndrome

Author
Korsakova, Elena

Publication Date
2021

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Understanding the Mechanisms of Stress Related Pathways in Rett Syndrome

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular Biology

by

Elena Korsakova

2021

ABSTRACT OF THE DISSERTATION

Understanding the Mechanisms of Stress Related Pathways in Rett Syndrome

by

Elena Korsakova

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2021

Professor William Edward Lowry, Chair

Rett syndrome is a severe neurodevelopmental disorder that arises due to a mutation in methyl-CpG-binding protein 2 (MECP2). Rett syndrome patients suffer delay in mental, physical and cognitive development. The mechanism underlying the onset of Rett syndrome remains poorly understood. We used Rett patient derived human induced pluripotent stem cells (hiPSCs) to model Rett syndrome *in vitro* in an attempt to elucidate the pathways implicated in Rett phenotype. We discovered that Rett neurons undergo neuronal stress resulting in an increased expression of OCT1 and P53 target genes. In addition, mutant neurons exhibit premature senescence accompanied by elevated levels of DNA damage. Analyzing neuronal phenotype revealed a significant decrease in the complexity of dendritic arborization. This led us to explore these neuronal stress signatures further by analyzing Rett patient brain samples with single-cell RNA sequencing. We discovered that lack of MECP2 leads to misregulated synaptic genes as

well as abnormal metabolism. We confirmed faulty metabolism and mitochondrial respiration in our *in vitro* model, and showed that various types of neuronal stress lead to induction of OCT1 *in vitro*.

The dissertation of Elena Korsakova is approved.

April Pyle

Bennett G. Novitch

Douglas L. Black

William Edward Lowry, Committee Chair

University of California, Los Angeles

2021

This dissertation is dedicated to

My Mom:

Natalia Plotin

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I never thought that I would be here. Getting a PhD from UCLA would've seemed absolutely impossible if not ridiculous 14 years ago. Having just immigrated to the US I did not believe I was smart enough or had what it takes to pursue career in science, let alone apply to a top school such as UCLA. One person did believe though, my Mom. So, to my Mom, I owe you everything I have. Thank you for being strong, supporting us and carrying all the weight for so many years. I admire your courage, you are my best friend forever. To Valentin, thank you for being the best father a girl could ask for these past 14 years. Finally, to my husband, Tom, thank you for being a constant source of inspiration and support. You've helped me grow so much, I will forever cherish hours we spent discussing science.

I would also like to thank the people that I was lucky enough to meet throughout these years that lead me to this point. To Jheem Medh, thank you for accepting me into your laboratory, when I was just an inexperienced undergrad, you were always kind and patient. And thank you for introducing me to the CSUN-UCLA Bridges to Stem Cell Research Program. To Cindy Malone, thank you for accepting me into the program and giving me a chance to do stem cell research at UCLA. To Kathrin Plath, thank you for accepting me into your laboratory for the duration of the CSUN-UCLA Bridges to Stem Cell Research Program. Your mentorship was invaluable and encouraging. It is the work in your lab that inspired me to continue stem cell research and apply for a PhD. To Bill Lowry, thank you for being my mentor all these years. You have been incredibly understanding and supportive no matter what happened. Thank you for always being there for me and the whole lab. You are the reason we have such an incredible lab

environment, where each member is an inspiration, advisor and most importantly, a friend. Thank you to all the past and present members of the Lowry lab. Finally, thank you to my committee members, Douglas Black, April Pyle and Bennet Novitch, your input has tremendously helped me in my research.

The work presented here was funded by the Broad Stem Cell Research Center, Rose Hills Foundation.

VITA

EDUCATION

University of California, Los Angeles 2016

Ph.D. Candidate, Molecular Biology (Cell and Developmental Biology)

California State University Northridge, Northridge 2014

B.S., Biochemistry

RESEARCH EXPERIENCE

University of California, Los Angeles 2016 - present

Graduate Student Researcher; Advisor: William E. Lowry, PhD

University of California, Los Angeles 2015 - 2016

Visiting Student Researcher; Advisor: Kathrin Plath, PhD

California State University Northridge, Northridge 2013 - 2015

Student Researcher; Advisor: Jheem Medh, PhD

PUBLICATIONS

Minori Ohashi, **Elena Korsakova**, Denise Allen *et al*. …William E. Lowry. Loss of MECP2 Leads to Activation of P53 and Neuronal Senescence. *Stem Cell Reports* (2018) 10:1453–1463.

Korsakova E, Morales A, McDaniel T, *et al*. …Lowry WE. Defining dysfunction due to loss of MECP2 in Rett Patient Brain. **In preparation.**

SELECTED HONORS AND AWARDS

BSCRC Training Grant 2018 – 2021

Sandra L. Jewett Scholarship in Biochemistry 2015

California State University Northridge, Dean's List 2014

California State University Northridge, Dean's List 2013

INTELLECTUAL PROPERTY

International Application No. PCT/US2020/035643

Title: PIFITHRIN ANALOGUES AND METHODS OF TREATING RETT SYNDROME

Date: June, 2, 2020

Inventor(s): William E. Lowry *et al.*

Chapter 1: Introduction

Understanding the Mechanisms of Stress Related Pathways in Rett Syndrome

Rett Syndrome Overview

Intellectual disability (ID) syndromes result from gene mutations that cause impaired development of the central nervous system, leading to abnormal physical and mental development of affected individuals. These individuals often require assistance maintaining their daily routine and have a shortened life span. The first test to ever diagnose ID was a cognitive capabilities test designed in 1911 and meant to identify children with special needs. Nowadays, ID is diagnosed based on the IQ score being less than 70, delayed development and adaptation, and the onset of the symptoms manifesting before the individual turns 18 years old[1]. ID syndromes are considered to be rare, for e.g. the most common ID syndrome, Downs Syndrome, affects 1 in 1000 individuals. Overall, about 2% of the whole population suffers from a form of ID syndrome. Due to the relatively low numbers of affected individuals, studying ID syndromes has remained challenging. Doctors are likely to never encounter several people suffering the same ID syndrome, which leads to the lack of data and missed shared phenotypes[2]. To this day, the mechanisms underlying the onset of numerous ID syndromes remain poorly understood, emphasizing the importance of uncovering contributing pathways for the eventual development of treatments.

In 1966 an Austrian doctor, Andreas Rett published a paper describing a syndrome that was only observed in females of different ages. All the patients exhibited similar uncontrolled hand gestures and a decline in cognitive abilities[3]. Today, we know that Rett syndrome affects 1 in 10,000 individuals and is one of the most prevalent intellectual disability syndromes in females[4]. Rett syndrome patients experience severe life altering symptoms such as delays in motor skill development, distinctive impaired hand

movements, irregular breathing, seizures, low intellectual capacity amongst many others[5]. Affected individuals appear to develop normally for the first six to eighteen months, a period called preregression, followed by a progressive onset of Rett phenotype, regression and postregression[6][7]. While the preregression stage appears to be asymptomatic, young children have been reported to exhibit delay in reaching and playing with objects[8]. The regression stage is more apparent in Rett patients, since they fail to meet specific millstones such as the ability to walk and talk. The postregression stage lasts through adulthood with surviving patients[7][9].

In 1999 Huda Y. Zoghbi's group identified a methyl-CpG-binding protein 2 (MECP2), which is responsible for the onset of 90% of Rett syndrome cases[10]. MECP2 is an X-linked gene; thus, due to random inactivation of one X chromosome during development, Rett syndrome patients are mostly females exhibiting mosaicism[11]. On average, 50% of cells express a healthy MECP2 allele, while 50% express the mutant copy (Figure 1). Severity of the Rett phenotype usually depends on the degree of X-chromosome inactivation skewing and the type of de novo mutation on the paternal X-chromosome[12][6]. Familial cases of Rett syndrome account for only 2% of all Rett cases, with MECP2 mutations in males being lethal due to them possessing only one X chromosome[13]. In these cases, mutant MECP2 is passed down from the mother, while the X-chromosome harboring the mutation undergoes a non-random X-chromosome inactivation[14]. Non classic Rett syndrome has also been linked to mutations in other genes, including a cyclin-dependent kinase-like 5 (CDKL5), forkhead box protein G1 (FOXG1), myocyte-specific enhancer factor 2C (MEF2C), and transcription factor 4 (TCF4)[15].

Structure and Function of MECP2

MECP2 has two isoforms, E1 and E2, producing a highly disordered protein, capable of adopting various secondary structures, whereby, E1 and E2 differ only in their N-terminus. Although two isoforms exist, E1 is more prevalent in the brain, and mutations in E1 are responsible for the onset of Rett syndrome[16]. Lack of secondary structure allows MECP2 to perform a variety of functions and interact with numerous partners. For example, it is known to bind DNA and RNA, serve as transcriptional repressor and activator amongst other functions. Thus, it is not surprising that mutations within MECP2 lead to severe alterations in multiple pathways within the human body[17]. MECP2 consists of five domains, with two of them playing a critical role in the onset of Rett syndrome. Methyl CpG binding domain (MBD) facilitates MECP2 binding to DNA, in particular, to 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC)[18]. Interestingly, binding to 5mC points to MECP2s role as a transcriptional repressor, since 5mC has been reported to be highly abundant within the heterochromatic nuclei of mouse cerebellum. While binding to 5hmC is weaker, it renders MECP2 a transcriptional activator, since 5hmC is enriched in the euchromatic regions of mouse cerebellum[19]. Gene repression is achieved by the transcriptional repression domain (TRD) which facilitates MECP2 interaction with histone deacetylase, co-repressors, receptors and silencers to promote repression[6]. In fact, our own work has demonstrated that lack of MECP2 in Rett patient brain samples leads to the dysregulation of both active and inactive genes[20]. Although MECP2 consists of a total of 5 structural domains (Figure 2), mutations in MBD and TRD account for 70% of all Rett cases, demonstrating the importance of these domains in MECP2s function[21]. In addition to participating in gene

regulation, MECP2 has been reported to modify chromatin structure. Mouse myoblasts overexpressing Mecp2 show a significant increase of pericentric heterochromatin, while Mecp2 deficient mouse brain lacks chromatin loops[22][23]. MECP2 also plays a role in alternative splicing potentially through the interaction with the Y box-binding protein 1. It has been shown that mouse brains lacking Mecp2 exhibit abnormal levels of alternative splicing[24]. Finally, MECP2 has been reported to bind and regulate miRNA expression. Lack of Mecp2 causes overexpression of miR-37, involved in regulation of neural stem cells, and changes in the epigenetic landscape surrounding miR-37[25]. Lots of questions remain about the function and targets of MECP2. However, the multitude of known interactions MECP2 is involved in highlights the historical difficulties and subsequent importance of uncovering the complex underlying network of mechanisms essential to understanding Rett syndrome.

Modeling Rett Syndrome

Ascertaining the mechanistic underpinnings of Rett syndrome requires the use of robust tools. Mouse models have been essential in understanding Rett phenotype, although there are significant caveats to using model organisms. For example, it is not the female but male mice that are often used in research. Female mice, heterozygous for Mecp2 mutation, do not exhibit consistent Rett phenotypes due to varying degree of X-chromosome inactivation. Thus, male hemizygous mice are often used in research. These mice have a shortened life span but live long enough to conduct experiments. Around 4 weeks of age they start to exhibit some of the classic Rett symptoms such as breathing issues, anxiety, impaired movement, microcephaly and obesity (Figure 3)

[26][27]. However, some mouse symptoms do not exactly recapitulate those of humans. Whilst hand clasping in Rett mice is thought to be equivalent to the specific hand gestures observed in Rett patients, loss of speech in Rett patients can never be accessed in mouse models. Additionally, Rett patients tend to be anxious and socially withdrawn, however, the opposite has been observed in Rett mice[28]. Finally, mouse models do not provide physiologically accurate information about the molecular changes due to a complete lack of functional Mecp2, and may show species specific differences to that of the human.

Studying Rett phenotype at the transcriptional level requires the use of human cells that more accurately reflect the mutational burden of Rett patients. Thus, many researchers including our team have turned to modeling disease in the dish, by using human pluripotent stem cells (hPSCs)[29]. The concept of stem cells arose in 1981, when studies of mouse teratocarcinomas, or embryonal carcinoma stem cells, revealed that these cells can give rise to cells of each primary germ layer solidifying the idea that a unique subset of cells were capable of generating every cell type within the adult body. However, mouse embryonal carcinoma stem cells were often limited in their differentiation capacity and karyotypically abnormal. This prompted studies demonstrating that it is possible to derive embryonic stem cells (ESCs) from mouse blastocysts. These ESCs were pluripotent, and could differentiate into any of the three germ layers[30]. In 1998 James Thomson published his work describing the derivation of ESC culture from human blastocysts[31]. This provided a remarkable tool for the field, however, there were many ethical issues associated with using ESCs. Thus, it was established that only tissue donated by couples undergoing *in vitro* fertilization was acceptable for the generation of ESCs, and no blastocysts could be created *in vitro* for scientific purposes[32]. In 2007,

the ethical issue of stem cell research was resolved, when Takashi and his colleagues published a protocol for generating induced pluripotent stem cells (iPSCs) from human fibroblasts through the transient over-expression of OCT4, c-MYC, KLF4 and SOX2 transcription factors[33]. This revolutionized the field, making it possible to generate stem cells without the need for human embryos, but also allowed for a new era in disease modeling through the direct reprogramming of patient cells, harboring patient specific mutations. Importantly, these patient specific iPSCs can be differentiated into unlimited quantities of patient specific cell types of interest, including cells of the central nervous system. Since then, iPSCs have been used to model various disorders in a two-dimensional culture, as well as in organoids[29][34].

For our studies, we took mutant and wild type fibroblasts from Rett patients, and reprogrammed them to iPSCs. This resulted in a mixed population of cells containing control iPSCs harboring a healthy MECP2 allele and Rett iPSCs, where cells express a mutant copy of MECP2[35]. After single-cell cloning, we were then able to generate cell lines either 100% wild-type or harboring the MECP2 mutation. Critically, this method ensures the same genetic background between wild-type and mutant iPSC lines, as they are both derived from the same patient. Armed with the clinical manifestations of the disease from patients, we are now able to couple this with an understanding of the genetic factors responsible for the disease.

Transcriptomic Studies of Rett Syndrome

Numerous attempts have been made to elucidate molecular changes due to the loss of MECP2, in various cell types. Most of the studies employed bulk RNA sequencing,

a technique that provides in depth analysis of the changes in gene expression. It provides information about mutations present within the RNA-sequence as well as the levels of RNA expression in a tissue of interest[36]. Analysis of mutant and wild type neurons derived from human embryonic stem cells revealed an overall downregulation of gene expression in mutant neurons, as well as mitochondrial dysfunction and diminished protein synthesis due to lack of MECP2[37]. We also employed bulk RNA sequencing to profile our patient derived interneurons and discovered dysregulation of synaptic signaling and extracellular remodeling in Rett neurons[38]. Bulk RNA sequencing of mouse cerebellum confirmed global downregulation of gene expression as well as misregulated synaptic signaling in Rett model mice[39]. While these findings contribute greatly to the field, analysis of Rett patient tissue remains the most essential and primary source of information about the molecular changes due to loss of MECP2. In 2018, Gogliotti's group performed bulk RNA sequencing of six Rett patient cerebellum and nine motor cortex samples. Contrary to previously mentioned data, Gogliotti's group found that lack of MECP2 leads to an overwhelming amount of upregulated genes in the patient brain. Analysis of misregulated pathways revealed faulty synaptic transmission and found CHRM4 to be a potential therapeutic target[40]. While bulk RNA sequencing has proven to be an essential tool in understanding molecular changes in the whole tissue, it doesn't provide information about gene expression within individual cell types, which is a crucial aspect of better understanding the mechanistic nature of Rett syndrome.

As mentioned previously, Rett patients are mosaic for MECP2 expression due to random X-chromosome inactivation[11]. Thus, it is not only important to distinguish between different cell types, it is essential to consider cellular heterogeneity and be able

to profile individual wild type and mutant cells. Analyzing single cells from the same tissue ensures the same genetic background and eliminates any factors that could influence the comparison of two different individuals. This has been achieved with the invention of single-cell RNA sequencing, a technique that captures changes in gene expression at a single cell level, thus providing essential information about the processes each cell undergoes due cell-autonomous, as well as cell-non-autonomous effects, the information lost during bulk RNA sequencing[41]. So far, the only semi informative single-cell RNA sequencing analysis performed on three Rett patient occipital cortex samples was done using SNPs to assign wild type versus mutant status to the cells. This analysis reveals differences in gene expression, however, does not provide in depth information about the misregulated pathways, due to the mutation[42]. Thus, we FACS sorted wild type and Rett patient brain samples, performed single-cell RNA sequencing and identified numerous pathways and processes that are affected due to loss of MECP2. We found strong misregulation of synaptic transmission, supported by the previously reported data, as well metabolic dysfunction in Rett patient brain[20].

MECP2 Importance Throughout the Body

Although MECP2 mutations are often linked with neurodevelopmental disorders, the genes' widespread expression throughout tissues within the body also impacts various other organ functions. Mice lacking Mecp2 in the peripheral tissue but retaining it in the brain show mild signs of Rett syndrome. These mice exhibit less activity, fatigue and reduced bone stiffness[43]. Lack of Mecp2 in mouse liver only, manifests syndromes such as fatty liver, glucose intolerance and abnormal levels of cholesterol[44]. In addition,

MECP2 is important for maintenance and organization of skeletal muscle, as knocking out Mecp2 leads to fibrosis and morphological abnormalities of the muscle[45]. Finally, Rett patients commonly suffer from dyslipidemia, gallbladder disease, scoliosis, gastrointestinal disorders and pneumonia[46][47][48]. While this demonstrates MECP2s importance in the peripheral tissue, most of the research throughout the years has been focused on the role of MECP2 in the central nervous system. This is due to MECP2s expression being much higher in the brain compared to other tissues[43].

MECP2 mutations affect various cell types within the brain to a different extent. Analysis of neurons lacking MECP2 points to a condition known as neuronal stress, which results in decreased dendritic arborization and axon length[49][50][51]. In addition, mouse neurons lacking Mecp2 exhibit abnormal levels of chromatin acetylation[52]. Lack of Mecp2 in mouse oligodendrocytes leads to motor dysfunction, while normal Mecp2 levels in astrocytes are essential for normalized breathing[53][54]. Restoring normal Mecp2 levels in the microglia of Rett mice prolongs their lifespan, rescues breathing and motor function[55]. Interestingly, lack of Mecp2 in the brain has a non-cell autonomous effect on neurons. Co-culturing wild type neurons with either Rett astrocytes or glia causes the neurons to exhibit aberrant morphology, reduced dendritic branching and decreased soma size[56][57].

Within the brain, MECP2 is highly enriched in neurons, compared to other cell types, and the expression increases as neurons mature[16]. This suggests that MECP2 is essential for the proper neuronal function, as lack of MECP2 in neurons only causes the onset of Rett phenotype. Knocking down 60% of Mecp2 function in post mitotic neurons results in mice exhibiting obesity, impaired motor function and microcephaly. In

addition, neurons lacking Mecp2 have decreased soma size and reduced spontaneous action potential rates[58][59]. Re-expressing Mecp2 in 70% of neurons, leads to improved morphology of the brain, including an increase in neuronal soma size, brain mass and number of dendritic spines. In addition, these mice exhibit normalized breathing, improved balance and increased grip strength[60].

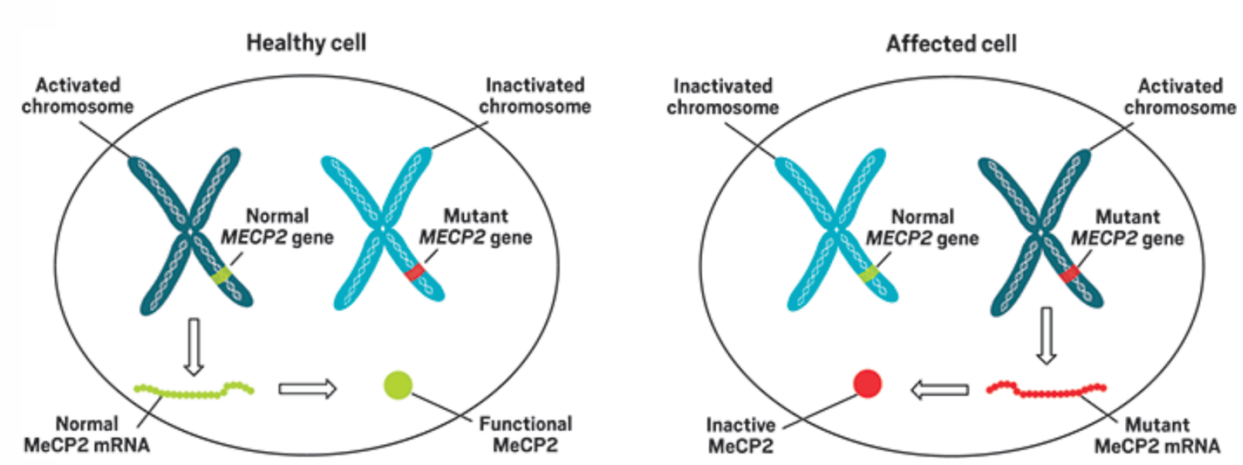
Interestingly, MECP2 plays distinct roles within different neuronal subtypes. Mice lacking Mecp2 in glutamatergic neurons suffer from tremor, anxiety and have shortened life span. Restoring Mecp2 function in glutamatergic neurons rescues neuron activity by normalizing the firing rate frequency[61]. Deleting Mecp2 from a different excitatory neuron subtype, serotonergic neurons, causes the mice to exhibit aggression[62]. Lack of Mecp2 in GABAergic neurons causes the mice to exhibit repetitive behaviors, motor dysfunction, decreased activity, memory problems, abnormal breathing and apneas[63]. Knocking down Mecp2 in somatostatin interneurons, specifically, leads to seizures and repetitive behaviour, such as nose poking. Conversely, lack of Mecp2 in parvalbumin interneurons causes mice to develop impaired motor function, obesity, social and memory problems[64]. The re-expression of Mecp2 in GABAergic interneurons of Mecp2 null mice rescues most of Rett syndromes symptoms. Mice exhibit decrease in body mass, prolonged lifespan, improved social behaviour, decrease occurrence of seizures, improved coordination, grip strength and stabilized inhibitory signaling[65]. Taken together, this data emphasizes the importance of MeCP2 in the brain, versus peripheral tissue, and implicates neurons as being primary contributors to the onset of Rett syndrome.

Rett Syndrome Therapies

While many challenges remain in understanding the mechanism underlying the onset of Rett syndrome, multiple avenues are being explored in treating this disorder. Insulin-like growth factor 1 (IGF-1) and brain-derived neurotrophic factor (BDNF) expression levels are severely downregulated due to lack of MECP2. This prompted attempts in rescuing Rett phenotype by supplementing these growth factors. Mecp2 deficient mice receiving IGF-1 showed improved life expectancy, more coordinated movement, better breathing pattern and improved synaptic plasticity[66]. However, no significant results have been achieved in early clinical trials using IGF-1[67]. Overexpressing BDNF in Mecp2 deficient mice had a similar effect to IGF-1, with mice living longer, exhibiting more coordinated movement and showing improved electrophysiological activity[68]. Early clinical trials showed varying degrees of success with a number of patients developing allergic reactions to a BDNF stimulator[69][70]. Another avenue being explored is gene therapy. Rett mice that were administered a virus containing wild-type Mecp2, showed signs of reversed Rett phenotype. However, gene therapy poses a threat of delivering the wrong dose of MECP2. Every affected female has varying degrees of mutant MECP2 protein due to random X-chromosome inactivation. It has been found that expressing too much MECP2 leads to a neurodevelopmental disorder, called MECP2 duplication syndrome that causes a number of symptoms similar to the Rett syndrome[71]. Thus, it is essential to get MECP2 dosage right if attempting gene therapy. Multiple research groups are exploring other treatment options, however, none have been successful in clinical trials so far. Too many questions about Rett syndrome remain. It is still unclear how mutations in MECP2 lead to the array

of phenotypes in Rett patients, primarily because the exact mechanism of MECP2 function is still poorly understood. It is hard to discern between cause and effect given the multitude of targets that MECP2 interacts with, as well as distinguish between cell-autonomous and non-cell-autonomous effects. However, we hope that our work will contribute to understanding the underlying pathways and causes of Rett phenotype and eventually lead to alleviating it, thus, promoting healthy development and hopefully rescuing the symptoms.

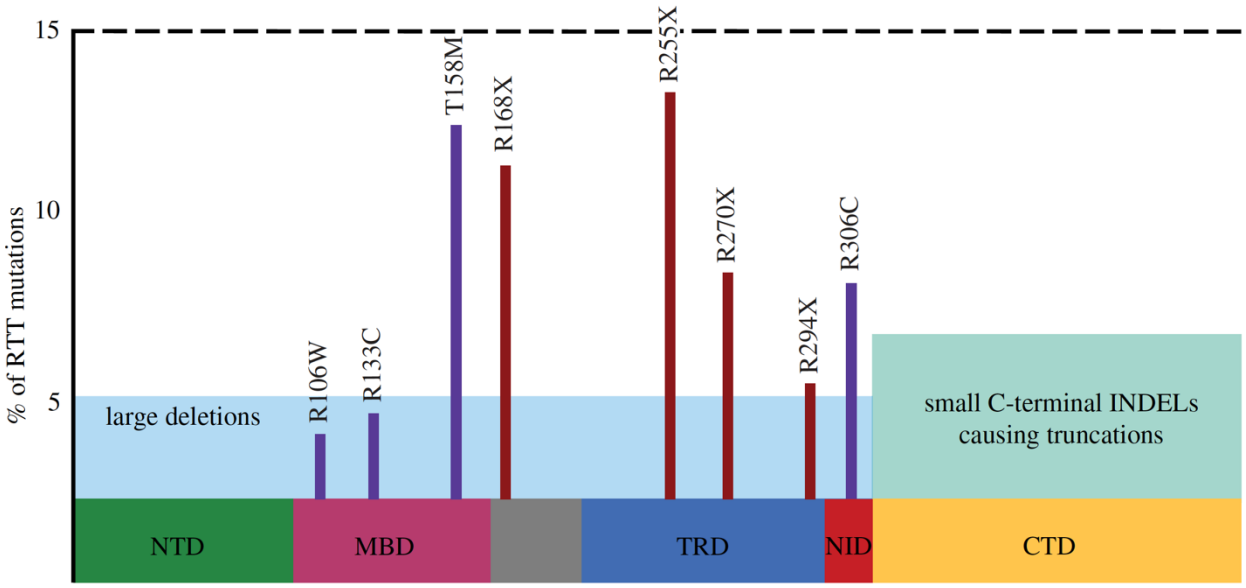
Figure 1



Adapted from C&EN. 2017.

Figure 1. Random X-chromosome Inactivation in Rett Patients. One X-chromosome in a female cell undergoes inactivation at random. If the inactivated chromosome contains a mutated MECP2 allele, silencing this allele ensures that a functioning MECP2 copy is produced. However, if the chromosome containing a healthy MECP2 allele is inactivated, then this cell contributes to the Rett syndrome by expressing mutant MECP2 copy.

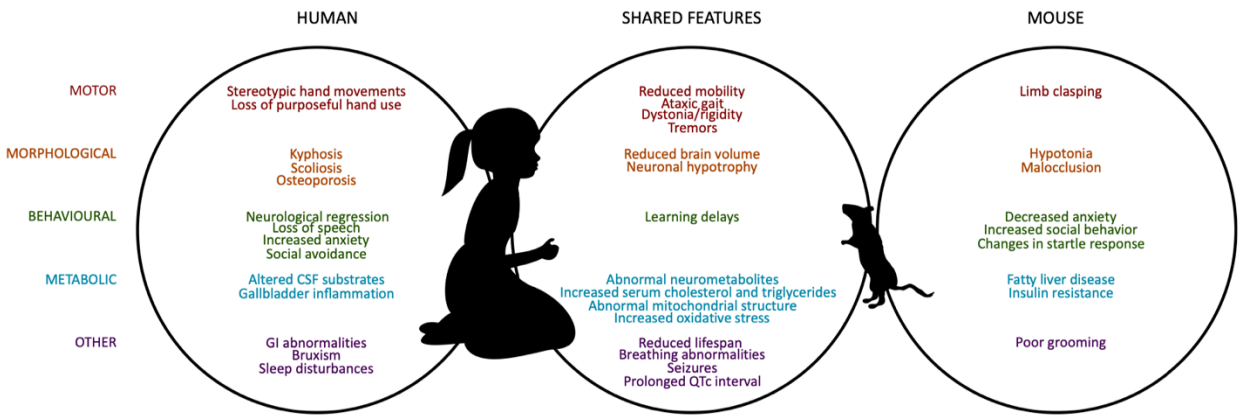
Figures 2



Adapted from Kyle et al. 2018

Figure 1. Structure of MECP2. MECP2 consists of five domains with mutations in MBD and TRD accounting for 70% of all Rett cases. Most common nonsense and missense mutations are indicated in red and purple respectively. Y-axis indicates the percent of individuals suffering the indicated mutation.

Figure 3



Adapted from Vashi et al.

Figure 3. Human Versus Mouse Phenotype due to Rett Syndrome. While many features of Rett syndrome are shared between a mouse and a human, many phenotypes differ. This emphasizes the caveats of using mouse models and highlights the importance of understanding Rett syndrome in humans.

References

1. K. Yeates, “Intellectual Disability Syndromes”, *Pediatric Neuropsychology*, second edition, pp. 447-470, 2010.
2. J. Waite, “The importance of understanding the behavioural phenotypes of genetic syndromes associated with intellectual disability”, *Paediatrics and Child Health*, vol. 24, no.10, pp. 468-472, 2014.
3. A. Rett, “Über ein eigenartiges hirnatrophisches Syndrom bei Hyperammonämie im Kin- desalter,” *Wien Med Wochenschr*, vol. 116, pp. 723–728, 1966.
4. D. Armstrong, “Neuropathology of Rett Syndrome,” *J. Child Neurol.*, vol. 20, no. 9, pp. 747–753, 2005.
5. Y. Nomura, “Early behavior characteristics and sleep disturbance in Rett syndrome,” *Brain Dev.*, vol. 27, pp. 35–42, 2005.
6. M. Lyst, “Rett syndrome: a complex disorder with simple roots,” *Nat. Rev. Genet.*, vol. 16, pp. 261–274, 2015.
7. Y. Nomura, “Natural History of Rett Syndrome”, *Journal of Child Neurology*, vol. 20, no.9, pp. 764-768, 2005.
8. Y. Nomura, “Characteristics of Motor Disturbances of the Rett Syndrome”, *Brain & Development*, vol. 12, no 1, 1990.
9. R. Kirby, “Longevity in Rett Syndrome: Analysis of the North American Database”, *J Pediatr*, vol.156, no.1, pp. 135–138, 2010.
10. R. Amir, “Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2,” *Nat. Genet.*, vol. 23, pp. 185–188, 1999.
11. R. Amir, “Influence of mutation type and X chromosome inactivation on Rett syndrome phenotypes,” *Ann. Neurol.*, vol. 47, no. 5, 2000.
12. Enikanolaiye, “Suppressor mutations in Mecp2-null mice implicate the DNA damage response in Rett syndrome pathology,” *Genome Res.*, vol. 30, pp. 1–13, 2020.
13. H. Zoghbi, “Genetic Aspects of Rett Syndrome,” *J. Child Neurol.*, vol. 3, pp. 76–78, 1988.

14. H. Zoghbi, Alan K Percy, Rebecca J Schultz, Carolyn Fill, "Patterns of X chromosome inactivation in the rett syndrome", *Brain and Development*, vol.12, no.1, pp. 131-135, 1990.
15. S. Alashti, " Two novel mutations in the MECP2 gene in patients with Rett syndrome", *Gene*, vol. 732, 2020.
16. K. Sharma, “MeCP2 in central nervous system glial cells: current updates”, *Acta Neurobiol Exp*, vol. 78, pp. 30-40, 2018.
17. J. Ausió, “MeCP2: the long trip from a chromatin protein to neurological disorders,” *Trends Mol. Med.*, vol. 20, no. 9, pp. 487–498, 2014.
18. M. Itoh, “Methyl CpG-binding Protein Isoform MeCP2_e2 Is Dispensable for Rett Syndrome Phenotypes but Essential for Embryo Viability and Placenta Development,” *The Journal of Biological Chemistry*, vol. 287, no. 17, pp. 13859–13867, 2012.
19. M. Mellén, “MeCP2 binds to 5hmc enriched within active genes and accessible chromatin in the nervous system,” *Cell*, vol. 151, no. 7, pp. 1417–1430, 2012.
20. E. Korsakova, *Prep.*, 2021.
21. S. Alashti, “Two novel mutations in the MECP2 gene in patients with Rett syndrome,” *Gene*, vol. 732, 2020.
22. A. Brero, “Methyl CpG–binding proteins induce large-scale chromatin reorganization during terminal differentiation”, *JCB*, vol. 169, no. 5, pp. 733-743, 2005.
23. S. Horike, “Loss of silent-chromatin looping and impaired imprinting of DLX5 in Rett syndrome”, *Nature Genetics*, vol. 37, no. 1, pp. 31-40, 2005.
24. J. Young, “Regulation of RNA splicing by the methylation-dependent transcriptional repressor methyl-CpG binding protein 2,” *PNAS*, vol. 102, no. 49, pp. 17551–17558, 2005.
25. E. Szulwach, “Cross talk between microRNA and epigenetic regulation in adult neurogenesis,” *J. Cell Biol*., vol. 189, no. 1, pp. 127–141, 2010.
26. A. Enikanolaiye, “Suppressor mutations in Mecp2-null mice implicate the DNA damage response in Rett syndrome pathology”, *Genome Research*, 2020.

27. P. Moretti, “Learning and Memory and Synaptic Plasticity Are Impaired in a Mouse Model of Rett Syndrome”, *The Journal of Neuroscience*, vol. 26, no. 1, pp. 319 – 327, 2006.
28. N. Vashi, “Treating Rett syndrome: from mouse models to human therapies”, *Mammalian Genome*, vol. 30, pp. 90–110, 2019.
29. P. Srikanth, “Stem Cells on the Brain: Modeling Neurodevelopmental and Neurodegenerative Diseases Using Human Induced Pluripotent Stem Cells”, *Journal of Neurogenetics*, vol. 28, no. 1-2, pp. 5-29, 2014.
30. M. Evans, “Establishment in culture of pluripotential cells from mouse embryos”, *Nature*, vol. 292, pp. 154-156, 1981.
31. J. Thomson, “Embryonic stem cell lines derived from human blastocysts”, Science, vol. 282, pp. 1145-1147, 1998.
32. H. Rippon, “Embryonic stem cells”, *Cell Prolif*, vol. 37, pp. 23-34, 2004.
33. K. Takahashi, “Stem Cells from Adult Human Fibroblasts by Defined Factors”, C*ell*, vol. 131, no. 5, pp. 861–872, 2007.
34. M. Lancaster, “Generation of cerebral organoids from human pluripotent stem cells”, *Nature Protocols*, vol. 9, no. 10, pp. 2329-2340, 2014.
35. A. Sahakyan, “Human Naive Pluripotent Stem Cells Model X Chromosome Dampening and X Inactivation”, *Cell Stem Cell*, vol. 20, pp. 87–101, 2017.
36. M. Kuksin, “Applications of single-cell and bulk RNA sequencing in onco-immunology”, *European Journal of Cancer*, vol. 149, pp. 193-210, 2021.
37. Y. Li, “Global transcriptional and translational repression in human embryonic stem cells-derived Rett Syndrome neurons”, *Cell Stem Cell*, vol. 3, no. 13, pp. 446–458, 2013.
38. M. Ohashi, “Loss of MECP2 Leads to Activation of P53 and Neuronal Senescence,” *Stem Cell Rep.*, vol. 40, pp. 1453–1463, 2018.
39. S. Ben-Shachar, “Mouse models of MeCP2 disorders share gene expression changes in the cerebellum and hypothalamus”, *Human Molecular Genetics*, vol. 18, no. 13, pp. 2431–2442, 2009.

40. R. Gogliotti, “Total RNA Sequencing of Rett Syndrome Autopsy Samples Identifies the M4 Muscarinic Receptor as a Novel Therapeutic Target”, *J Pharmacol Exp Ther*, vol. 365, pp. 291–300, 2018.
41. L. Zhu, “A UNIFIED STATISTICAL FRAMEWORK FOR SINGLE CELL AND BULK RNA SEQUENCING DATA”, *Ann Appl Stat*, vol. 12, no. 1, pp. 609–632, 2018.
42. W. Renthal, “Characterization of human mosaic Rett syndrome brain tissue by single-nucleus RNA sequencing”, *Nat Neurosci*, vol. 21, no. 12, pp. 1670–1679, 2018.
43. P. Ross, “Exclusive expression of MeCP2 in the nervous system distinguishes between brain and peripheral Rett syndrome-like phenotypes”, *Human Molecular Genetics*, vol. 25, no. 20, pp. 4389–4404, 2016.
44. S. Kyle, “MeCP2 co-ordinates liver lipid metabolism with the NCoR1/HDAC3 corepressor complex,” *Hum. Mol. Genet.*, vol. 25, no. 14, pp. 3029–3041, 2018.
45. V. Conti, “MeCP2 Affects Skeletal Muscle Growth and Morphology through Non Cell-Autonomous Mechanisms,” *PLOS ONE*, vol. 10, no. 16, 2015.
46. M. Justice, “A role for metabolism in Rett syndrome pathogenesis”, *Rare Diseases*, vol. 1, 2014.
47. M. Freilinger, “Prevalence, clinical investigation, and management of gallbladder disease in Rett syndrome”, *DMCN*, vol. 56, no. 8, pp. 756-762, 2014.
48. A. Anderson, “Twenty years of surveillance in Rett syndrome: what does this tell us?”, *Orphanet Journal of Rare Diseases*, vol. 8, no. 87, 2014.
49. C. Chapleau, “Dendritic spine pathologies in hippocampal pyramidal neurons from Rett syndromebrain and after expression of Rett-associatedMECP2mutations”, *Neurobiology of Disease*, vol. 35, pp. 219–233, 2009.
50. D. Armstrong, “Selective dendritic alterations in the cortex of Rett syndrome”, *J Neuropathol Exp Neurol*, vol. 54, pp. 195–20, 1995.
51. M. Farley, “Intrinsic Neuronal Stress Response Pathways in Injury and Disease”, *Annual Review of Pathology: Mechanisms of Disease*, vol. 13, pp. 93-116, 2018.
52. P. Skene, “Neuronal MeCP2 is expressed at near histone-octamer levels and globally alters the chromatin state”, *Mol Cell*, vol. 37, no. 4, pp. 457-468, 2010.

53. M. Nguyen, “Oligodendrocyte Lineage Cells Contribute Unique Features to Rett Syndrome Neuropathology”, *The Journal of Neuroscience*, vol. 33, no.48, pp. 18764 –18774, 2013.
54. D. Lioy, “A role for glia in the progression of Rett's syndrome”, *Nature*, vol. 475, no. 7357, pp. 497-500, 2011.
55. N. Dereck, “Wild type microglia arrest pathology in a mouse model of Rett syndrome”, *Nature*, vol. 484, no. 7392, pp. 105–109, 2012.
56. E. Williams, “Mutant astrocytes differentiated from Rett syndrome patients-specific iPSCs have adverse effects on wild- type neurons”, *Human Molecular Genetics*, vol. 23, no. 11, pp. 2968–2980, 2014.
57. N. Ballas, “Non-cell autonomous influence of MeCP2-deficient glia on neuronal dendritic morphology”, *Nat Neurosci*, vol. 12, no. 3, pp. 311–317, 2009.
58. R. Chen, “Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice,” *Nat. Genet.*, vol. 27, pp. 327–331, 2001.
59. V. Dani, “Reduced cortical activity due to a shift in the balance between excitation and inhibition in a mouse model of Rett Syndrome”, *PNAS*, vol. 102, no. 35, pp. 12560–12565, 2005.
60. L. Robinson, “Morphological and functional reversal of phenotypes in a mouse model of Rett syndrome,” *Brain*, vol. 135, no. 9, pp. 2699–2710, 2012.
61. X. Meng, “Manipulations of MeCP2 in glutamatergic neurons highlight their contributions to Rett and other neurological disorders,” *eLIFE*, vol. 5, 2016.
62. R. Samaco, “Loss of MeCP2 in aminergic neurons causes cell-autonomous defects in neurotransmitter synthesis and specific behavioral abnormalities”, *PNAS*, vol. 106, no. 51, pp. 21966–21971, 2009.
63. H. Chao, “GABAergic dysfunction mediates autism-like stereotypies and Rett syndrome phenotypes”, *Nature*, vol. 468, no. 7321, pp. 263–269, 2010.
64. A. Ito-Ishida, “Loss of MeCP2 in Parvalbumin-and Somatostatin-Expressing Neurons in Mice Leads to Distinct Rett Syndrome-like Phenotypes,” *Neuron*, vol. 88, no. 4, pp. 651–658, 2015.

65. K. Ure, “Restoration of Mecp2 expression in GABAergic neurons is sufficient to rescue multiple disease features in a mouse model of Rett syndrome,” *eLIFE*, vol. 5, 2016.
66. J. Castro, “Functional recovery with recombinant human IGF1 treatment in a mouse model of Rett Syndrome”, *PNAS*, vol. 111, no. 27, pp. 9941–9946, 2014.
67. H. O’Leary, “Placebo-controlled crossover assessment of mecasermin for the treatment of Rett syndrome”, *Annals of Clinical and Translational Neurology*, vol. 5, no. 3, pp. 323–332, 2018.
68. Q. Chang, “The Disease Progression of *Mecp2* Mutant Mice Is Affected by the Level of BDNF Expression”, *Neuron*, vol. 49, no. 3, pp. 341-348, 2006.
69. A. Djukic, “Pharmacologic treatment of Rett syndrome with glatiramer acetate”, *Pediatr Neurol*, vol. 6, pp. 51–57, 2016.
70. A. Nissenkorn, “A potential life-threatening reaction to glatiramer acetate in Rett syndrome”, *Pediatr Neurol*, vol. 68, pp. 40–43, 2017.
71. A. Sandweiss, “Advances in understanding of Rett syndrome and MECP2 duplication syndrome: prospects for future therapies”, *The Lancet Neurology*, vol. 19, no.8, pp. 689-698, 2020.
72. 80. C&EN, “LOOK BACK AT 2007”, vol. 95, no. 49, 2017.

Chapter 2

Loss of MECP2 Leads to Activation of P53 and Neuronal Senescence

Stem Cell Reports
Report
ISSCR
OPEN ACCESS

Loss of MECP2 Leads to Activation of P53 and Neuronal Senescence

Minori Ohashi,[1,3,6] Elena Korsakova,[4,6] Denise Allen,[1] Peiyee Lee,[1] Kai Fu,[1] Benni S. Vargas,[1] Jessica Cinkornpumin,[1] Carlos Salas,[1] Jenny C. Park,[1] Igal Germanguz,[1] Justin Langerman,[2] Contantinos Chronis,[2] Edward Kuoy,[2] Stephen Tran,[5] Xinshu Xiao,[5] Matteo Pellegrini,[1] Kathrin Plath,[2,3,*] and William E. Lowry[1,3,4,*]

[1]Department of Molecular Cell and Developmental Biology, UCLA, Los Angeles, CA 90095, USA
[2]Department of Biological Chemistry, UCLA, Los Angeles, CA 90095, USA
[3]Eli and Edythe Broad Center for Regenerative Medicine, UCLA, Los Angeles, CA 90095, USA
[4]Molecular Biology Institute, UCLA, Los Angeles, CA 90095, USA
[5]Department of Integrative Biology and Physiology, UCLA, Los Angeles, CA 90095, USA
[6]Co-first author
*Correspondence: kplath@mednet.ucla.edu (K.P.), blowry@mcdb.ucla.edu (W.E.L.)
https://doi.org/10.1016/j.stemcr.2018.04.001

SUMMARY

To determine the role for mutations of *MECP2* in Rett syndrome, we generated isogenic lines of human induced pluripotent stem cells, neural progenitor cells, and neurons from patient fibroblasts with and without MECP2 expression in an attempt to recapitulate disease phenotypes *in vitro*. Molecular profiling uncovered neuronal-specific gene expression changes, including induction of a senescence-associated secretory phenotype (SASP) program. Patient-derived neurons made without MECP2 showed signs of stress, including induction of P53, and senescence. The induction of P53 appeared to affect dendritic branching in Rett neurons, as P53 inhibition restored dendritic complexity. The induction of P53 targets was also detectable in analyses of human Rett patient brain, suggesting that this disease-in-a-dish model can provide relevant insights into the human disorder.

INTRODUCTION

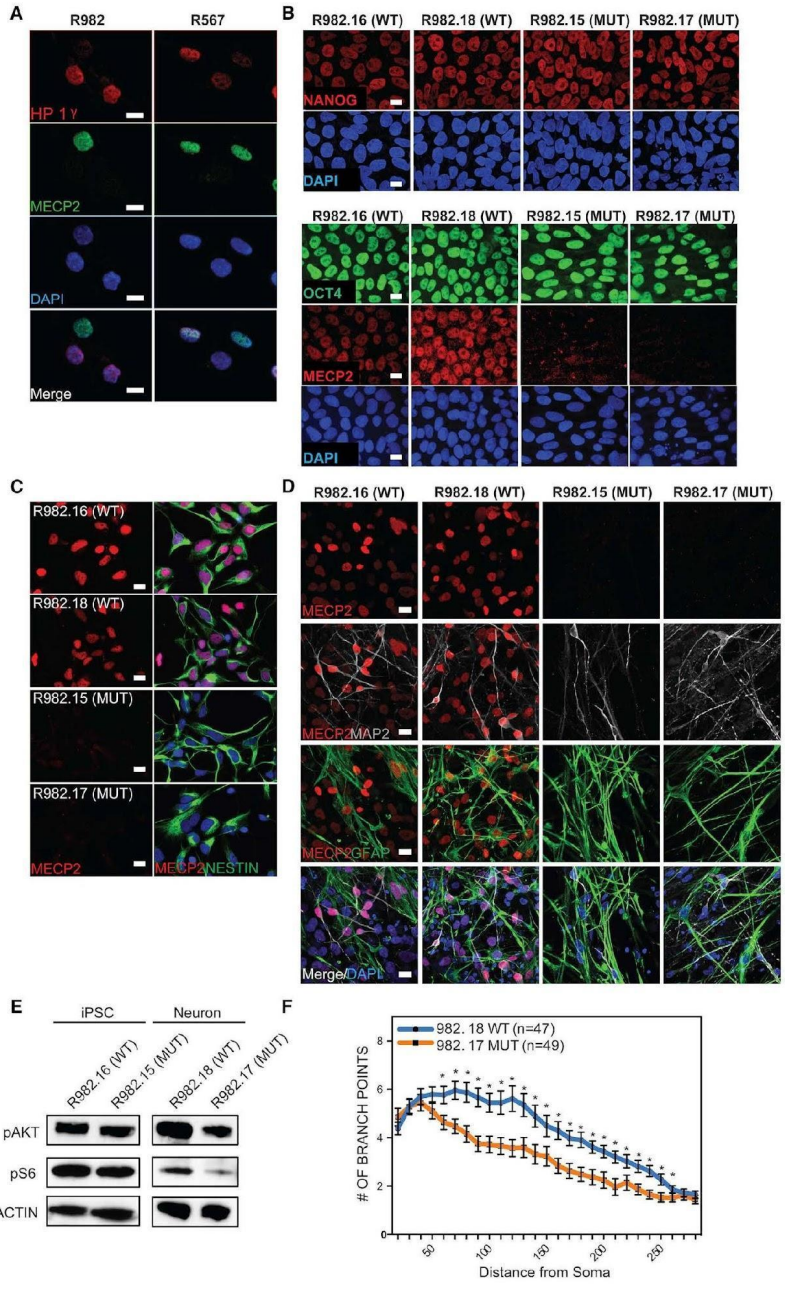
Rett syndrome is a disease associated with loss of function mutations in the gene MECP2, which was originally identified as encoding a methylated DNA binding protein (Chen et al., 2001; Meehan et al., 1992). Patient symptoms include microcephaly, intellectual disability, facial dysmorphia, and seizure activity (Bird, 2008). Studies in murine models recapitulate many of the patient phenotypes and have recently identified a role for Mecp2 particularly in inhibitory neurons (Tomassy et al., 2014). These studies demonstrated that loss of MECP2 can lead to defects in transcription (Chen et al., 2003; Lee et al., 2014), dendritic branching (Zhou et al., 2006), nuclear size (Chen et al., 2001), and AKT signaling (Li et al., 2013).

MECP2 has also been described as a transcription factor with specific targets (Chen et al., 2003; Zhou et al., 2006), and more broadly as either a transcriptional activator (Li et al., 2013) or repressor (Cross et al., 1997; Nan et al., 1997). However, despite decades of research on MECP2, it is still unclear how mutations in this protein lead to patient symptoms (Chen et al., 2001; Marchetto et al., 2010). To confirm findings made in other models and further study these in a human system, some have turned to modeling Rett syndrome *in vitro* by taking advantage of disease-in-a-dish approaches. This involves making human induced pluripotent stem cells (hiPSCs) from patient somatic cells, or using genome engineering to introduce mutations into wild-type (WT) human pluripotent stem cells. In the current study, we also sought to mitigate the effect of genetic background and variability of differentiation by taking advantage of several isogenic lines of hiPSCs that either express the WT allele or the mutant allele leading to cells that express or lack MECP2 (Tchieu et al., 2010). This allowed for detailed molecular analyses of hiPSCs, neural progenitor cells (NPCs), and neurons with and without MECP2 under the same genetic background. In comparing neurons from Rett patients as well as those with *MECP2* silenced by small interfering RNA (siRNA), it is clear that loss of MECP2 leads to induction of P53 and senescence, potentially opening an avenue of investigation for this intellectual disability syndrome.

RESULTS

A Human Model of Rett Syndrome *In Vitro*

Cognizant of the fact that differentiation from hPSCs is highly variable across individual lines, culture conditions, and time, we developed an isogenic model to study Rett syndrome *in vitro* to remove the confound of genetic background (Tchieu et al., 2010). Because female patients with Rett syndrome are usually heterozygous for mutant alleles of *MECP2*, fibroblasts isolated from these patients display a mosaic pattern where roughly half the cells express either the mutant or WT allele. This is shown in Figure 1A, where fibroblasts isolated from two patients with distinct mutant alleles of *MECP2* (R982 and R567) showed that roughly half



(legend on next page)

the cells expressed MECP2 while the other half lacked detectable amounts of this protein. Patient descriptions are provided in Figure S1. Reprogramming to iPSCs using a small set of transcription factors has been shown to happen at the clonal level, such that individual reprogramming events in single fibroblasts generate isolated hiPSC clones (Winkler et al., 2010). Therefore, reprogramming of mosaic fibroblast cultures from two different patients generated single hiPSC clones that either expressed MECP2 protein or lacked it (Figure 1B) (method described in a previous study; Sahakyan et al., 2016). In addition, our work and that of others has shown that under standard conditions, the inactive X chromosome in human fibroblasts does not reactivate upon reprogramming to the pluripotent state (Tchieu et al., 2010), which is distinct from murine reprogramming (Maherali et al., 2007).

Thus, we were able to create multiple lines of hiPSCs with and without MECP2 from individual patients and thereby control for differences in genetic background (shown in Figure 1B are clones made from patient 982; clones from 567 look similar). The hiPSCs generated from fibroblasts of both patients appeared to be unaffected by the lack of MECP2, expressed all appropriate markers, and successfully generated teratomas upon injection into the testes of immunocompromised mice, consistent with previous hiPSC models for loss of MECP2 (Figure S2A) (Cheung et al., 2011; Hotta et al., 2009). Lack of MECP2 in patient-derived cells and specificity of antibody was also confirmed by western blot (Figure S2B).

Importantly, we never observed reactivation of the silenced X chromosome that would have resulted in re-expression of the WT allele of *MECP2* in any cultures regardless of differentiation status or passage. This is consistent with previous data showing that, despite evidence for erosion of isolated portions of the silenced X chromosome (Mekhoubad et al., 2012), many portions of the inactivated X remain silenced even through reprogramming or differentiation (Patel et al., 2017; Tchieu et al., 2010). To measure the effect of any potential XCI erosion, we performed a DNA methylation analysis on the X chromosome on the lines from patient 982. This analysis showed that while some erosion of XCI was detectable across the X chromosome, there was not a significant difference between any of the lines (Figure S3A), and methylation at the *MECP2* locus specifically was unchanged between the lines (Figure S3B).

As Rett syndrome primarily afflicts the nervous system and MECP2 is most highly expressed in neurons, we first generated NPCs from all of the hiPSCs lines following standard protocols (Patterson et al., 2012). Across at least two lines per patient with and without MECP2, we measured the rate of neuralization, the morphology of NPCs, and expression of typical marker genes. We were unable to detect consistent differences in these properties between multiple clones of both WT and MECP2 null lines derived from both patients (Figures 1C and S2C). Furthermore, the growth rate of NPCs with and without MECP2 was not consistently different in NPCs made from either patient (Figure S2D). Next, the NPCs were further differentiated by a non-directed differentiation approach that yields both neurons and glia (growth factor withdrawal; Patterson et al., 2012) (Figure 1D). All NPCs from both patients produced neurons and glia at the same rate (Figures S2E and S2F).

Previous studies have also shown that loss of MECP2 in neurons can lead to a decrease in AKT signaling (Li et al., 2013). A similar pattern was observed here in mutant neurons generated from Rett patient hiPSCs as measured by phosphorylation of AKT and S6, while hiPSCs themselves did not seem to be affected by loss of MECP2 (Figure 1E). Dendritic complexity has been shown extensively to be reliant on MECP2 expression in various models of Rett syndrome, and we found a statistically significant decrease in complexity in neurons made in the absence of MECP2 by Sholl assay (Figure 1F). In addition, we observed qualitative differences in basic neuronal morphology between WT and mutant neurons, where the neurons lacking MECP2 had shorter, thicker processes, and their soma was not as well defined.

Figure 1. Generation of the Isogenic Model of Rett Syndrome *In Vitro*

(A) Fibroblasts isolated from Rett syndrome patients (R982 and R567) heterozygous for MECP2 mutations exhibit a mosaic pattern of MECP2 expression due to random XCI.

(B) Multiple isogenic hiPSC lines were produced from patient 982 with a typical Yamanaka protocol yielding individual isogenic clones with and without MECP2 expression from the same patient, as judged by NANOG and OCT4 staining.

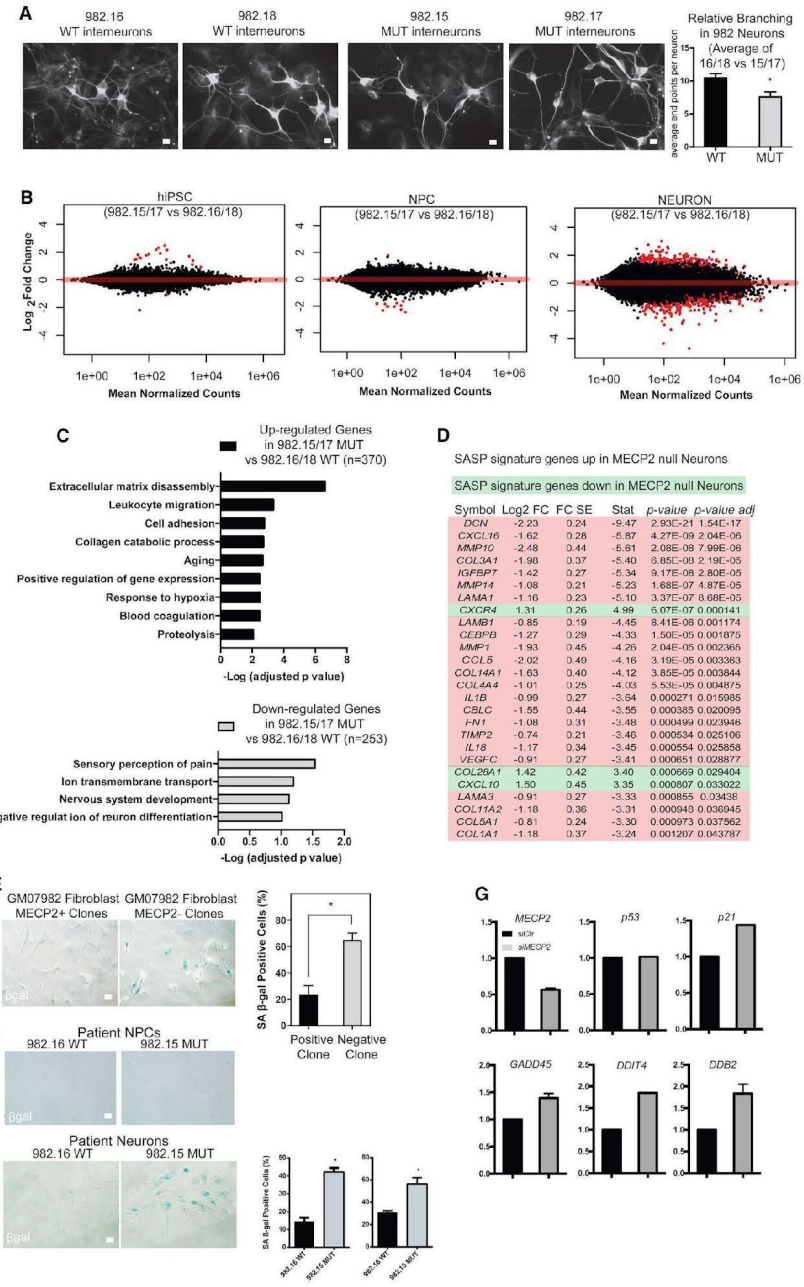
(C) Specification of hiPSCs derived from patient 982 toward neural progenitor cells yielded homogeneous cultures of NPCs with and without MECP2.

(D) Terminal differentiation of NPCs derived from patient 982 toward neurons and glia by growth factor withdrawal as measured by immunostaining for MAP2 and GFAP.

(E) MECP2+ and MECP2− hiPSCs and neurons were assayed by western blot with antibodies that recognize the active forms of Akt and its downstream target S6.

(F) Sholl assay of dendritic complexity was performed on WT versus MUT neurons derived from patient 982.

*$p < 0.05$ according to Student's t test. Bar graphs represent means ± SEM. Scale bars on images indicate 10 μm.



(legend on next page)

Loss of MECP2 Affects the Transcriptome of Neurons

It has been suggested that loss of MECP2 only affects gene expression in neurons as opposed to the hPSCs and NPCs from which they were derived (Li et al., 2013). We sought to determine whether gene expression was affected in hiPSCs, NPCs, or neurons in this patient-derived *in vitro* model. To optimize the search for molecular effects of loss of MECP2 in neurons, we generated defined neuronal cultures by following the newly established 3i (three inhibitor) method to create interneurons (Figure 2A) (Maroof et al., 2013). Interneurons are particularly relevant in the study of Rett syndrome as interneuron-specific deletion of *Mecp2* in mice recapitulates many of the disease symptoms (Ito-Ishida et al., 2015; Tomassy et al., 2014). We validated the quality of differentiation at each step by immunostaining for markers typical of particular cell types (SOX2, SOX1, and NESTIN as well as FOXG1 and NKX2.1 for NPCs; and Tuj1, MAP2, and GABA for interneurons) in both WT and MUT cultures followed by quantification (Figures S4A and S4B). While methods for derivation from pluripotent stem cells are effective at making interneurons, these cultures are not pure. As such, we first ensured that the proportion of neurons present in the cultures for comparison were not consistently different (Figure S4C). We then assessed whether interneurons lacking MECP2 also showed diminished dendritic branching. In fact, in patient-derived interneurons made by 3i, defects in dendritic branching as measured by the number of endpoints were clearly observed (Figure 2A).

We therefore proceeded with deep RNA sequencing (RNA-seq; >120 million reads per sample) of hiPSC, NPC, and interneuron cultures. With such sequencing depth, it was possible to analyze the RNA-seq reads for the known mutations present in the patients from which these lines were made (Figure S4D). This analysis demonstrated that each line studied expressed strictly either the WT or mutant allele of *MECP2*, and that XCI status was unchanged even after extensive differentiation to neurons. We quantified the expression level of *MECP2* in WT cells across these three stages of development and found that the average reads per kilobase of transcript per million mapped reads RPKM was 3.1 for hiPSCs, 4.3 for NPCs, and 7.75 for interneuron-enriched cultures. This is consistent with consensus that MECP2 is enriched in neuronal cells, but also demonstrates that it could potentially be relevant to hiPSC and NPC physiology as well. However, high stringency analyses (false discovery rate <0.05) of the RNA-seq data yielded very few gene expression changes due to loss of MECP2 in hiPSCs or NPCs derived from Rett patients (Figure 2B), consistent with Li et al. (2013). On the other hand, interneuron cultures made from patient 982 showed many gene expression changes when comparing two individual WT and MUT clones (Figure 2B). Gene ontology analysis uncovered many neuronal physiology-related pathways that were downregulated due to loss of MECP2 in neurons, while genes associated with extracellular remodeling and cell migration appeared to be induced (Figure 2C).

Probing the RNA-seq data, we also found that MECP2 null interneuron cultures showed a strong increase in a group of genes that are known to be induced by senescent cells, known as the senescence-associated secretory program (SASP). The vast majority of SASP genes that were changed in MECP2 null neurons were upregulated as opposed to downregulated, suggesting a robust pattern of SASP induction (Figure 2D). The only previous report linking MECP2 loss to senescence was performed by partial silencing of this protein in mesenchymal stem cells, but the results were consistent with those shown here for patient-derived MECP2 null fibroblasts (Squillaro et al., 2010). The induction of SASP was intriguing in light of the fact that, while attempting to make clones of fibroblasts from patients with Rett syndrome, we repeatedly found that clones lacking MECP2 did not expand well after passage (14 MECP2 null clones were created, none expanded), while clones expressing the WT allele expanded without a problem (42 MECP2+ clones were created, we attempted to expand four of them, and all four expanded).

To determine whether MECP2 null fibroblasts encounter senescence, we performed an assay to detect endogenous

Figure 2. Loss of MECP2 Is Associated with Differential Gene Expression in Neurons

(A) Immunostaining neurons generated from patient 982 for TuJ1, a neuronal-specific marker. Right: quantification of dendritic complexity by counting endpoints.

(B) Volcano plots of differentially expressed genes (DEGs) in hiPSCs, NPCs, and neurons.

(C) Gene ontological analysis of DEGs increased versus decreased in MECP2 null neurons.

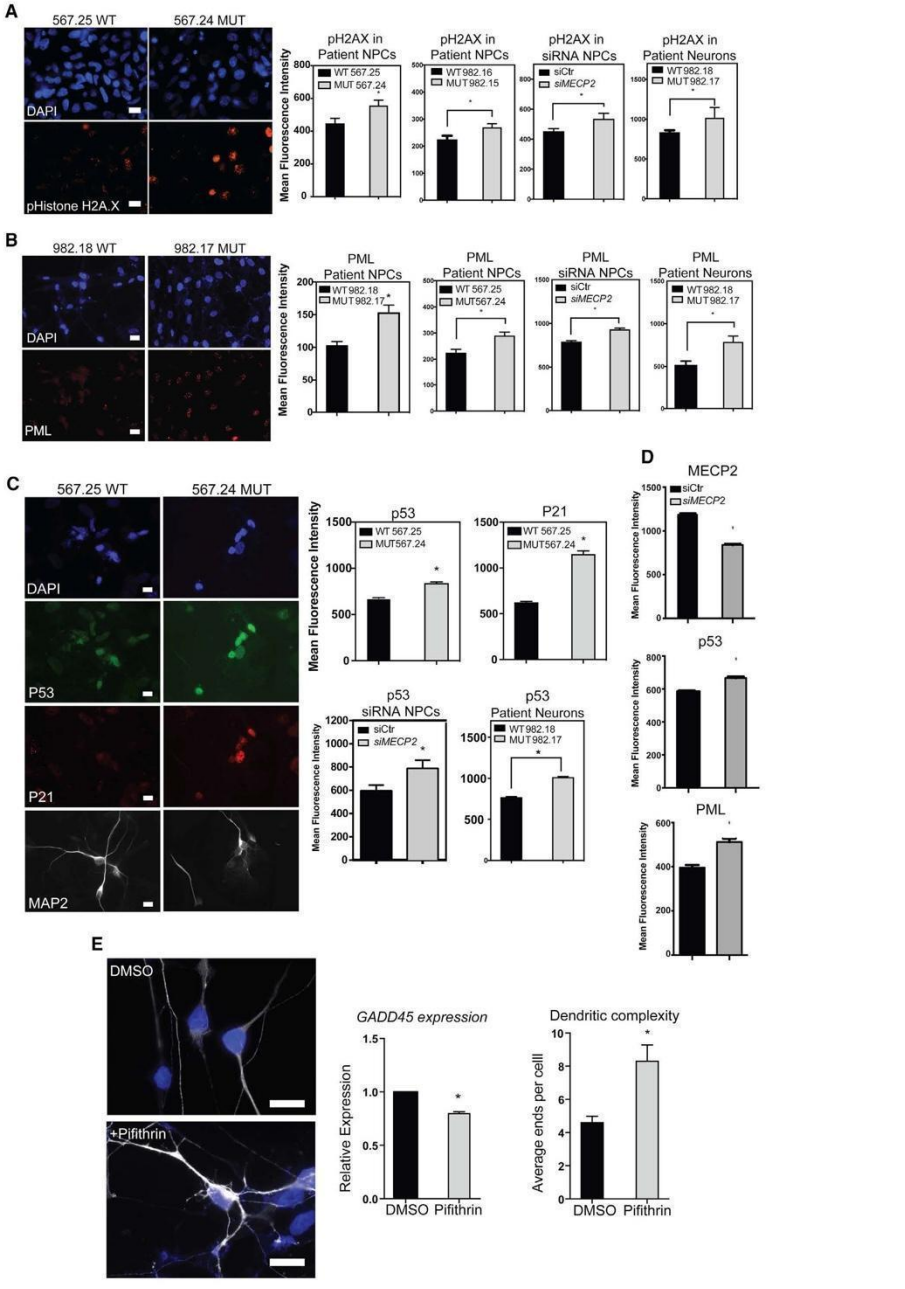
(D) An examination of SASP genes in neurons.

(E) Patient skin-derived clones of fibroblasts lacking MECP2 showed strong β-gal activity, while those of WT fibroblasts did not.

(F) Top: the senescence assay applied to neural progenitors derived from Rett patients did not show significant senescence activity. Bottom: patient-derived neuronal cultures showed a strong increase in the absence of MECP2 (quantification across independent lines shown on the right).

(G) RT-PCR for P53 targets after siRNA treatment of WT neurons.

Bar graphs represent means ± SEM. *p < 0.05. Scale bars on images indicate 10 μm.



(legend on next page)

beta-galactosidase activity, which is known to be a hallmark of this process (Wang et al., 2013). Indeed, MECP2 null fibroblasts showed strong activity in this senescence assay (Figure 2E). We did not encounter such difficulties with clonal expansion once hiPSCs or hiPSC-derived NPCs were made from patients, presumably because during reprogramming, telomerase is strongly induced to restore telomere length at least beyond the critical threshold (Marion et al., 2009; Suhr et al., 2009). In fact, our RNA-seq data showed that hiPSCs made from patients had very high expression of *TERT*, and NPCs still expressed moderate levels, while neurons did not express appreciable levels (average RPKM for *TERT*: hiPSC, 8.8; NPC, 1.6; neuron, 0.006). Importantly, the same endogenous galactosidase activity assay on interneurons showed a dramatic increase in senescence activity in neurons lacking MECP2 (Figure 2F). These data indicate that loss of MECP2 leads to not only induction of SASP but also a *bona fide* senescence program in neurons.

Induction of P53 in the Absence of MECP2

Cellular senescence programs are known to be regulated by P53, which can then activate various response pathways downstream, such as DNA repair and apoptosis (Vaziri and Benchimol, 1996). Interestingly, P53 induction due to telomere shortening was previously shown to cause defects in dendritic branching (Ferron et al., 2009), which is also the dominant phenotype in Rett syndrome. To begin to look for hallmarks of P53 induction in the absence of MECP2, we performed RT-PCR for P53-related targets in cells with silencing of *MECP2* by siRNA (Figure S4E). This assay suggested that decreased MECP2 levels led to induction of P53-related target genes such as *P21*, *GADD45*, *DDIT4*, and *DDB2* (Figure 2G).

To determine the effect of loss of MECP2 in relation to cell-stress pathways at the protein level, we performed immunostaining for H2AX, PML, P53, and P21 in neurons with and without MECP2. Staining for each of these markers showed strong increases in expression/levels of these markers of cell stress in patient-derived NPCs, neurons, and also after silencing of *MECP2* in both NPCs and neurons (Figures 3A–3D). WT NPCs with silencing of *MECP2* by siRNA and neurons lacking MECP2 also showed clear induction of these marks.

Blocking Induction of P53 Can Rescue Dendritic Branching Defects Due to Loss of MECP2

Previous evidence from a murine model of telomere shortening as a result of loss of telomerase complex (TERT) led to defects in dendritic branching, and this effect was strictly dependent on induction of P53 (Ferron et al., 2009). A more recent study also showed that experimentally aging the neural lineage with telomerase inhibition led to neurons with signs of aging, including reduced dendritic branching (Vera et al., 2016). Therefore, we posited that inhibition of P53 in MECP2 null neurons could potentially restore appropriate dendritic branching. To determine whether blocking the action of P53 could improve dendritic branching in MECP2 null interneurons, we took advantage of Pifithrin-α, a potent inhibitor of P53 target gene activation (Bassi et al., 2002). Treatment of MECP2 null interneurons with Pifithrin-α showed evidence of P53 inhibition as measured by RT-PCR for *GADD45* (Vaziri and Benchimol, 1996), a target gene important for DNA repair (Figure 3E). After 24–48 hr of P53 inhibition by Pifithrin-α, MECP2 null interneurons appeared to adopt an improved neuronal morphology typified by increased physical distinction between the soma and neurites, longer, thinner neurites, as well as increased dendritic branching as shown and quantified in Figure 3E. These data provide evidence that neurons without MECP2 induce P53 activity, which then inhibits the formation of complex neuronal processes.

To determine whether any of the phenotypes discovered in this *in vitro* model of Rett syndrome have relevance to patients afflicted with the disease, we acquired tissue specimens from Rett patients and age-matched controls. We first quantified the degree of chimerism of female Rett patient neurons due to skewing of X chromosome inactivation to determine the relative ratio of neurons that express MECP2 versus those that did not. Some of the Rett patient brains showed roughly 75% of neurons lacked MECP2, while others appeared to have less than 25% MECP2 null neurons (Figure 4A).

Figure 3. Loss of MECP2 Leads to Induction of DNA Damage and P53

(A) Immunostaining of patient NPCs, NPCs with siRNA against MECP2, and patient neurons showed a strong increase in H2aX in the absence of MECP2.

(B) Immunostaining of patient NPCs, NPCs with siRNA against MECP2, and patient neurons.

(C) Immunostaining for P53 and p21, a target of P53.

(D) Immunostaining after siRNA silencing of MECP2 in WT neuronal cultures.

(E) Treatment of MECP2 null neurons with DMSO or Pifithrin, followed by immunostaining with antibody for TuJ1 shows a change in dendritic branching. Bottom left: RT-PCR for *GADD45*, a P53 target gene, showed that Pifithrin reduced P53 activity. Bottom right: quantification of branching phenotype across three independent experiments.

*$p < 0.05$ according to Student's t test. Bar graphs represent means ± SEM. Scale bars on images indicate 10 μm.

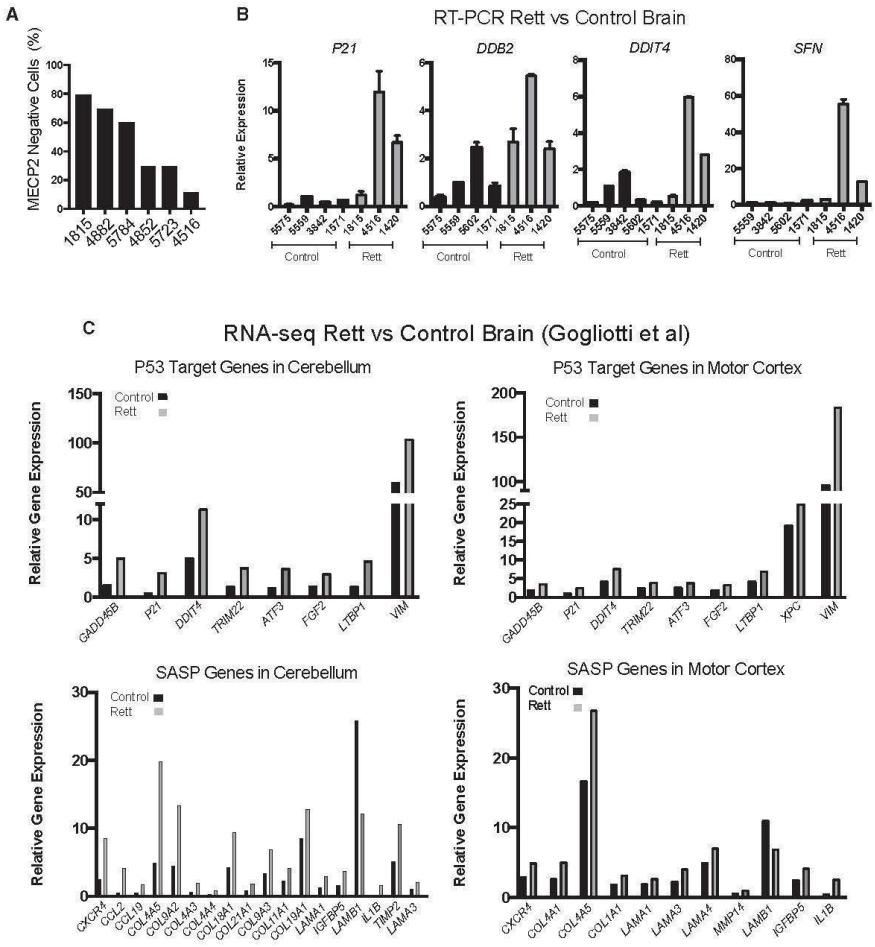


Figure 4. Evidence for P53 Induction in Rett Patient Neurons in Human Brain

(A) Each Rett brain sample was assessed for the percentage of neurons with and without expression of MECP2 by immunostaining.

(B) RT-PCR on RNA isolated from Rett brain versus age-matched control brains for P53 targets.

(C) A re-analysis of data published by Gogliotti et al. (2018). Shown are a sample of P53 targets and SASP genes found on lists of genes upregulated in Rett brain. All of these differentially expressed genes were derived using a corrected p value (false discovery rate) <0.05 from at least n = 5 samples from control and Rett brains.

Bar graphs represent means ± SEM.

We performed RT-PCR on samples from some of these brains to determine whether they showed signs of increased P53 activity. To ensure accurate RNA representation, we first assessed the quality of the RNA from these frozen tissues, and only proceeded with RT-PCR in samples that showed an RIN (RNA integrity number) value above 5. All the Rett patient brains we processed for RT-PCR showed induction of canonical P53 target genes (identified in Wei et al., 2006), consistent with what was observed in the patient-derived neurons *in vitro* (Figure 4B). Recently, a new study was published that isolated brain tissue from Rett patients and control subjects to

perform an RNA-seq profile to characterize changes specific to Rett motor cortex or cerebellum. Using data provided in that study, we found that many direct P53 targets (as defined in Wei et al., 2006) and SASP signature genes were upregulated in the Rett brains in both the motor cortex and the cerebellum (Figure 4C).

DISCUSSION

Taken together, these data demonstrate that loss of MECP2 leads to clear signs of stress such as H2AX deposition, P53/P21 induction, and initiation of a senescence program, all of which suggest that neurons in Rett syndrome could be in suboptimal health, leading to neurophysiological defects such as dendritic arborization (Zhou et al., 2006). While one paper suggested that RNAi-mediated silencing of *MECP2* could promote senescence in mesenchymal cells (Squillaro et al., 2010), decades of work on Rett syndrome have not uncovered a role for MECP2 in relation to senescence in a wide variety of models such as various transgenic mouse lines, human patient postmortem analyses, and *in vitro* human models.

These results also raise the question of whether senescence could be common to the etiologies of other intellectual disability syndromes. The phenotypes described here show a striking similarity to those observed in hiPSCs and neural derivatives made from patients with immunodeficiency, centromeric region instability, and facial anomalies syndrome (ICF) syndrome (Yehezkel et al., 2013). Two independent studies showed that ICF patient-derived hiPSCs displayed telomere shortening that was coupled to senescence of somatic derivatives such as fibroblasts. ICF syndrome only partially overlaps with Rett syndrome in terms of patient phenotypes, but is caused by mutations in DNMT3B, a *de novo* DNA methyltransferase (Linhart et al., 2007). These findings together are highly relevant as DNMT3B is a key *de novo* methyl transferase to create methylated DNA (5mC), which is the substrate for Tet oxygenase's to create 5-hydroxmethylated DNA (5hmC), which is known to be strongly bound by MECP2 (Mellen et al., 2012). Recently, another study showed that deletion of Tet enzymes, which are critical to generate the 5hmC mark, led to shortened telomeres (Yang et al., 2016), which is known to lead to P53 activation.

Another possible interpretation of these data is that instead of a failure to mature, Rett syndrome neurons instead show aspects of premature aging. The fact that MECP2 null neurons show induction of aging-related genes, including P53 targets, and induce senescence pathways are consistent with this idea (Tan et al., 2014). On the other hand, while Rett patients suffer from a post-natal cognitive decline, and long-term survivors show phenotypes associated with Parkinson disease (Zoghbi, 2016), the typical phenotypes presented in young female patients are not consistent with premature aging. Whether the physiological response to loss of MECP2 is truly akin to premature aging or whether patients suffer from effects that are unrelated to aging is worthy of continued investigation.

EXPERIMENTAL PROCEDURES

Generation of Isogenic Rett Syndrome iPSCs

Reprogramming was performed as described (Lowry et al., 2008).

Generation of Teratomas

Generation of teratomas was previously described (Lindgren et al., 2011).

Differentiation *In Vitro* and Analysis

Neural specification with neural rosette derivation, neuroprogenitor (NPC) purification, and further differentiation to neurons and glia were performed as described previously (Patterson et al., 2012, 2014).

Immunofluorescence and Image Quantification

Immunofluorescence was performed as described previously (Patterson et al., 2014) and is described in detail in the Supplemental Information.

RT-qPCR

RT-PCR with real-time PCR measurement was carried out on a Roche 480 as described (Patterson et al., 2012, 2014). The primer sequences are available in the Supplemental Information.

siRNA Gene Silencing

All knockdown experiments were performed as described previously (Patterson et al., 2014).

β-Galactosidase Senescence Assay

β-Galactosidase senescence assay was performed using the Senescence β-Galactosidase Staining Kit from Cell Signaling. The number of blue cells and number of total cells were quantified using the Cell Counter plugin in ImageJ.

Quantification of Dendritic Arborization

The stained cells were then imaged at 20×, and dendritic arbors of individual cells were traced using the Simple Neurite Tracer plugin for ImageJ. The number of process ends per cell were counted using the Cell Counter plugin for ImageJ. The number of process ends per cell are presented as mean ends per cell ± SEM.

RNA Expression Profiling

RNA-seq was performed as described previously (Gu et al., 2016). These data are available from NIH dataset GEO: GSE107399.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.04.001.

AUTHOR CONTRIBUTIONS

M.O., E.K., D.A., P.L., K.F., B.S.V., J.C., C.S., J.C.P., I.G., J.L., C.C., E.K., and S.T. provided data through experimentation. M.O., E.K., and W.E.L. contributed to writing the manuscript. X.X., M.P., K.P., and W.E.L. provided financial support for this work.

ACKNOWLEDGMENTS

We would like to acknowledge the helpful discussions and insight on this manuscript with Gail Mandel (OHSU). This work was funded by training grants to M.O. (NIH-Virology and Gene Therapy, UCLA), P.L. (CIRM, UCLA), C.S. (CIRM-Bridges, Cal-State-Northridge), and D.A. (HHURP, UCLA). W.E.L. was supported by a Rose Hills Scholar award through the Eli and Edythe Broad Center for Regenerative Medicine. W.E.L. and K.P. were supported by NIH (P015P01GM099134). This research was also supported by the Allen Distinguished Investigator Program and the Paul G. Allen Frontiers Group, and a March of Dimes Scholar Award (6-FY17-406).

Received: September 15, 2017
Revised: March 30, 2018
Accepted: April 3, 2018
Published: May 8, 2018

REFERENCES

Bassi, L., Carloni, M., Fonti, E., Palma de la Pena, N., Meschini, R., and Palitti, F. (2002). Pifithrin-alpha, an inhibitor of p53, enhances the genetic instability induced by etoposide (VP16) in human lymphoblastoid cells treated in vitro. Mutat. Res. *499*, 163–176.

Bird, A. (2008). The methyl-CpG-binding protein MeCP2 and neurological disease. Biochem. Soc. Trans. *36*, 575–583.

Chen, R.Z., Akbarian, S., Tudor, M., and Jaenisch, R. (2001). Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. Nat. Genet. *27*, 327–331.

Chen, W.G., Chang, Q., Lin, Y., Meissner, A., West, A.E., Griffith, E.C., Jaenisch, R., and Greenberg, M.E. (2003). Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. Science *302*, 885–889.

Cheung, A.Y., Horvath, L.M., Grafodatskaya, D., Pasceri, P., Weksberg, R., Hotta, A., Carrel, L., and Ellis, J. (2011). Isolation of MECP2-null Rett syndrome patient hiPS cells and isogenic controls through X-chromosome inactivation. Hum. Mol. Genet. *20*, 2103–2115.

Cross, S.H., Meehan, R.R., Nan, X., and Bird, A. (1997). A component of the transcriptional repressor MeCP1 shares a motif with DNA methyltransferase and HRX proteins. Nat. Genet. *16*, 256–259.

Ferron, S.R., Marques-Torrejon, M.A., Mira, H., Flores, I., Taylor, K., Blasco, M.A., and Farinas, I. (2009). Telomere shortening in neural stem cells disrupts neuronal differentiation and neuritogenesis. J. Neurosci. *29*, 14394–14407.

Gogliotti, R., Fisher, N., Stansley, B., Jones, C., Lindsley, C., Conn, J., and Niswender, C. (2018). Total RNA-sequencing of Rett syndrome autopsy samples identifies the M4 muscarinic receptor as a novel therapeutic target. J. Pharmacol. Exp. Ther. *365*, 291–300.

Gu, W., Gaeta, X., Sahakyan, A., Chan, A.B., Hong, C.S., Kim, R., Braas, D., Plath, K., Lowry, W.E., and Christofk, H.R. (2016). Glycolytic metabolism plays a functional role in regulating human pluripotent stem cell state. Cell Stem Cell *19*, 476–490.

Hotta, A., Cheung, A.Y., Farra, N., Vijayaragavan, K., Seguin, C.A., Draper, J.S., Pasceri, P., Maksakova, I.A., Mager, D.L., Rossant, J., et al. (2009). Isolation of human iPS cells using EOS lentiviral vectors to select for pluripotency. Nat. Methods *6*, 370–376.

Ito-Ishida, A., Ure, K., Chen, H., Swann, J.W., and Zoghbi, H.Y. (2015). Loss of MeCP2 in parvalbumin-and somatostatin-expressing neurons in mice leads to distinct Rett syndrome-like phenotypes. Neuron *88*, 651–658.

Lee, W., Yun, J.M., Woods, R., Dunaway, K., Yasui, D.H., Lasalle, J.M., and Gong, Q. (2014). MeCP2 regulates activity-dependent transcriptional responses in olfactory sensory neurons. Hum. Mol. Genet. *23*, 6366–6374.

Li, Y., Wang, H., Muffat, J., Cheng, A.W., Orlando, D.A., Loven, J., Kwok, S.M., Feldman, D.A., Bateup, H.S., Gao, Q., et al. (2013). Global transcriptional and translational repression in human-embryonic-stem-cell-derived Rett syndrome neurons. Cell Stem Cell *13*, 446–458.

Lindgren, A.G., Natsuhara, K., Tian, E., Vincent, J.J., Li, X., Jiao, J., Wu, H., Banerjee, U., and Clark, A.T. (2011). Loss of Pten causes tumor initiation following differentiation of murine pluripotent stem cells due to failed repression of Nanog. PLoS One *6*, e16478.

Linhart, H.G., Lin, H., Yamada, Y., Moran, E., Steine, E.J., Gokhale, S., Lo, G., Cantu, E., Ehrich, M., He, T., et al. (2007). Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing. Genes Dev. *21*, 3110–3122.

Lowry, W.E., Richter, L., Yachechko, R., Pyle, A.D., Tchieu, J., Sridharan, R., Clark, A.T., and Plath, K. (2008). Generation of human induced pluripotent stem cells from dermal fibroblasts. Proc. Natl. Acad. Sci. USA *105*, 2883–2888.

Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R., et al. (2007). Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. Cell Stem Cell *1*, 55–70.

Marchetto, M.C., Carromeu, C., Acab, A., Yu, D., Yeo, G.W., Mu, Y., Chen, G., Gage, F.H., and Muotri, A.R. (2010). A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. Cell *143*, 527–539.

Marion, R.M., Strati, K., Li, H., Tejera, A., Schoeftner, S., Ortega, S., Serrano, M., and Blasco, M.A. (2009). Telomeres acquire embryonic stem cell characteristics in induced pluripotent stem cells. Cell Stem Cell *4*, 141–154.

Maroof, A.M., Keros, S., Tyson, J.A., Ying, S.W., Ganat, Y.M., Merkle, F.T., Liu, B., Goulburn, A., Stanley, E.G., Elefanty, A.G., et al. (2013). Directed differentiation and functional maturation

of cortical interneurons from human embryonic stem cells. Cell Stem Cell *12*, 559–572.

Meehan, R.R., Lewis, J.D., and Bird, A.P. (1992). Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. Nucleic Acids Res. *20*, 5085–5092.

Mekhoubad, S., Bock, C., de Boer, A.S., Kiskinis, E., Meissner, A., and Eggan, K. (2012). Erosion of dosage compensation impacts human iPSC disease modeling. Cell Stem Cell *10*, 595–609.

Mellen, M., Ayata, P., Dewell, S., Kriaucionis, S., and Heintz, N. (2012). MeCP2 binds to 5hmC enriched within active genes and accessible chromatin in the nervous system. Cell *151*, 1417–1430.

Nan, X., Campoy, F.J., and Bird, A. (1997). MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. Cell *88*, 471–481.

Patel, S., Bonora, G., Sahakyan, A., Kim, R., Chronis, C., Langerman, J., Fitz-Gibbon, S., Rubbi, L., Skelton, R.J.P., Ardehali, R., et al. (2017). Human embryonic stem cells do not change their X inactivation status during differentiation. Cell Rep. *18*, 54–67.

Patterson, M., Chan, D.N., Ha, I., Case, D., Cui, Y., Handel, B.V., Mikkola, H.K., and Lowry, W.E. (2012). Defining the nature of human pluripotent stem cell progeny. Cell Res. *22*, 178–193.

Patterson, M., Gaeta, X., Loo, K., Edwards, M., Smale, S., Cinkornpumin, J., Xie, Y., Listgarten, J., Azghadi, S., Douglass, S.M., et al. (2014). let-7 miRNAs can act through notch to regulate human gliogenesis. Stem Cell Reports *3*, 758–773.

Sahakyan, A., Kim, R., Chronis, C., Sabri, S., Bonora, G., Theunissen, T.W., Kuoy, E., Langerman, J., Clark, A.T., Jaenisch, R., et al. (2016). Human naive pluripotent stem cells model X chromosome dampening and X inactivation. Cell Stem Cell *20*, 87–101.

Squillaro, T., Alessio, N., Cipollaro, M., Renieri, A., Giordano, A., and Galderisi, U. (2010). Partial silencing of methyl cytosine protein binding 2 (MECP2) in mesenchymal stem cells induces senescence with an increase in damaged DNA. FASEB J. *24*, 1593–1603.

Suhr, S.T., Chang, E.A., Rodriguez, R.M., Wang, K., Ross, P.J., Beyhan, Z., Murthy, S., and Cibelli, J.B. (2009). Telomere dynamics in human cells reprogrammed to pluripotency. PLoS One *4*, e8124.

Tan, F.C., Hutchison, E.R., Eitan, E., and Mattson, M.P. (2014). Are there roles for brain cell senescence in aging and neurodegenerative disorders? Biogerontology *15*, 643–660.

Tchieu, J., Kuoy, E., Chin, M.H., Trinh, H., Patterson, M., Sherman, S., Aimiuwu, O., Lingren, A., Zack, J.A., Clark, A., et al. (2010). Female human iPS cells retain an inactive X-chromosome. Cell Stem Cell *7*, 329–342.

Tomassy, G.S., Morello, N., Calcagno, E., and Giustetto, M. (2014). Developmental abnormalities of cortical interneurons precede symptoms onset in a mouse model of Rett syndrome. J. Neurochem. *131*, 115–127.

Vaziri, H., and Benchimol, S. (1996). From telomere loss to p53 induction and activation of a DNA-damage pathway at senescence: the telomere loss/DNA damage model of cell aging. Exp. Gerontol. *31*, 295–301.

Vera, E., Bosco, N., and Studer, L. (2016). Generating late-onset human iPSC-based disease models by inducing neuronal age-related phenotypes through telomerase manipulation. Cell Rep. *17*, 1184–1192.

Wang, Z., Wei, D., and Xiao, H. (2013). Methods of cellular senescence induction using oxidative stress. Methods Mol. Biol. *1048*, 135–144.

Wei, C.L., Wu, Q., Vega, V.B., Chiu, K.P., Ng, P., Zhang, T., Shahab, A., Yong, H.C., Fu, Y., Weng, Z., et al. (2006). A global map of p53 transcription-factor binding sites in the human genome. Cell *124*, 207–219.

Winkler, T., Cantilena, A., Metais, J.Y., Xu, X., Nguyen, A.D., Borate, B., Antosiewicz-Bourget, J.E., Wolfsberg, T.G., Thomson, J.A., and Dunbar, C.E. (2010). No evidence for clonal selection due to lentiviral integration sites in human induced pluripotent stem cells. Stem Cells *28*, 687–694.

Yang, J., Guo, R., Wang, H., Ye, X., Zhou, Z., Dan, J., Wang, H., Gong, P., Deng, W., Yin, Y., et al. (2016). Tet enzymes regulate telomere maintenance and chromosomal stability of mouse ESCs. Cell Rep. *15*, 1809–1821.

Yehezkel, S., Shaked, R., Sagie, S., Berkovitz, R., Shachar-Bener, H., Segev, Y., and Selig, S. (2013). Characterization and rescue of telomeric abnormalities in ICF syndrome type I fibroblasts. Front. Oncol. *3*, 35.

Zhou, Z., Hong, E.J., Cohen, S., Zhao, W.N., Ho, H.Y., Schmidt, L., Chen, W.G., Lin, Y., Savner, E., Griffith, E.C., et al. (2006). Brain-specific phosphorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic growth, and spine maturation. Neuron *52*, 255–269.

Zoghbi, H.Y. (2016). Rett syndrome and the ongoing legacy of close clinical observation. Cell *167*, 293–297.

Supplemental information

Extended Materials and Methods

Generation of isogenic Rett Syndrome iPSCs

Two primary fibroblast lines GM17567 (1461A>G in the gene encoding methyl-CpG binding protein 2 (MECP2)), and GM07982 (frameshift mutation, 705delG, in the gene encoding methyl-CpG binding protein 2 (MECP2)), from patients with Rett Syndrome were obtained from Coriell Cell Repositories (Described in Fig S1). After 8-12 hours, the cells were infected with reprogramming lentivirus that harbors polycystronic human Yamanaka factors (Oct4, Klf4, Sox2, cMyc) in DMEM medium containing 10ug/ml of polybrene and incubated overnight at 37°C in 5% CO2 incubator. On day 6, the culturing media was changed to human ES media containing DMEM/F12 supplemented with L-glutamine, nonessential amino acids (NEAA), penicillin-streptomycin, knockout serum replacement (Invitrogen), and 10 ng/ml basic FGF. Cells were cultured in hiPSC media until iPSC-like colonies were formed.

Generation of teratomas

Generation of teratoma was previously described(Lindgren et al., 2011). Briefly, a single incision was made in the peritoneal cavity of adult SCID mice and the testis was explanted through the incision site. Approximately 60,000 iPSC in a volume of 50 ml 0.5X Matrigel (BD) were transplanted into the testis using a 27-gauge needle. Four to six weeks after surgery, mice were euthanized and the tumors removed for histology.

Differentiation in vitro and analysis

Neural specification with neural rosette derivation, neuroprogenitor (NPC) purification, and further differentiation to neurons and glia were performed as described previously (Karumbayaram et al., 2009; Patterson et al., 2011; Patterson et al., 2014). For spontaneous terminal neuronal differentiation by growth factor withdrawal, NPC cultures were subjected to growth factor withdrawal (removal of EGF and FGF) and cultured in basic medium (DMEMF12 + N2 + B27) with three quarter exchange of media every three days. Neural differentiation efficiency was analyzed four weeks after growth factor withdrawal by counting the number of cells positive for neuronal markers (MAP2 and Tuj1) over the total number of cells visualized by DAPI. NPCs were transfected with *DCX-GFP* reporter one day prior to differentiation using Lipofectamine 2000 (Invitrogen). Sholl analysis of DCX-GFP positive neurites were also measured using ImageJ. All data values were presented as mean +/- SEM. For directed differentiation of interneurons, iPSCs were grown on plates coated with matrigel (Corning) until 80% confluency with mTeSR (Stem Cell Technologies). Cells were then treated with DMEM/F12 (GIBCO) containing NEAA (GIBCO), GlutaMAX (GIBCO), bovine serum albumin (Sigma-Aldrich), ß-mercaptoethanol (Sigma-Aldrich), N2 (GIBCO), B27 (GIBCO), SB431542 (10uM; Cayman Chemical), LDN-193189 (100nM; Cayman Chemical) and XAV939 (2uM; Cayman Chemical) later transitioning to the media containing sonic hedgehog (20ng/mL; R&D) and purmorphamine (1uM; Cayman Chemical) as previously described (Maroof et al., 2013). Cells were further differentiated into interneurons with neurobasal medium (GIBCO) containing N2 (GIBCO), B27 (GIBCO), ascorbic acid (Sigma-Aldrich), GlutaMAX (GIBCO), bovine serum albumin (Sigma-Aldrich), ß-mercaptoethanol (Sigma-Aldrich),

neurotrophin-3 (10ng/mL; R&D), brain-derived neurotrophic factor (10ng/mL; R&D), and glial cell-derived neurotrophic factor (10ng/mL; R&D).

Western blot

Cells were lysed on ice with RIPA buffer (Pierce) that contains Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). The total protein concentration was measured using BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's protocol. Supernatant was electrophoresed onto NuPAGE 4-12% Bis-Tris Protein Gels (Invitrogen) using MOPS running buffer (Invitrogen). The membrane was blocked with 5% non-fat milk for 1 hr and incubated overnight with primary antibodies at 4°C.

Immunofluorescence and image quantification

Cells on coverslips were washed with PBS, fixed in 4% paraformaldehyde for 15 min at room temperature, blocked for 1 hr at room temperature with 10% serum and 0.1% Triton-X-100, then incubated overnight at 4 °C with primary antibodies. Following primary antibody incubation, the coverslips were incubated with Alexa Fluor (Invitrogen) or Jackson Immunoresearch secondary antibodies at room temperature for 1 hr. Cells were counterstained with DAPI and mounted in Prolong Gold (ThermoFisher). Antibodies used include the following: mouse anti-OCT3/4 (1:100, Santa Cruz Biotechnology Inc., sc-5279), rabbit anti-SOX2 (1:300, Cell Signaling Technology, 3579), rabbit anti-Nanog (1:100, Cell Signaling Technology, 4903), mouse anti-Tra-1-81 (1:250, Chemicon, MAB4381), mouse anti-NESTIN (1:1000, Neuromics, MO15012), chicken anti-MAP2 (1:2000, Biolegend, PCK-554P), chicken anti-GFAP (1:2000, Abcam, ab4647), rabbit

anti-Tubulin β3 (1:500, Covance, MMS-435P), mouse anti-P53 (1:500, Cell Signaling, 2524), rabbit anti-p21 (1:250, Santa Cruz, sc-397), mouse anti-PML (1:100, Santa Cruz, sc-9862), mouse anti-phospho-Histone H2A.X (1:2000, EMD Millipore, 05-636), rabbit anti-5hmc (1:100, Active Motif, 39791), rabbit anti MECP2 (1:1000, Diagenode, pAb-052-050), rabbit anti Foxg1 (1:1000, Abcam, ab18259), and mouse anti NKX2.1 (1:300, Novocastra, NCL-L-TTF-1). Secondary antibodies conjugated with Alexa 488, 568, 594, 647 (1:500, Life Technologies, A-21203, A21202, A31571, A-21207) were used. Mean intensity or a number of foci were quantified using ImageJ (http://rsb.info.nih.gov/ij/). At least 100 cells per condition were used for each independent experiment.

RT-qPCR

RNA from cultured cells was collected using the RNeasy Mini Kit from Qiagen according to the manufacturer's instructions. RNA with an A260/A280 ratio in between 1.8 and 2.0 as well as an A260/A230 ratio in between 2.0 and 2.2 was used. RNA was then reverse transcribed using the Super Script III First-Strand cDNA Synthesis kit with Random Hexamers (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed using SYBR Green master mix (Roche). Reactions were performed in duplicate and duplicate CT values were averaged and then used for standard $\Delta\Delta$CT analysis. Expression levels were normalized to beta actin.

Primer Sequences

MECP2	*GCTCTGCTGGGAAGTATGATG*
MECP2	*ATGTGTCGCCTACCTTTTCG*
P53_F	*GCCCAACAACACCAGCTCCT*
P53_R	*CCTGGGCATCCTTGAGTTCC*

P21-F	*AAAGAAGAACGGAGCGAACA*
P21-R	*CTCCGCTCAATTTCCAAGAG*
GADD45G-F	*TACGCTGATCCAGGCTTTCT*
GADD45G-R	*AACAGGCTGAGCTTCTCCAA*
DDIT4_F	*GTTTGACCTCTCCACCAGCCT*
DDIT4_R	*GCACACAAGTGTTCATCCTCAGG*
DDB2_F	*TCACTTCCAGCACCTCACAC*
DDB2_R	*ACGTCGATCGTCCTCAATTC*
SFN_F	*GTGTGTCCCCAGAGCCATGG*
SFN_R	*ACCTTCTCCCGGTACTCACG*

Data collection and statistical analysis

All the experimental data (RT-qPCR, immunostaining, ß-Galactosidase Senescence Assay) were presented as mean +/- SEM based on at least three biological replicates from independent experiments. Student's t-tests were applied to data with two groups. ANOVA analyses were used for comparisons of data with greater than two groups. A *p-value* < 0.05 was considered as statistically significant.

siRNA gene silencing

All knockdown experiments were performed using trilencer siRNAs (from OriGene Technologies) and RNAimax (ThermoFisher) in Opti-MEM media (ThermoFisher). Trilencers were used at a concentration of 20 nM. Transfection media was prepared and then 500,000 cells were plated on top of the transfection media in 6-well plates. The medium was changed to normal NPC media the next day and cells were collected for analysis at the time points indicated.

ß-Galactosidase Senescence Assay

ß-Galactosidase Senescence Assay was performed using the Senescence β-Galactosidase Staining Kit from Cell Signaling according to manufacturer's instructions. Briefly, the cells were fixed on coverslips, incubated with X-gal overnight at 37°C, then mounted on glass slides and imaged using a brightfield microscope. The number of blue cells and number of total cells were quantified using the Cell Counter plugin in ImageJ.

Quantification of Dendritic Arborization

Neuronal cultures were immunostained for Tuj1 in order to identify mature neurons and visualize entire cells. The stained cells were then imaged at 20x and dendritic arbors of individual cells were traced using the Simple Neurite Tracer plugin for ImageJ. The number of process ends per cell were counted using the Cell Counter plugin for ImageJ. The number of process ends per cell are presented as mean ends per cell +/- SEM. Means were compared using the Student's t-Test for data with two groups.

RNA expression profiling

Libraries were prepared according to the manufacturers guidelines using The TruSeq V2 kit (Illumina). For RNA sequencing, the datasets were mapped with RASER and HISAT2. Genes were defined by the exon union from the hg19 ensembl annotations. The function of DESeq in DESeq2 package was used to first normalize the gene read counts data and then identified the differentially expressed genes. The MA plot was generated with the function of plotMA in DESeq2 package. Q-value of 0.05 is regarded as the stringent cutoff of calling DEGs while p-value less than 0.05 is regarded as the low stringency cutoff. For the meta-chromosome plot of DEGs, all the chromosomes (except chromosome Y) were first divided equally into 20bins with different length, and then the number of DEGs in

each bin was counted. GO analysis was performed using DAVID. These data are available from NIH GEO Dataset GSE107399.

Figure S1. Description of patient source for fibroblasts for reprogramming. Supplementary to Figure 1.

Patient data, taken from NIH BioBrainBank repository

Patient 982

Description:
RETT SYNDROME; RTT
METHYL-CPG-BINDING PROTEIN 2; MECP2
Affected: Yes
Gender: Female
Age: 25 YR (At Sampling)

Remarks: Clinically affected; microcephaly; scoliosis diagnosed at age 12; severe kyphoscoliosis at age 25; early milestones were slow; started losing skills at age 2; currently severely retarded; behavioral phenotype includes hand wringing that began at age 2 and became more intense; no sleep problems; no self-injurious behavior; abnormal EEG; CT scan at age 25 showed evidence of atrophy; donor subject carries a frameshift mutation, 705delG, in the gene encoding methyl-CpG binding protein 2 (MECP2); see GM07983 lymphoblast.

Patient data 567

Description:
RETT SYNDROME; RTT
METHYL-CPG-BINDING PROTEIN 2; MECP2
Affected:Yes
Gender:Female
Age:5 YR (At Sampling)

Clinically affected; onset between 15-20 months of age; seizures began at age 3; never walked independently; began to develop repetitive hand movements at 28 months; no hand use; small feet; language regression at 18 months; some sleep problems; nonverbal; significantly abnormal EEG; swallowing difficulties, reflux, and breathing problems; teeth grinding; decelerating head circumference; growth retardation; seizures; donor subject has a missense mutation (A>G) at nucleotide 1461 (1461A>G) in the gene encoding methyl-CpG binding protein 2 (MECP2), resulting in a substitution of a tryptophan for a stop codon at codon 487 [TER487TRP (X487W)].

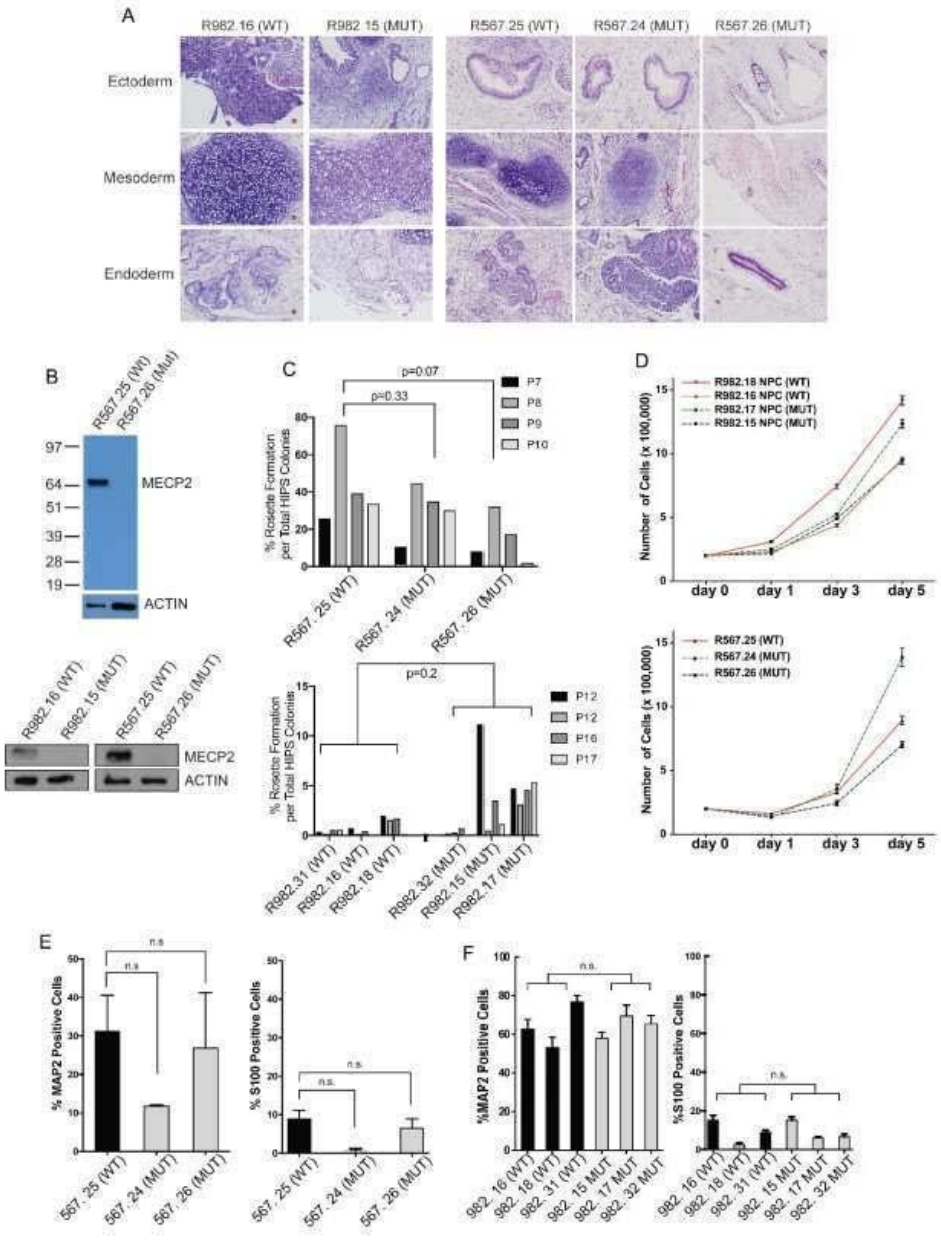

A
R982.16 (WT)
R982.15 (MUT)
R567.25 (WT)
R567.24 (MUT)
R567.26 (MUT)
Ectoderm
Mesoderm
Endoderm
B
R567.25 (WT)
R567.26 (Mut)
97
64
51
39
28
19
MECP2
ACTIN
R982.16 (WT)
R982.15 (MUT)
R567.25 (WT)
R567.26 (MUT)
MECP2
ACTIN
C
p=0.07
p=0.33
P7
P8
P9
P10
% Rosette Formation per Total HIPS Colonies
R567.25 (WT)
R567.24 (MUT)
R567.26 (MUT)
p=0.2
P12
P12
P16
P17
% Rosette Formation per Total HIPS Colonies
R982.31 (WT)
R982.16 (WT)
R982.18 (WT)
R982.32 (MUT)
R982.15 (MUT)
R982.17 (MUT)
D
R982.18 NPC (WT)
R982.16 NPC (WT)
R982.17 NPC (MUT)
R982.15 NPC (MUT)
Number of Cells (x 100,000)
day 0
day 1
day 3
day 5
R567.25 (WT)
R567.24 (MUT)
R567.26 (MUT)
Number of Cells (x 100,000)
day 0
day 1
day 3
day 5
E
n.s
n.s
% MAP2 Positive Cells
567.25 (WT)
567.24 (MUT)
567.26 (MUT)
n.s.
n.s.
% S100 Positive Cells
567.25 (WT)
567.24 (MUT)
567.26 (MUT)
F
n.s.
%MAP2 Positive Cells
982.16 (WT)
982.18 (WT)
982.31 (WT)
982.15 MUT
982.17 MUT
982.32 MUT
n.s.
%S100 Positive Cells
982.16 (WT)
982.18 (WT)
982.31 (WT)
982.15 MUT
982.17 MUT
982.32 MUT

Figure S2. Validation of disease in a dish model for Rett Syndrome. Supplementary to Figure 1.

A, Teratoma assay was performed to establish pluripotency of hiPSCs made from Rett patient fibroblasts. The resulting tumors each showed evidence of differentiation towards all three embryonic germ layers. **B,** NPCs were produced from isogenic hiPSCs of Rett patient, and assessed by western blot to validate loss of MECP2 and specificity of antibody. Top panel shows that the antibody only recognizes MECP2. Bottom panel shows that in NPCs from both patients, individual clones either express or lack MECP2. **C**, The ability of hiPSCs to generate NPCs was assayed in Rosette formation assay. Lack of MECP2 did not affect rosette formation across multiple lines from both patients. N=4 independent experiments. *p value < 0.05 according to student's t test (for patient R567) or ANOVA (for patient R982). Bar graphs represent mean +/- SEM. **D**, Growth curves show that loss of MECP2 does not affect proliferation of NPCs made from either patient. **E**, 3 weeks of growth factor withdrawal drives NPCs to differentiate into neurons and glia as measured here by immunostaining for MAP2/Tuj1 or S100/GFAP in patient 567 derived cultures. There was no consistent difference in differentiation potential across lines from either patient. N=2 independent experiments. Bar graphs represent mean +/- SEM. **F**, Patient 982 derived cultures also do not show dramatic differences in the presence of neurons or astrocytes as measured by MAP2 and S100. N=3 independent experiments. Bar graphs represent mean +/- SEM. Scale Bars represent 20 microns.

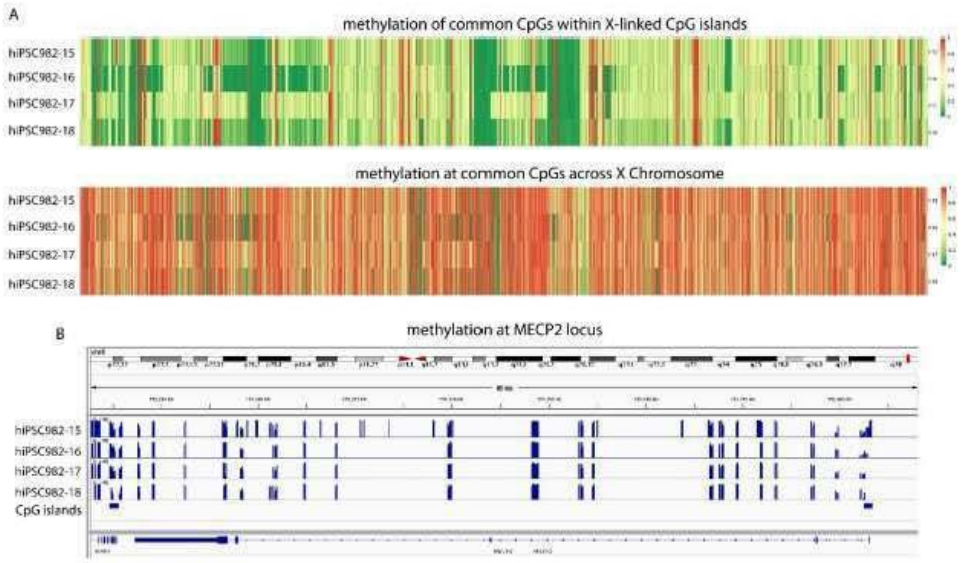


Figure S3. Methylation analysis to measure XCI erosion in hiPSC lines made from Rett Patients. Supplementary to figure 1.

A, The silencing of the X chromosome is accompanied by the gain of DNA methylation at CpG islands (CGIs). We utilized reduced representation bisulfite sequencing (RRBS) to examine methylation levels across the X chromosome in indicated hiPSC lines. Methylation level of 1 indicates 100% methylation and 0 absence of methylation. Top: Heatmap of RRBS-based methylation levels for CpGs within X-linked CGIs showing that most CGIs are hemi-methylated (intermediate methylation level) indicative of XCI on one X chromosome and no methylation on the other (which results in hemi-methylation when measured across both X chromosomes). Erosion (no methylation) is only seen for a small subset of CGIs (compare to data in (Patel et al., 2017)). Only CpGs with coverage across all samples are shown (including across Patel et al samples). Bottom: As above, except for all CpGs on the X chromosome with coverage in all samples, demonstrating the globally high methylation level in all hiPSCs. **B,** IgV view of the

methylation data within the MECP2 locus. The CGI of MECP2 is indicated and hemi-methylated in all hiPSC lines, indicative of XCI at this locus. RRBS and data analysis was performed as described in(Patel et al., 2017).

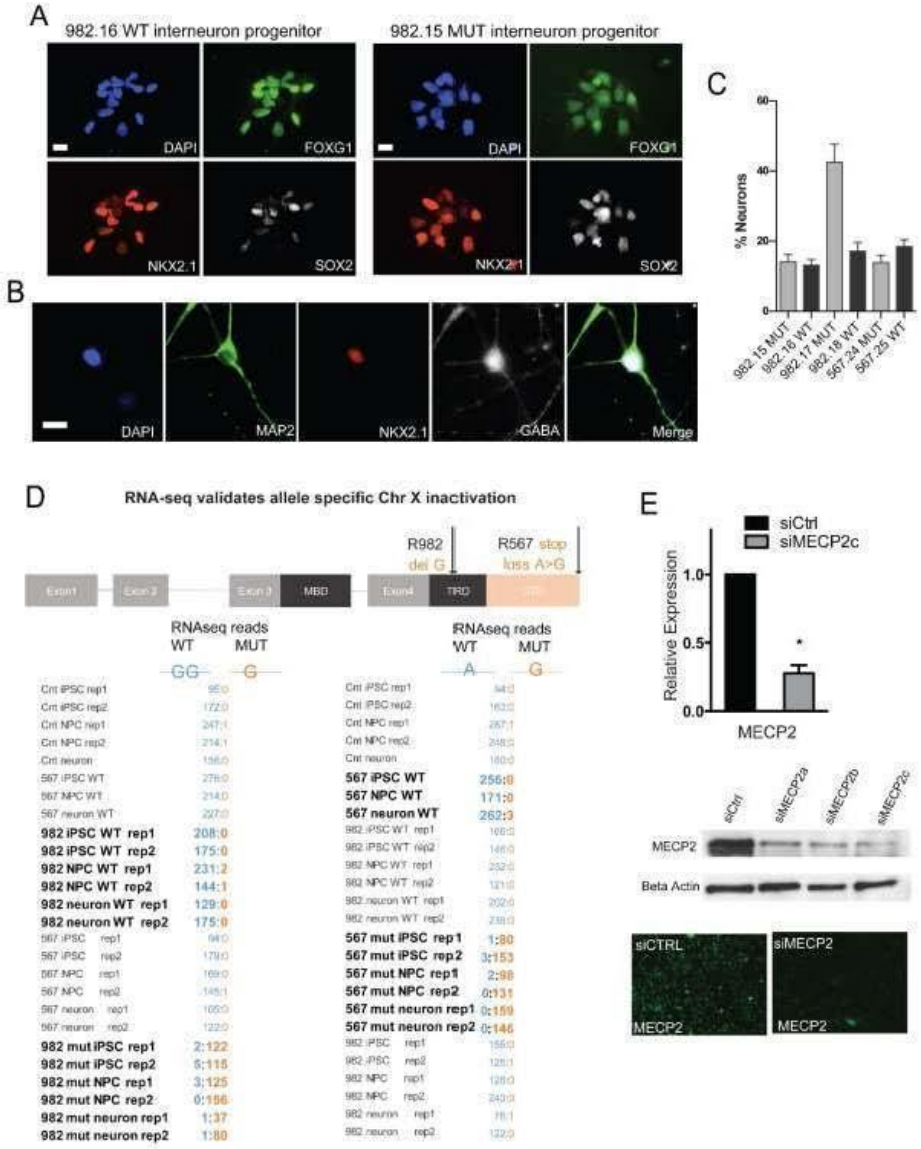


Figure S4. RNA-seq analysis to determine the relative ration of WT versus MUT transcripts of MECP2 in Rett patient derived lines. Supplementary to figure 2.

A and B, immunostaining to demonstrate the efficiency of directed differentiation towards neural progenitors (A) and then onto interneurons (B) with markers typical of each stage. **C,** Quantification of the percentage of interneurons in the cultures used to perform the RNA-seq, as measured by immunostaining. **D,** Detection of WT and MUT transcripts from each of the lines indicated demonstrated a clear bias towards individual alleles in each patient derived line. This analysis indicates XCI status for each allele, and demonstrates that XCI status is unchanged, even after differentiation to neurons. **E**, MECP2 was downregulated by RNA interference, quantified by RT-PCR (left), for protein by western blot (middle), and as demonstrated by immunostaining for MECP2 (right). N=3 independent experiments. *p value < 0.05 according to student's t test. Bar graphs represent mean +/- SEM. Scale bars represent 10 microns.

Patel, S., Bonora, G., Sahakyan, A., Kim, R., Chronis, C., Langerman, J., Fitz-Gibbon, S., Rubbi, L., Skelton, R.J.P., Ardehali, R., *et al.* (2017). Human Embryonic Stem Cells Do Not Change Their X Inactivation Status during Differentiation. Cell Rep *18*, 54-67.

Contributions:

EK contributed to the genomic analysis, bulk RNA-seq analysis, interpretation of the results and manuscript writing.

Chapter 3

Defining Dysfunction due to Loss of MECP2 in Rett Patient Brain

Defining Dysfunction due to Loss of MECP2 in Rett Patient Brain

Korsakova E, Morales A, McDaniel T, Lund A, Cooper B, Ma F, Allison TA, Bhaduri A and Lowry WE

1, Molecular Biology Institute, UCLA, Los Angeles, CA 90095

2, Department of Molecular Cell and Developmental Biology, UCLA, Los Angeles, CA 90095

3, Division of Dermatology, DGSOM, UCLA, Los Angeles, CA 90095

4, Broad Center for Regenerative Medicine, UCLA, Los Angeles, CA 90095

5, Jonsson Comprehensive Cancer Center, UCLA, Los Angeles, CA 90095

To whom correspondence should be addressed

William E Lowry

615 Charles Young Drive South

Los Angeles, CA 90095

blowry@ucla.edu

and

Aparna Bhaduri

621 Charles Young Drive South

Los Angeles, CA 90095

ABhaduri@mednet.ucla.edu

Abstract

Rett Syndrome is characterized by a postnatal loss of neurophysiological function and regression of childhood development. Because the syndrome is X-linked and males with MECP2 mutations generally do not survive birth, the study of this syndrome has been complicated by the fact that in female brain, a portion of neurons express wild type MECP2, and another portion express a non-functional allele of MECP2. Therefore, bulk-RNA-sequencing of Rett brain is confounded by the presence of chimerism in neurons for expression of functional MECP2. Here we present an approach that allows for transcriptional profiling of individual neurons and a direct comparison between neurons that express functional MECP2 with those that express the disease-causing allele. Taking advantage of single-nuclei-RNA-Seq and FACS isolation of neurons that express or lack MECP2, we find that mutant neurons from Rett brain show patterns of defects in expression of synaptic and metabolic genes, both of which can be detected in *in vitro* models of Rett Syndrome. In addition, these patterns in young adult Rett brain show similarity with changes found in both the aging brain as well as brain with Alzheimer's disease. We used these resources to identify a role for POU2F1/OCT1 as a potential stress response transcription factor in mediating the response to loss of MECP2. Together, these data highlight defective molecular pathways and metabolic pathways in Rett brain neurons and suggest that *in vitro* models could serve as valuable tools to further study this syndrome and potentially for development of novel therapeutics.

Introduction

The disruption of the methyl-CpG-binding protein 2 (MECP2), encoded on the X chromosome, is known to cause a severe neurodevelopmental disease called Rett Syndrome[1]. It is an X-linked dominant disorder observed mostly in female heterozygotes, as males with this mutation on their only X chromosome typically fail to survive birth[1]. Female patients with Rett Syndrome present with short stature overall, but have a relatively more profound microcephaly phenotype, suggesting a prominent role for MECP2 in the brain[2]. Accordingly, while the MECP2 protein is present in all tissues, the expression of MECP2 is particularly high in all types of mature neurons of the CNS[3]. Brain-specific deletion of MECP2 in mice phenocopies MECP2-null animals[4-7], and subsequent studies showed that neuronal specific deletion of MECP2 recapitulated phenotypes of MECP2-null animals.

MECP2 has been described as both a transcriptional stimulator and inhibitor, a regulator of RNA splicing, and a regulator of DNA methylation or reader of methylation[8-10]. Moreover, loss of MECP2 has been argued to lead to a variety of cell physiological defects such as mitochondrial permeability, diminished dendritic branching, altered electrophysiological activity, and changes in nuclear and nucleolar size, etc[11, 12]. Despite all these interesting observations, it is still not clear which of these are relevant for human Rett Syndrome patients, and which should serve as proxies for experimentally targeting and developing therapeutic strategies. The vast majority of these observations have been made *in vitro* with cell-based models of the disease or in murine models of the syndrome lacking MECP2[12]. Therefore, the identification of which of these phenotypes

occur in actual human patient brain is critical to understand the etiology of the disease and to uncover which *in vitro* or murine models most accurately reflect the human condition.

We previously created an isogenic *in vitro* system to model how the loss of MECP2 impacts development of human neural cell types by exploiting reprogramming of patient fibroblasts to a pluripotent state to create human induced pluripotent stem cells (hiPSCs), and differentiation towards particular neural lineages (Neural Progenitor Cells (NPCs) and interneurons)[13]. Our previously published work with this *in vitro* model of Rett Syndrome indicated that specifically postmitotic neurons lacking MECP2 show defects in dendritic branching coincident with induction of p53 and cellular senescence. Blocking senescence with P53 inhibition restored dendritic branching in Rett-patient-derived neurons[13]. These results were consistent with those of the Galderisi group who also showed senescence phenotypes in various Rett models[14, 15].

The study of human Rett brain until very recently was limited to pathological examination of tissue or bulk molecular methods to understand the course of the disease. For example, Gogliotti et al performed bulk RNA-seq on a number of Rett brain samples versus unaffected controls, and found 100s of differentially expressed genes. However, the methods employed precluded identification of cell type specific changes and is complicated by the fact that the female Rett brain is chimeric for both MECP2 WT and MECP2 mutant cells based on random X-inactivation during development. A more recent study took advantage of differential SNPs between maternal and paternal alleles coupled

with sc-RNA-seq to define gene expression changes in specific cell types[16]. While this study did identify differentially expressed genes in MECP2- cells, the analysis did not implicate particular dysfunctional pathways to shed light on the effect of loss of MECP2 in neurons, perhaps due to a shallow depth of sequencing.

Here we employ sc-RNA-seq on nuclei from Rett brain and normal brain to understand the effect of loss of MECP2 on neurons compared to both MECP2hi neurons in isogenic Rett brain, as well as to WT brain to determine which changes are due directly to loss of MECP2. In addition, we re-analyze data from previous Rett brain studies, as well as various *in vitro* models to corroborate our findings and highlight effective *in vitro* models of Rett Syndrome. We used these methods to determine that while loss of *MECP2* leads to differential synaptic and metabolic gene expression patterns, which are reminiscent of neurons in aging brain, the ratio of subtypes of neurons within Rett brain is not dramatically altered. We also identify *POU2F1/OCT1* as a potential key transcription factor in the stress response of *MECP2*-null neurons, and demonstrate that some of the key phenotypes found in Rett brain are recapitulated in *in vitro* models of the disease.

Results

Method to isolate and profile neurons with and without expression of MECP2 from individual Rett patient brains

Rett syndrome is caused by a heterozygous mutation in MECP2 gene. MECP2 is an X-linked gene, thus half of MECP2 alleles are turned off in a female brain due to random X chromosome inactivation, resulting in roughly half of the expressed alleles being wild type

and the other half harboring MECP2 mutation[1, 17]. By isolating cells from a single brain, we can directly compare wild type cells to the mutant cells. Any differences found between the two will be attributed solely to the mutation, since all the cells will come from the same genetic background and environment. Thus, age, lifestyle and other factors that typically are taken into consideration during such comparison would not affect the results.

We started by taking advantage of the frozen brain sections of Rett patients to investigate differences in gene expression between $MECP2^{hi}$ and $MECP2^{low}$ cells. We chose to isolate the nuclei from these sections, since they tend to stay intact and preserve genetic material following tissue thawing (Fig 1A)[18]. Nuclei isolation was performed utilizing sucrose gradient accompanied by a series of washes and centrifugations. The quality of nuclei isolation was assessed visually and quantitatively using a hemocytometer. We were particularly interested in the role of MECP2 in neurons. Thus, we chose NeuN, a mature neuron nuclear marker, to isolate neuronal populations from Rett nuclei. We stained the nuclei with MECP2, NeuN and DAPI and subjected them to FACS. We collected $DAPI^{+}/Neun^{+}$ intact neuronal nuclei, which were either $MECP2^{hi}$ or $MECP2^{low}$. To confirm the nuclei sorting strategy was successful, we used cytospin, immunostaining, and western blotting which both confirmed that the expression level of MECP2 was highly diminished in the MECP2low population of neurons (Fig 1B and C).

Wild type and mutant neuronal nuclei were separately analyzed by both Drop-SEQ (3 individual samples from independent Rett patients)[19] and 10x RNA sequencing (Illumina)(2 individual samples from independent Rett patients). While both methods

detected numerous clusters related to various neuronal subtypes, the 10X were much deeper and thus became the focus of our follow-on analyses. The drop-seq data were sufficient to identify neuronal subtypes and were thus used to explore cell proportion changes in response to loss of MECP2 but were not deep enough to accurately calculate differentially expressed genes. UMAP analysis on the 10X data generated 19 transcriptionally unique clusters in both the WT and mutant populations. Further analysis of the gene expression revealed several types each of inhibitory and excitatory neuronal populations (Fig 1D).

This sorting and transcriptomic approach allowed for a determination of whether the proportion of cell types was affected by MECP2 expression. Because NeuN expression tracked nearly identically with MECP2 expression, namely all 18 subtypes of neurons expressed NeuN and MECP2 to a similar degree, we were able to construct a proportional analysis of cells types between $MECP2^{hi}$ and MECP2low neurons. In addition, we generated data from 5 individual brains (with both Drop-seq and 10X), and we re-analyzed data from the Greenberg lab that used SNP analysis to define WT vs mutant neurons from the Rett brain. Taken together, this analysis from 8 Rett samples did not reveal a significant difference in the ratio of any neuronal subtypes amongst all these Rett patient brains (Fig 1E). These data suggest that neurological deficits in Rett patients are probably not due to different proportions of neurons.

We next explored the data to identify differentially expressed genes. We applied a *p-value* cut off of 0.01 to the data set to identify differentially expressed genes (DEGs) between

MECP2^{hi} and MECP2low neurons from each brain. We first compared DEGs identified in our own data versus those uncovered from the Greenberg data to identify overlap between the two methods. In fact, DEGs from our 10X analysis overlapped significantly with data from the Greenberg group with independent brain samples despite the differences in methods (identification of WT vs Mutant and sc-RNA-seq approaches) (Figure 2A). We next showed that there are overlapping DEGs patterns found in both excitatory and inhibitory neurons in Rett brain (Figure 2B), suggesting general physiological defects due to loss of MECP2. Looking at DEGs across various neuronal subtypes from our own data, we noticed that only a subset of neurons appear to be affected transcriptionally by loss of MECP2, suggesting there is something of a selective vulnerability of neuronal subtypes to loss of MECP2 (Figure 2C).

We then used Gene Ontological (GO) analysis to determine which patterns of gene expression are upregulated and downregulated in either excitatory or inhibitory subclasses of neurons in the absence of MECP2. Remarkably, in the two major classes of excitatory neurons (SLC17A6+ and SCL17A7+), nearly all the gene expression changes induced in MECP2low neurons appeared to be related specifically to neuronal physiology such as 'Neuron Projection Morphogenesis', 'Synapse Organization', 'Dendrite Development', 'Neuron Differentiation' etc. This is an important observation considering recent data from our own group showing that Rett patient derived neural organoids show elevated levels of synapses and synaptic genes (Samarsinghe et al, 2021). On the other hand, the majority of gene expression changes downregulated, appeared to be related to cellular metabolism, such as 'Translocation of GLUT4 to

membrane', 'Mitochondrion Organization', 'Gluconeogenesis' etc. Considering the overlapping pattern of DEGs shown in Figure 2B, it is not surprising that the same GO analysis on inhibitory neuron subtypes (RELN+, SST+, VIP+) yielded similar results, where the same categories of DEGs were found in both the up- and downregulated expression patterns. While it is perhaps not surprising that genes related to neuronal physiology were perturbed in MECP2low neurons in the Rett brain, considering previously reported phenotypes from both *in vivo* and *in vitro* phenotypes, the alteration of metabolism in Rett brain is relatively unexplored and warranted further investigation. This pattern of metabolic disruption at the RNA level could portend metabolic defects in the neurons, as it has been previously established that metabolic dysfunction can present at the level of RNA expression of important metabolic enzymes.

Numerous attempts to model Rett Syndrome have taken advantage of deletion of Mecp2 via homologous recombination[4, 5, 20-28]. These models recapitulate some, but not all phenotypes of human Rett Syndrome. We re-analyzed a dataset from murine Rett brain and found first that many of the same neuronal-specific categories of genes were downregulated instead of upregulated as in the human Rett brain, and that most of the upregulated genes were related to inflammatory responses such as 'Inflammatory Response', 'Leukocyte Migration', 'Wound Healing' etc. It is tempting to speculate that the differences observed between the human and murine cases is due to the design of the mouse model, whereby Mecp2 was deleted as opposed to mutated, which could have induced a more profound phenotype than that observed in human.

Next, we looked at the published RNA sequencing data set for the aging brain and saw another resemblance to the Rett brain. Aging brain showed a strong induction of inflammatory gene signatures similar to what we observed in the murine Rett brain such as ‘Inflammatory Response’, ‘Positive Regulation of NFKB’, ‘Microglial Activation’ etc. Conversely, downregulated gene categories were almost entirely related to metabolism such as ‘Mitochondrion Organization’, ‘Inner Mitochondrion Membrane Organization’ and ‘Aerobic Respiration’, nearly identical to what was observed in the human Rett brain.

POU2F1/OCT1 binding sites are enriched in genes affected by loss of MECP2 function

To gain more insight into the molecular nature changes in the Rett patient brain, we analyzed transcription factor binding sites enriched within the promoter of significantly altered genes (Figure 3A). A similar pattern emerged from analysis of the Greenberg Rett sc-RNA-seq data (Figure 3C), as well as in the Gogliotti bulk RNA-seq data from Rett brain (Figure 3D). Furthermore, looking across the aging brain, Rett Brain, and *in vitro* culture, in every case, POU2F1/OCT1 binding sites were also among the most enriched in both up and downregulated genes (Figure 3, and 4). This suggests that POU2F1/OCT1 could be playing an outsized role in neuronal aging and in the response of neurons to loss of MECP2 in a variety of settings. This finding is interesting in light of extensive data suggesting that POU2F1/OCT1 is important in mediating stress responses in a variety of tissues[29-32], and the previous work from our group and the Galderisi group that MECP2low neurons undergo upregulated of stress response pathways downstream of P53[13-15].

To corroborate our findings and identify valid *in vitro* models of Rett Syndrome, we investigated whether similar perturbations are present in a cell culture model of Rett. We analyzed bulk RNA sequencing results of interneurons generated in our lab. Lack of *MECP2* caused downregulation of 'Axon development', 'Synaptic signaling', 'Synapse assembly' and 'Cerebellum development'. In addition, MECP2low neurons showed induction of stress pathways such as 'Wound healing' and 'Negative regulation of cell proliferation', similar to what was seen in Aging brain. Comparing isogenic neurons both wild type to mutant for *MECP2* expression revealed that *POU2F1/OCT1* was significantly upregulated at the protein level in the absence of *MECP2* by western blotting and immunofluorescence (Figure 4A, B).

Potential routes to activation of POU2F1/OCT1

POU2F1/OCT1 is known to be regulated post-translationally through covalent modification and stabilization from degradation[33, 34]. Several studies have suggested that DNA damage or lack of DNA repair is a driving factor to stabilize POU2F1/OCT1 levels in the cell. Therefore, to determine what types of stress in human neurons can stabilize POU2F1/OCT1 protein, we treated wildtype human neurons with various stress inducing insults and measured POU2F1/OCT1 protein levels. In addition, the sc-RNA-seq data from Rett brain showed evidence of neuronal dysfunction due to metabolic stress. Therefore, we attempted to see which of these could potentially be relevant in *in vitro* models of Rett Syndrome, and which could potentially induce POU2F1/OCT1 as a result.

In Ohashi et al we showed evidence that MECP2low neurons potentially have increased DNA damage by staining for H2Ax, even in the absence of deliberate DNA damage[13]. We found that pATR was significantly upregulated upon UV induced DNA damage, and that $MECP2^{+}$ and $MECP2^{-}$ neurons responded similarly to the damage (Figure 5A). In addition, we found that inducing DNA damage in WT neurons led to a strong increase in OCT1 protein expression (Figure 5A and B), possibly indicating a role for OCT1 as a stress sensor also in human neurons. Next, we attempted to disrupt mitochondrial function to ascertain whether metabolic stress could induce POU2F1/OCT1 protein stabilization in human neurons. In fact, disruption of metabolic homeostasis through the use of uncoupling agents or inhibitors of the electron transport chain led to a significant induction of POU2F1/OCT1 protein in otherwise normal neurons to nearly the level observed in Rett mutant neurons (Figure 5C, D).

We next used glucose tracing and metabolomics by Mass Spectrometry to assess the utilization of glucose in neurons with and without MECP2 function, and found defects in several TCA metabolites, which would be consistent with mitochondrial dysfunction (Figure 6A). Repeated experiments were compiled and organized into pathway enrichment scores for the observed metabolic changes measured by metabolomics (similar to Gene Set Enrichment Analysis (GSEA) for RNA data) leading to categories such as ‘Pyruvate metabolism’ (Figure 6B). To functionally measure whether Rett neurons lacking MECP2 expression show defective cellular metabolism, we used

Seahorse assay to determine their oxygen consumption rate, a direct output of mitochondrial function. The Seahorse assay showed that Rett mutant neurons from two distinct Rett patients are defective at all stages of the electron transport chain (Figure 6C). Further analysis of Seahorse data from these experiments show that these same Rett neurons from two patients had a reduced ATP consumption rate relative to isogenic neurons expressing MECP2 (Figure 6D).

We also reanalyzed the DEGs shown in Figure 3 to isolate just those called out as 'Metabolism related' by gene ontology, and used TF binding site enrichment analysis to identify TFs that potentially regulate differences in metabolic gene expression. In the Metabolic genes upregulated, OCT1 again stood out as a potential regulator (Figure 6E). Together, these data are consistent with mitochondrial dysfunction in the absence of MECP2 function, which perhaps sheds light on some of the transcriptional differences observed in Rett brain. As others have already identified metabolic deficiencies in murine and human Rett experimental models[35-37], the single cell analysis performed here on human Rett brain suggests a similar dysfunction potentially driving Rett phenotypes in patients.

Discussion

This study sheds new light on the etiology of Rett Syndrome by molecular interrogation of individual neurons from Rett patient brain tissue. From these data, several important observations were made about selective susceptibility of individual subtypes of neurons,

the proportions of neuronal subtypes, effects on expression of synaptic genes, and the nature of stress in neurons induced by loss of *MECP2* function. We then went on to identify a transcription factor with potentially a role in regulating the differential gene expression observed in some subtypes of neurons lacking *MECP2* function. We used *in vitro* models to both confirm what we had observed in Rett brain, but also to extend this work and determine the nature of the stress

Given their critical role in cognition, it is especially important that neurons be equipped to withstand various threats such as injury and disease. This is especially true for postmitotic cells, which bear special relevance to Rett Syndrome because MECP2 is most highly expressed in these specifically [6, 7, 38]. It is known that many forms of neuronal stress response lead ultimately to the activation of various transcription factors, which in turn act to determine cell fate[39-41]. Some hypothesize that the resulting transcriptional changes may be a cellular attempt to mitigate the initial stress or its downstream impacts, remove damaged neurons, alter brain activity to compensate for the stressor, or may even directly be a pathological symptom [42]. Further, neuroinflammation, a known consequence of chronic stress [43], has been linked to MECP2 mutations as measured by observed dysregulation of several acute-phase response (APR) proteins in a Rett mouse model [44]. The current study attempted to define which types of neurons in the Rett brain are undergoing stress and what are potential molecular or physiological triggers of that stress.

Studies from our group and others have suggested an important role of senescence in Rett Syndrome. It has been argued that senescence may be a cellular adaptation designed to conserve energy required for division and differentiation so the cell can

survive when facing stress[45]. The Galderisi group has published several studies demonstrating senescence in Rett patient mesenchymal stem cells (MSCs) [14, 15], partially MECP2 silenced human MSCs[14], MECP2 silenced human neuroblastoma cells [36], and heterozygous MECP2 mutant mouse mesenchymal stromal cells [35] and neural stem cells [36]. In our previous study on Rett derived hiPSCs, we found SASP induction in MECP2 null interneurons, consistent with these previous results[13]. While neurons from the human Rett brain did not show clear transcriptional evidence of a senescence program, there was evidence for metabolic stress.

Given their critical role in cellular energy production, mitochondrial function is highly important for normal cell function. We found strong evidence for mitochondrial disruption in the Rett brain via ontological analysis of differentially expressed genes (Fig 6). These data were consistent with an increasingly large body of evidence which connects Mecp2 deficiency to mitochondrial abnormalities, and in turn these abnormalities to further Rett phenotypes. Multiple studies in Rett mice models and human Rett samples have shown that Mecp2 interacts with genes encoding mitochondrial subunits which are abnormally expressed in its absence [46-48]. Additionally, analysis of male mice hemizygous for Mecp2 mutation revealed redox-imbalance across cytosol and mitochondria of neurons accompanied by increased oxygen consumption and generation of reactive oxygen species (ROS) [49]. Increased oxygen consumption was also confirmed in neural progenitor cells (NPCs) derived from Rett Syndrome patient cells in a separate study[50]. This is particularly interesting since another characteristic Rett mitochondrial alteration is a leaky inner membrane [51].

Our data suggest that MECP2low neurons have dysfunctional mitochondrial metabolism, which is consistent with previous studies. This is interesting in light of what is known about mitochondrial diseases caused by mutations in genes for mitochondrial proteins. These diseases share many of the characteristic clinical symptoms of Rett Syndrome including intellectual disability, motor problems, and seizures[52, 53]. In addition, they are accompanied by evidence of oxidative damage and increased blood lactate and pyruvate content [54] which have also been identified as features of Rett [49, 51, 55].

There is also extensive evidence of the role of abnormal mitochondria in oxidative stress, a type of neuronal stress heavily implicated in many diseases including Rett Syndrome which leads to the before mentioned oxidative damage. Oxidative stress is a cellular condition caused by excessive ROS. This therefore suggests a positive feedback mechanism in which mitochondrial defects (initially stemming from MeCP2 loss) lead to overproduction of ROS which in turn causes further mitochondrial defects due to oxidative stress. This cycle may explain the delayed onset of Rett as the initial mitochondrial deficiency caused by loss of MeCP2 may not produce a large enough effect to lead to symptoms, but accumulation of mitochondrial defects over time can. However, this hypothesis conflicts with the fact that unlike many other neurological diseases in which oxidative stress is implicated (such as Alzheimer's and Parkinson's) [42], Rett Syndrome is not degenerative. Additionally, reintroduction of Mecp2 into both Mecp2 null and deficient symptomatic mice models rescued wildtype levels of several oxidative stress markers[5, 56]. One possible explanation for this is that once the initial driver of

mitochondrial dysfunction is ameliorated, the brain is able to correct for the accumulation of mitochondrial defects, likely through the major mtDNA repair mechanism, base excision repair. However, further studies are required to determine if this is true, and more generally how the dynamics of cellular stress and cellular stress response work together to produce Rett symptoms without typical neurodegeneration. Critically, oxidative stress is known to induce senescence, p53 activation, and Oct1 upregulation, both of which we have identified as key features of MECP2low neurons. The Oct family, one branch of the POU transcription factors, is a group of transcription factors so named for their characteristic recognition of the octamer DNA element ATGCAAAT. Unlike any of its family members, Oct1 is expressed ubiquitously both temporally and spatially throughout the body [29, 30]. A study on Oct1 deficient mice revealed abnormal expression of many stress related genes as well as hypersensitivity to oxidative stress[30, 31, 57]. Based on the observation that many of the genes dysregulated in response to stress inputs contained human-conserved octamer sequences in their regulatory regions, it is hypothesized that Oct1 may play an important role in regulation of cell-stress response genes. However, other findings suggest that Oct1 may be a direct participant in cellular response pathways[29]. Oct1 has been shown through various studies to interact with multiple stress-response implicated factors. One of these studies found that Oct1 mediates the induction by BRCA1 of the gene GADD45[58]. This is of particular interest since GADD45 is a stress-inducible DNA repair gene regulated by p53[59]. In addition, we found that GADD45 was one of several p53 targets whose expression was upregulated following silencing of MECP2 in neuronal cultures.

Another question not answered by the current study is exactly how loss of MECP2 function can lead to metabolic dysfunction. While MECP2 is known to bind methylated DNA, it is also now clear that MECP2 can bind RNA as well, and this protein has been implicated in a panoply of different functions. One could speculate that loss of function of an epigenetic regulator such as MECP2 could disrupt gene regulation of important metabolic proteins; or perhaps induce a stress response that leads to P53 upregulation and subsequent diminishment of metabolic activity in an active effort to diminish ROS levels and allow for repair; or many have argued that MECP2 can regulate gene expression as a DNA binding protein or more recently through its ability to directly bind RNA. Finally, its tempting to speculate that because epigenetic regulation and metabolism both share similar substrates and products, that perhaps epigenetic regulation in the nucleus leads to an imbalance of substrates available for metabolic enzymes, and therefore metabolic disruption.

Here we found that POU2F1/OCT1 is potentially a key regulator of gene expression in response to loss of MECP2 in Rett brain, and is clearly upregulated in MECP2-null neurons *in vitro*. Furthermore, we probed for potential instigators of OCT1 stabilization and found that metabolic stress and DNA damage can induce OCT1 in normal neurons. Whether OCT1 induction is required for the physiological manifestations associated with Rett disease in patients is an open question. While it does seem likely that OCT1 induction is due to increased stress in neurons lacking MECP2, it is not clear whether blocking OCT1 function in neurons would reverse synaptic transmission defects observed in other studies in response to loss of MECP2. It is also possible that increasing OCT1

further would promote the type of repair or abrogation of stress necessary to promote neurophysiological restoration of MECP2-null neurons. Future effort will be devoted to understanding how OCT1 functionally regulate neuronal stress in Rett brain.

Materials and Methods

Generation of *in vitro* neurons First, isogenic Rett Syndrome and wild type iPSCs were derived from Rett patient fibroblasts as described previously [13]. iPSCs were maintained on plates coated with matrigel (Corning) in mTeSR1 (StemCell Technologies) until 80% confluency. Neural progenitor cell (NPC) fate was induced following the manufacturer's protocol. Briefly, 2 million cells were passaged per each well using Accutase (StemCell Technologies) into the STEMdiff SMADi neural induction medium (StemCell Technologies). Cells were passaged this way once a week, two more times to induce NPC fate. Next, NPCs were differentiated to a Cajal-Retzius neuronal cell type using growth factor withdrawal method. EGF and FGF were removed from the media, and the cells were cultured in DMEMF12 supplemented with N2 and B27 (Thermo Fisher).

Western Blot Cell lysate was prepared using RIPA buffer (Pierce) supplemented with Halt Protease Inhibitor Cocktail (ThermoFisher Scientific) and Halt Phosphatase Inhibitor Cocktail (ThermoFisher Scientific). Total protein concentration was determined using BCA Protein Assay Kit (ThermoFisher Scientific) following the manufacturer's protocol. Equal protein concentrations were loaded onto the NuPAGE 4-12% Bis-Tris gel (ThermoFisher Scientific) and run at 150 Volts for 90 minutes in running buffer, containing 25 mL of 20x NuPAGE MOPS SDS Running Buffer (ThermoFisher Scientific) and 475 mL of mili-Q water. Next, the protein was transferred onto the nitrocellulose membrane at

30 Volts for 60 minutes in transfer buffer, containing 25 mL 20x NuPAGE Transfer Buffer (ThermoFisher Scientific), 100 mL Methanol (ThermoFisher Scientific), 375 mL mili-Q water. The membrane was blocked overnight at 4°C in OneBlock Western-FL Blocking Buffer (Genesee Scientific), then incubated in the primary antibody at 4°C overnight. The following primary antibodies were used: rabbit MECP2 (Diagenode C15410052, 1:1000), rabbit anti-histone H3 (Abcam ab1791, 1:1000), rabbit OCT1 (Novus Biologicals NBP2-21584, 1:500), mouse ®-actin (SCBT sc-47778, 1:500). Membrane was washed twice with 0.1% PBST and incubated in anti-rabbit or anti-mouse secondary HRP-labeled secondary antibody (ThermoFisher Scientific 31460, 31430 1:100000) for 1 hour at room temperature. Membrane was washed twice with 0.1% PBST and SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific) was added to the membrane and subjected to film exposure.

Immunofluorescence and image quantification Cells grown on coverslips were washed with PBS and fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 15 minutes at room temperature. Next, the cells were washed with 0.1 % PBST three times and blocked in MAXblock Blocking Medium (Active Motif) for 1 hour at room temperature, then incubated overnight at 4°C in the primary antibody. The following primary antibodies were used: rabbit MECP2 (Diagenode C15410052, 1:1000), rabbit OCT1 (Novus Biologicals NBP2-21584, 1:500), rabbit pATR (Abcam ab227851 1:500). Next, the slides were washed three times with 0.1% PBST and secondary antibody conjugated with Alexa 488, 568, 594 or 647 (1:500, Life Technologies A-21203, A21202, A31571, A-21207) was used, accompanied by DAPI (Invitrogen 1:500). Slides were then

washed three times with 0.1% PBST and mounted using Prolong Gold (Invitrogen). Mean fluorescent intensity was quantified using ImageJ.

Cytospin Sorted nuclei were injected into the cytofunnel and spun down at 600 rpm for 15 minutes. Next, the nuclei were fixed with 4% paraformaldehyde following the immunofluorescence procedure.

UV irradiation Cell culture media was exchanged for PBS and the cells were placed in the GS GENE LINKER UV chamber (Bio-Rad). The irradiation was set to 100 mJ for 45 seconds. Next, the cells were washed with PBS and growth factor withdrawal media was applied. 4 hours later the cells were fixed for immunofluorescence.

Disruption of mitochondrial function Cells were washed with PBS and treated with either DMSO, 1 uM Phenformin, 20 uM Phenformin, 0.1 uM Rotenone or 5 uM Rotenone for three days. Media was changed every day.

C13 labeled glucose incorporation Cells were fed with DMEM (ThermoFisher Scientific) supplemented with 4 mM glutamine (ThermoFisher Scientific), 1 mM pyruvate (ThermoFisher Scientific), and 10 mM C13 labeled glucose (Cambridge Isotope Laboratories). 24 hours later the cells were washed twice with ammonium acetate (ThermoFisher Scientific) on ice and 80% methanol (ThermoFisher Scientific) was added to the cells. The plates were placed in -80C for 15 minutes. Next, the cells were scraped off the plate into eppendorf tubes, vortexed and centrifuged at 17000 g for 10 minutes at

4C. The methanol was then evaporated using the EZ-Lite evaporator. Dried samples were analyzed using Mass Spectrometry.

Seahorse Assay Cell were plated at a density 50,000-90,000 cells per well in a XF96 microplate (Agilent) and placed in the 37oC 5% CO2 incubator overnight. The next day, the cells were washed twice with the assay medium (Dulbecco′s Modified Eagle′s Medium supplemented with 10 mM glucose, 2 mM L-glutamine, 1 mM pyruvate and 5 mM HEPES, pH 7.4), and the microplate was placed in a 37oC incubator without CO2. 30 minutes later, the plate was loaded into the Seahorse XF96 Extracellular Flux Analyzer (Agilent Technologies). The following compounds were Injected during the assay: 2 uM oligomycin, 0.75 and 1.35 uM FCCP; 2 uM rotenone and antimycin A. When the measurements were done, cells were fixed with 4% paraformaldehyde, stained with Hoechst, and cell number per well was determined using an Operetta High-Content Imaging System (PerkinElmer). Oxygen consumption rates (OCR) were normalized to cell number per well.

Nuclei isolation from frozen brain samples Nuclei were isolated as described previously (Krishnaswami et al. 2016). Briefly, brain tissue was cut on ice using a scalpel and homogenized in a glass dounce. The homogenate was then filtered through a 40 um cell strainer and centrifuged at 1000g for 8 min at 4ºC. The pellet was resuspended in a homogenization buffer and mixed with equal amounts of iodixanol. This mixture was then gently placed in a new tube over a 29% iodixanol. The nuclei were centrifuged at 13500g for 20 min at 4ºC. The pellet was resuspended in the immunostaining buffer and incubated for 15 min at 4ºC. Next, primary antibody was added to the nuclei pellet and incubated on a rotator at

4ºC for 40 min. The following primary antibodies were used: rabbit MECP2 (Diagenode C15410052, 1:250), chicken Neun (Millipore Sigma MAB377 1:250). Nuclei were centrifuged at 400 g for 5 min at 4 ºC and washed with PBS/BSA twice. Secondary antibodies conjugated with Alexa 488 and 594 (1:500, Life Technologies A-21203, A21202) were added, accompanied by DAPI (Invitrogen 1:500), the nuclei were incubated on a rotator at 4ºC for 30 min. Immunostained nuclei were subjected to FACS.

Library Preparation and Sequencing Sorted nuclei were delivered to the UCLA Technology Center for Genomics & Bioinformatics where libraries for RNA sequencing were prepared. The samples were sequenced using NovaSeq 6000 S2 PE 2x50 with 50,000 reads per cell.

Single-cell RNA-sequencing analysis Aligned counts were clustered using Seurat v3 and the standard clustering pipeline and parameters. Clustermarkers were calculated using the Wilcoxon rank sum test to compare distributions across individual clusters and the rest of the population. All differential expression analyses were also calculated using the Wilcoxon rank sum test. Venn diagrams were generated using bioVenn. Cite: BioVenn - a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams T. Hulsen, J. de Vlieg and W. Alkema, BMC Genomics 2008, 9 (1): 488. Gene ontology visualizations were generated using ggplot2, with geometric points and a scaled color gradient.

Transcription factor binding site enrichment analysis Analysis was performed using the DIRE tool https://dire.dcode.org.

Gene ontology analysis Analysis was performed using Metascape, a Gene Annotation & Analysis Resource https://metascape.org/gp/index.html#/main/step1.

Figures and Legends

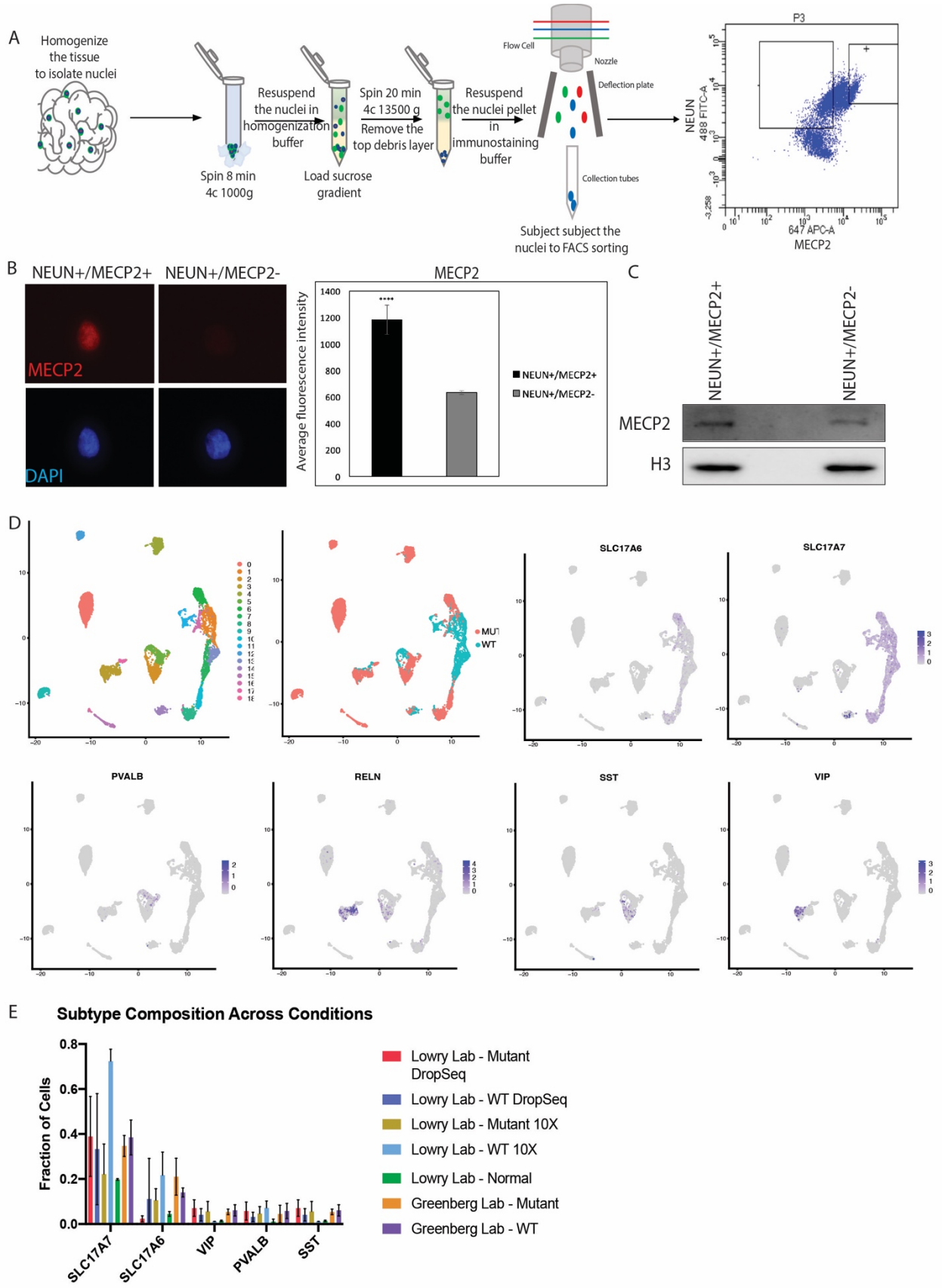

A
Homogenize the tissue to isolate nuclei
Spin 8 min 4c 1000g
Resuspend the nuclei in homogenization buffer
Load sucrose gradient
Spin 20 min 4c 13500 g
Remove the top debris layer
Resuspend the nuclei pellet in immunostaining buffer
Flow Cell
Nozzle
Deflection plate
Collection tubes
Subject subject the nuclei to FACS sorting
P3
NEUN 488 FITC-A
647 APC-A MECP2
B
NEUN+/MECP2+
NEUN+/MECP2-
MECP2
DAPI
MECP2
Average fluorescence intensity
****
NEUN+/MECP2+
NEUN+/MECP2-
C
NEUN+/MECP2+
NEUN+/MECP2-
MECP2
H3
D
SLC17A6
SLC17A7
PVALB
RELN
SST
VIP
E
Subtype Composition Across Conditions
Fraction of Cells
SLC17A7
SLC17A6
VIP
PVALB
SST
Lowry Lab - Mutant DropSeq
Lowry Lab - WT DropSeq
Lowry Lab - Mutant 10X
Lowry Lab - WT 10X
Lowry Lab - Normal
Greenberg Lab - Mutant
Greenberg Lab - WT

Figure 1. A method to isolate and molecularly profile MECP2hi and MECP2low neurons from Rett brain (**A**) The diagram depicts the process of nuclei isolation that employs sucrose gradient and a number of washes and centrifugations followed by immunostaining and FACS. (**B**) Immunostaining of the sorted MECP2high and MECP2low neuronal nuclei after cytospin from Rett patient brain shows a decrease in MECP2 expression in the MECP2low fraction, images taken at 40x. (right), quantification of IF signal. (**C**) Western blot analysis verifying a decrease in MECP2 expression in the MECP2low fraction isolated by FACS. (**D**) UMAP showing 19 clusters identified during the analysis of RNA-seq. MECP2low (MUT) and MECP2high (WT) nuclei contribute to multiple identified clusters, expressing excitatory and inhibitory neuronal subtypes. (**E**) UMAP plots of abundant neuronal subtypes as defined by the indicated markers. (**F**) Proportions of neuronal subtypes in MECP2high, MECP2low and WT nuclei from three independent experiments. Mutant (MECP2-Mutant nuclei from Rett brain), WT (MECP2-wild type nuclei from Rett brain), Normal (wild type nuclei from wild type brain).

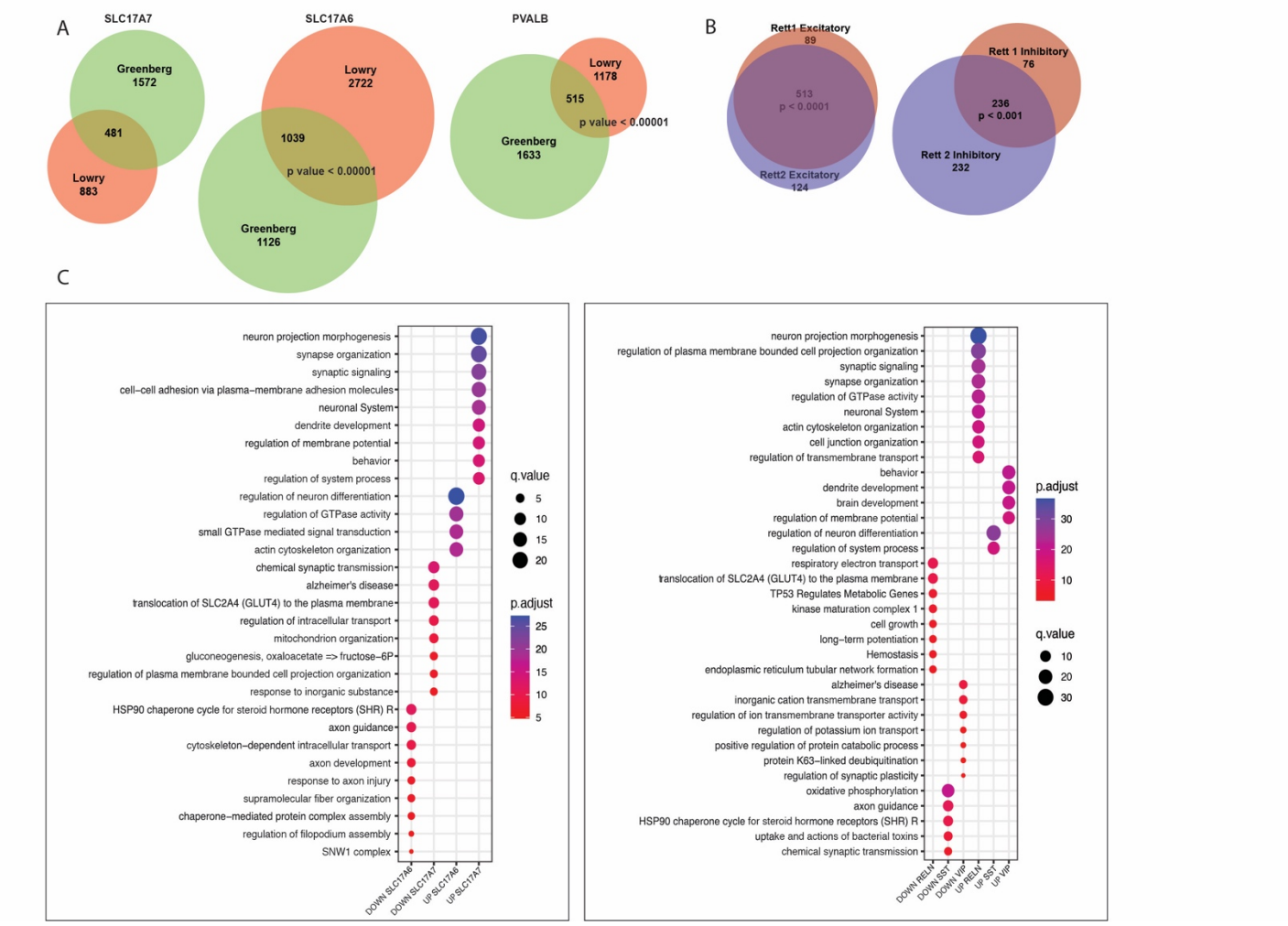


Figure 2. Transcriptome analysis reveals alteration in synaptic and metabolic gene expression due to lack of MECP2.

(**A**) Venn diagram showing the number of overlapping genes in several excitatory and inhibitory neuronal populations between our data and a published data set from the Greenberg lab. (**B**) Venn diagram showing the number of genes that overlap between our excitatory and inhibitory neuronal populations. (**C**) A summary of the number of Differentially Expressed Genes (DEGs) in several of the main cell types. (**D**) Gene ontology (GO) analysis of misregulated pathways indicate significant alterations in

synaptic and metabolic gene expression in both excitatory (left) and inhibitory (right) MECP2low neurons.

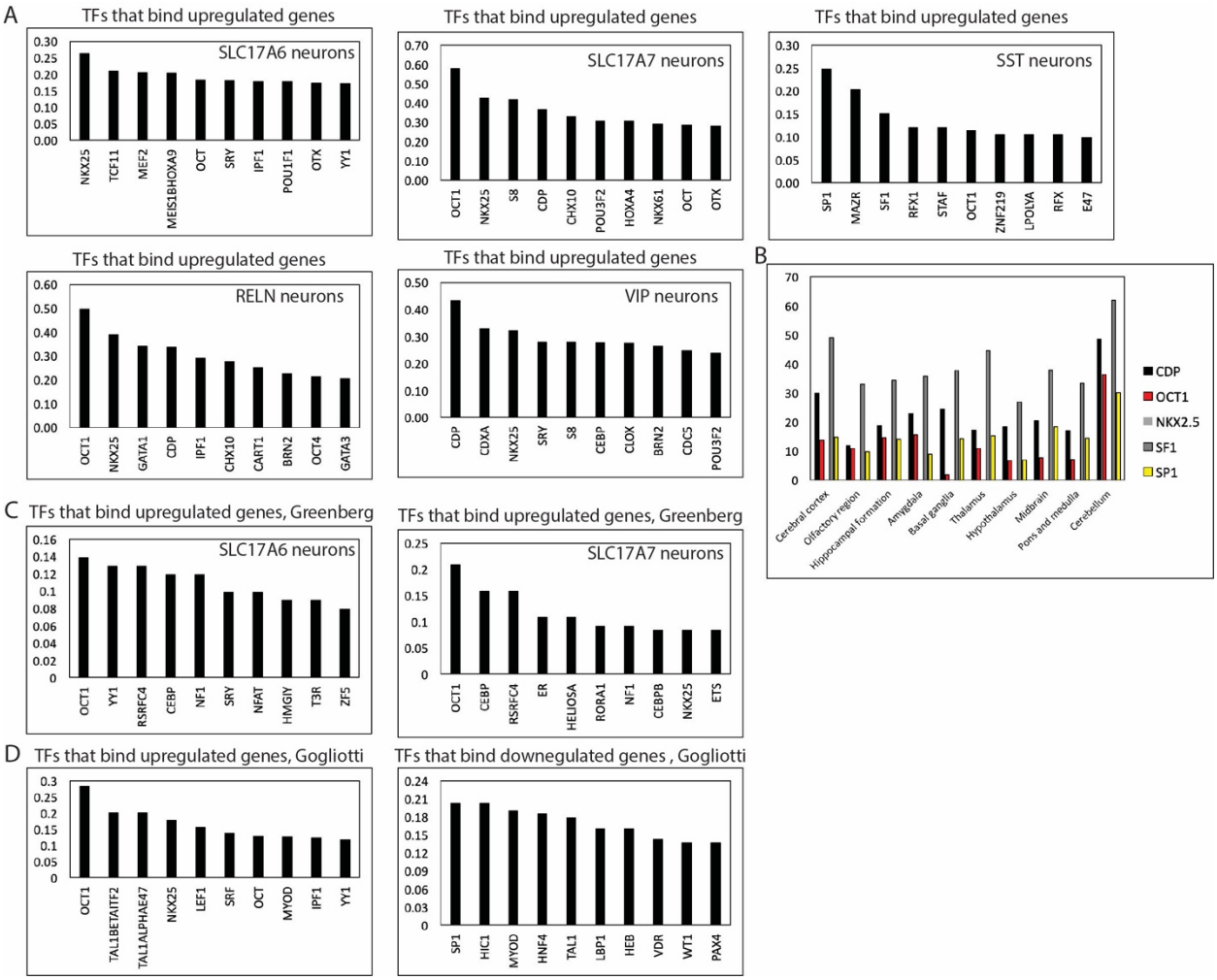


Figure 3. POU2F1/OCT1 is one of the most enriched potential regulators of genes altered in Rett patient brain MECP2low neurons.

(**A**) Analysis of the transcription factor binding sites within the genes significantly altered dues to lack of MECP2 shows POU2F1/OCT1 binding site enrichment (Measured by DIRE algorithm). y-axis indicates the percentage of genes containing a conserved binding site for a particular transcription factor. (**B**) Same analysis as in (**A**), but using the Greenberg Rett dataset. (**C**) Same analysis as in (A), but performed on bulk-RNA-seq

DEGs from Gogliotti et al. (**D**) Expression of the enriched potential regulators of genes altered in MECP2low neurons by brain region.

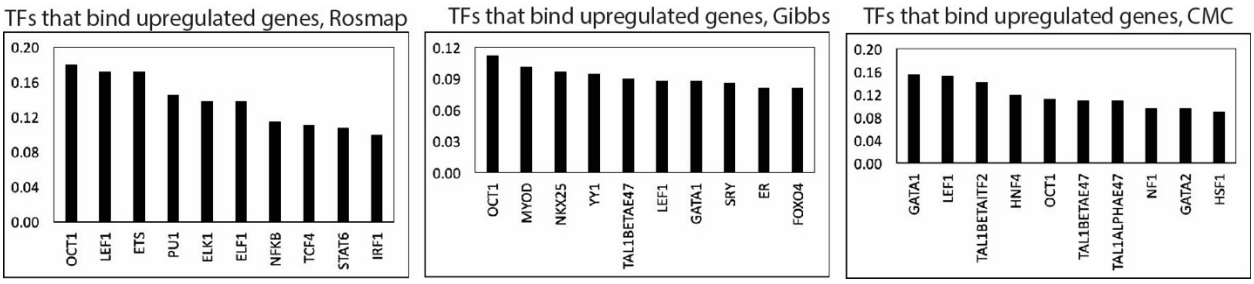


Figure 4. POU2F1/OCT1 is one of the most enriched potential regulators of genes altered in the aging brain.

Analysis of the aging brain from three independent cohorts shows enrichment of POU2F1/OCT1 binding sites within the differentially expressed genes.

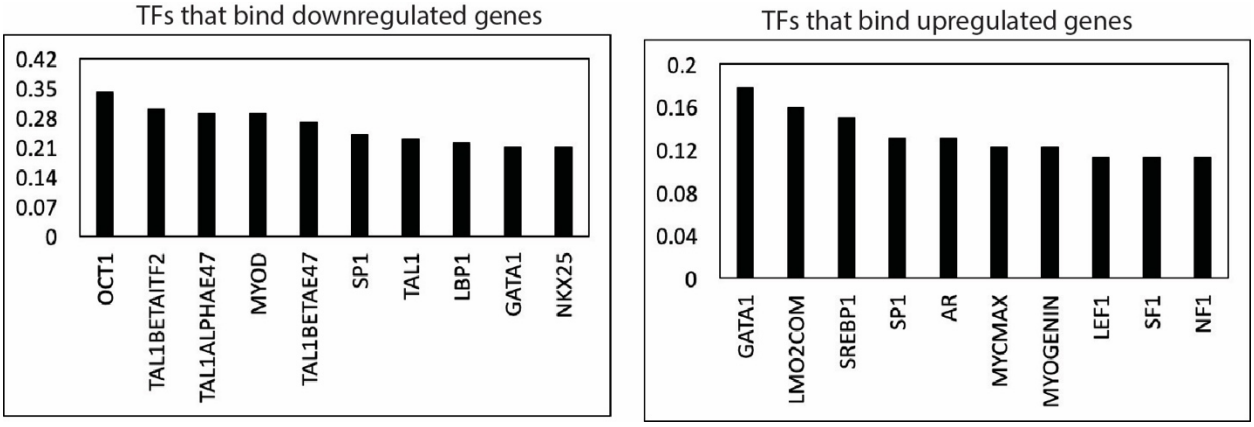


Figure 5. POU2F1/OCT1 is one of the most enriched potential regulators of genes altered in the neurons lacking MECP2 *in vitro*.

Analysis of bulk RNA sequencing of Rett patient derived *in vitro* neurons shows enrichment of POU2F1/OCT1 binding sites within the differentially expressed genes.

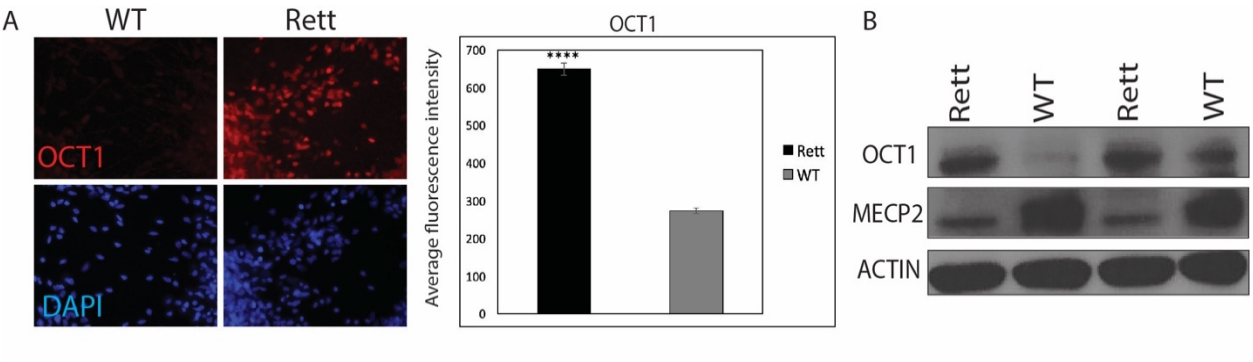


Figure 6. POU2F1/OCT1 expression is induced in neurons lacking MECP2 *in vitro*. (**A**) Left, immunostaining shows significantly higher expression of POU2F1/OCT1 in patient derived Rett neurons compared to the wild type, 40x. Right, quantification of the immunostaining. (**B**) Western blot shows that POU2F1/OCT1 is induced in two independently derived Rett cell lines compared to two wild types.

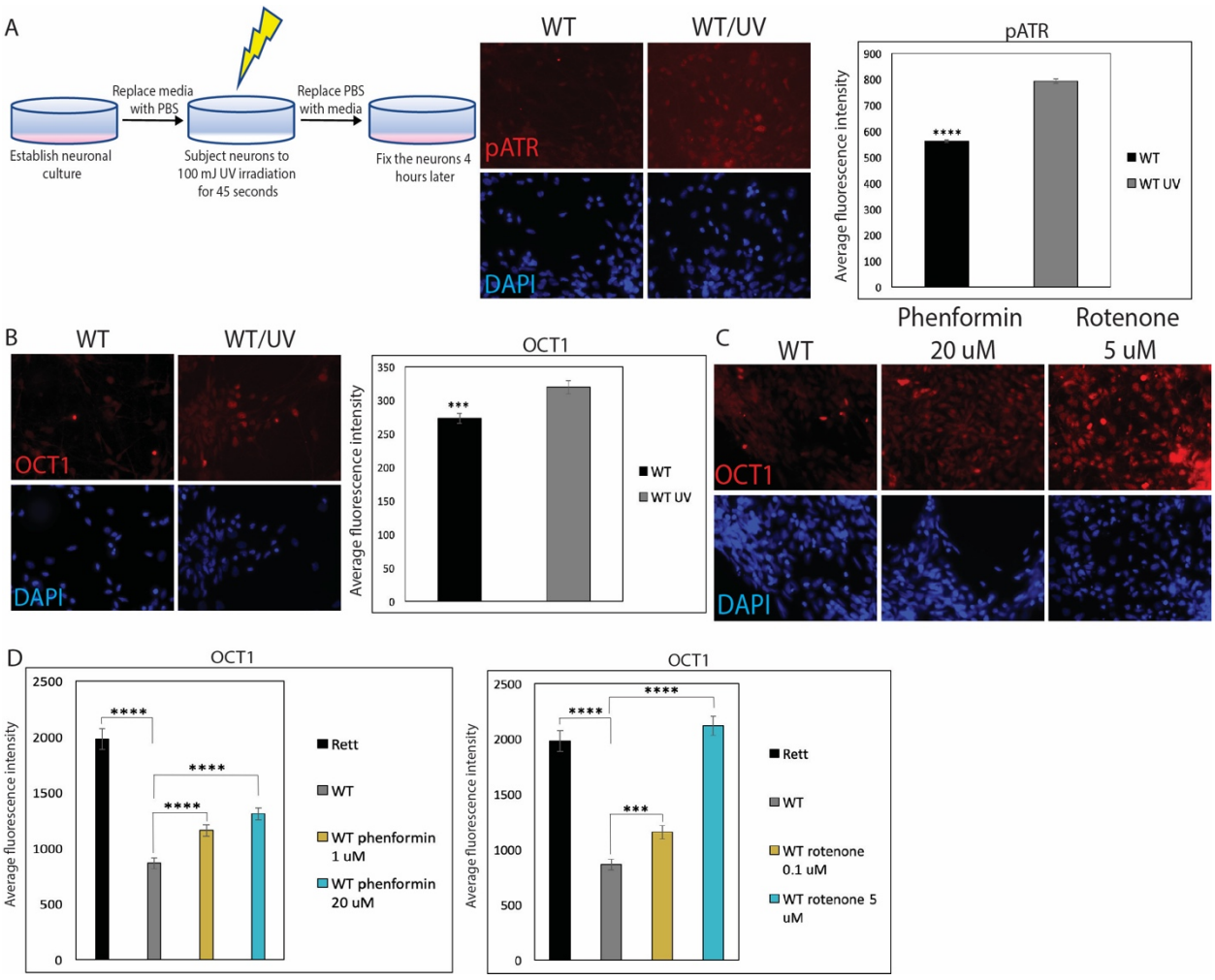


Figure 7. POU2F1/OCT1 is induced in wild type neurons upon DNA damage and disruption of mitochondrial function *in vitro*.

(**A**) Left, schematic of UV irradiation of wild type neurons. Middle, immunostaining showing that phosphorylation of ATR increases in neurons upon UV irradiation, indicating DNA damage, 40x. Right, quantification of the immunostaining. Statistical significance was checked using a paired t-test, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (**B**) Left, immunostaining showing that Induction of DNA damage in wild type neurons leads to elevated expression of POU2F1/OCT1 protein, 40x. Right, quantification

of the immunostaining (**C**) Immunostaining showing that wild type neurons treated with 20 uM Phenformin or 5 uM Rotenone which leads to a disruption of mitochondrial function, induce of POU2F1/OCT1 expression, 40x. (**D**) Quantification of the immunostaining, POU2F1/OCT1 is induced upon Phenformin or Rotenone treatment in a concentration dependent manner.

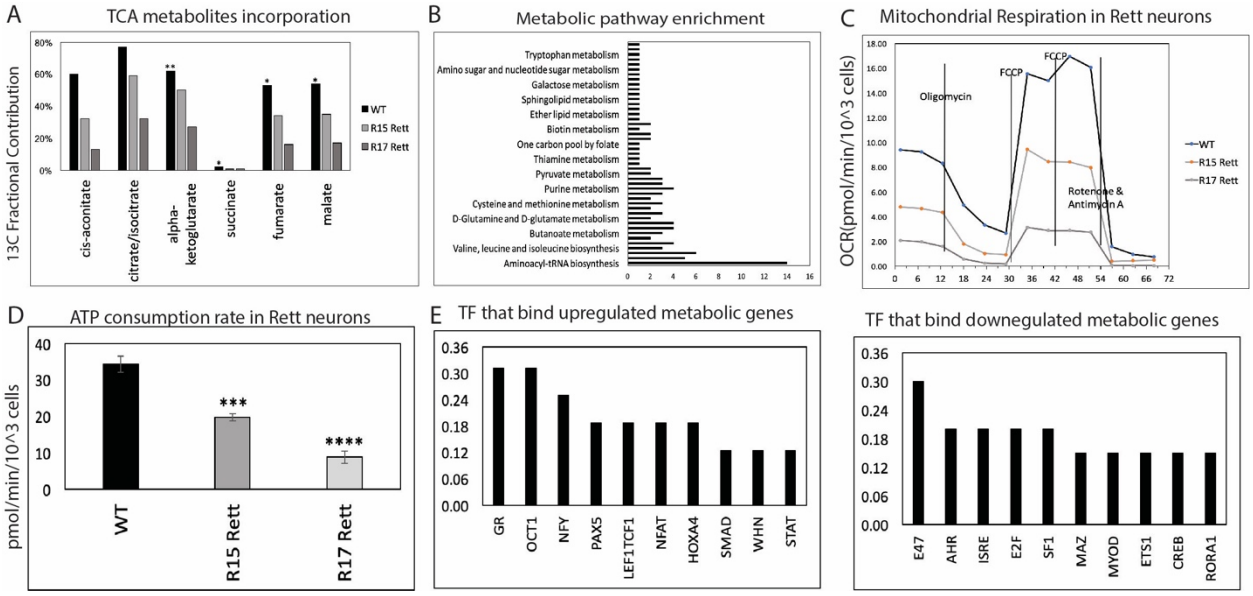


Figure 8. Rett neurons exhibit metabolic dysfunction *in vitro*.

(**A**) Lack of MECP2 in patient derived neurons leads to a decreased incorporation of C13 labeled glucose into the citric acid cycle. (**B**) Pathway enrichment showing the processes most affected by the metabolic dysfunction in Rett neurons. x-axis is the number of times a particular pathway came up as affected during the analysis. (**C**) Oxygen consumption rate is decreased in Rett neurons as detected by the Seahorse assay. (**D**) Rett neurons also exhibit decreased ATP consumption rate. (**E**) Analysis of transcription factor binding sites within significantly changed metabolic genes revealed POU2F1/OCT1 as being one of the potential regulators of these genes.

References

1. Amir, R.E., et al., *Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2.* Nat Genet, 1999. **23**(2): p. 185-8,
2. Moretti, P. and H.Y. Zoghbi, *MeCP2 dysfunction in Rett syndrome and related disorders.* Curr Opin Genet Dev, 2006. **16**(3): p. 276-81,
3. Akbarian, S., et al., *Expression pattern of the Rett syndrome gene MeCP2 in primate prefrontal cortex.* Neurobiol Dis, 2001. **8**(5): p. 784-91,
4. Fyffe, S.L., et al., *Deletion of Mecp2 in Sim1-expressing neurons reveals a critical role for MeCP2 in feeding behavior, aggression, and the response to stress.* Neuron, 2008. **59**(6): p. 947-58.PMC2597031
5. Giacometti, E., et al., *Partial rescue of MeCP2 deficiency by postnatal activation of MeCP2.* Proc Natl Acad Sci U S A, 2007. **104**(6): p. 1931-6.1794312
6. Luikenhuis, S., et al., *Expression of MeCP2 in postmitotic neurons rescues Rett syndrome in mice.* Proc Natl Acad Sci U S A, 2004. **101**(16): p. 6033-8.395918
7. Chen, R.Z., et al., *Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice.* Nat Genet, 2001. **27**(3): p. 327-31,
8. Kinde, B., et al., *Reading the unique DNA methylation landscape of the brain: Non-CpG methylation, hydroxymethylation, and MeCP2.* Proc Natl Acad Sci U S A, 2015. **112**(22): p. 6800-6.PMC4460470
9. Wen, L. and F. Tang, *Genomic distribution and possible functions of DNA hydroxymethylation in the brain.* Genomics, 2014. **104**(5): p. 341-6,
10. Guy, J., et al., *The role of MeCP2 in the brain.* Annu Rev Cell Dev Biol, 2011. **27**: p. 631-52,
11. Cohen, S., et al., *Genome-wide activity-dependent MeCP2 phosphorylation regulates nervous system development and function.* Neuron, 2011. **72**(1): p. 72-85.PMC3226708
12. Marchetto, M.C., et al., *A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells.* Cell, 2010. **143**(4): p. 527-39.3003590

13. Ohashi, M., et al., *Loss of MECP2 Leads to Activation of P53 and Neuronal Senescence.* Stem Cell Reports, 2018. **10**(5): p. 1453-1463.PMC5995366
14. Squillaro, T., et al., *Partial silencing of methyl cytosine protein binding 2 (MECP2) in mesenchymal stem cells induces senescence with an increase in damaged DNA.* FASEB J, 2010. **24**(5): p. 1593-603,
15. Squillaro, T., et al., *A case report: bone marrow mesenchymal stem cells from a Rett syndrome patient are prone to senescence and show a lower degree of apoptosis.* J Cell Biochem, 2008. **103**(6): p. 1877-85,
16. Renthal, W., et al., *Characterization of human mosaic Rett syndrome brain tissue by single-nucleus RNA sequencing.* Nat Neurosci, 2018. **21**(12): p. 1670-1679.PMC6261686
17. Quaderi, N.A., et al., *Genetic and physical mapping of a gene encoding a methyl CpG binding protein, Mecp2, to the mouse X chromosome.* Genomics, 1994. **22**(3): p. 648-51,
18. Habib, N., et al., *Massively parallel single-nucleus RNA-seq with DroNc-seq.* Nat Methods, 2017. **14**(10): p. 955-958.PMC5623139
19. Macosko, E.Z., et al., *Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets.* Cell, 2015. **161**(5): p. 1202-14.PMC4481139
20. Ito-Ishida, A., et al., *Loss of MeCP2 in Parvalbumin-and Somatostatin-Expressing Neurons in Mice Leads to Distinct Rett Syndrome-like Phenotypes.* Neuron, 2015. **88**(4): p. 651-8.PMC4656196
21. Tomassy, G.S., et al., *Developmental abnormalities of cortical interneurons precede symptoms onset in a mouse model of Rett syndrome.* J Neurochem, 2014. **131**(1): p. 115-27,
22. Baker, S.A., et al., *An AT-hook domain in MeCP2 determines the clinical course of Rett syndrome and related disorders.* Cell, 2013. **152**(5): p. 984-96.PMC3641682
23. Lang, M., et al., *Selective preservation of MeCP2 in catecholaminergic cells is sufficient to improve the behavioral phenotype of male and female Mecp2-deficient mice.* Hum Mol Genet, 2013. **22**(2): p. 358-71,

24. Panayotis, N., et al., *Morphological and functional alterations in the substantia nigra pars compacta of the Mecp2-null mouse.* Neurobiol Dis, 2011. **41**(2): p. 385-97,
25. Samaco, R.C., et al., *Loss of MeCP2 in aminergic neurons causes cell-autonomous defects in neurotransmitter synthesis and specific behavioral abnormalities.* Proc Natl Acad Sci U S A, 2009. **106**(51): p. 21966-71.PMC2799790
26. Smrt, R.D., et al., *Mecp2 deficiency leads to delayed maturation and altered gene expression in hippocampal neurons.* Neurobiol Dis, 2007. **27**(1): p. 77-89.2789309
27. Tudor, M., et al., *Transcriptional profiling of a mouse model for Rett syndrome reveals subtle transcriptional changes in the brain.* Proc Natl Acad Sci U S A, 2002. **99**(24): p. 15536-41.137752
28. Guy, J., et al., *A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome.* Nat Genet, 2001. **27**(3): p. 322-6,
29. Kang, J., et al., *A general mechanism for transcription regulation by Oct1 and Oct4 in response to genotoxic and oxidative stress.* Genes Dev, 2009. **23**(2): p. 208-22.PMC2648538
30. Kang, J., A. Shakya, and D. Tantin, *Stem cells, stress, metabolism and cancer: a drama in two Octs.* Trends Biochem Sci, 2009. **34**(10): p. 491-9,
31. Vazquez-Arreguin, K., et al., *Oct1/Pou2f1 is selectively required for colon regeneration and regulates colon malignancy.* PLoS Genet, 2019. **15**(5): p. e1007687.PMC6522070
32. Jafek, J.L., et al., *Transcription factor Oct1 protects against hematopoietic stress and promotes acute myeloid leukemia.* Exp Hematol, 2019. **76**: p. 38-48 e2,
33. Kang, J., et al., *Regulation of Oct1/Pou2f1 transcription activity by O-GlcNAcylation.* FASEB J, 2013. **27**(7): p. 2807-17.PMC3688745
34. Kang, J., et al., *Dynamic regulation of Oct1 during mitosis by phosphorylation and ubiquitination.* PLoS One, 2011. **6**(8): p. e23872.PMC3163677
35. Squillaro, T., et al., *Senescence Phenomena and Metabolic Alteration in Mesenchymal Stromal Cells from a Mouse Model of Rett Syndrome.* Int J Mol Sci, 2019. **20**(10).PMC6567034

36. Alessio, N., et al., *Neural stem cells from a mouse model of Rett syndrome are prone to senescence, show reduced capacity to cope with genotoxic stress, and are impaired in the differentiation process.* Exp Mol Med, 2018. **50**(3): p. 1.PMC6118406
37. Mucerino, S., et al., *Alterations in the carnitine cycle in a mouse model of Rett syndrome.* Sci Rep, 2017. **7**: p. 41824.PMC5288798
38. Alvarez-Saavedra, M., et al., *Cell-specific expression of wild-type MeCP2 in mouse models of Rett syndrome yields insight about pathogenesis.* Hum Mol Genet, 2007. **16**(19): p. 2315-25,
39. Lin, M., et al., *Heat shock alters the expression of schizophrenia and autism candidate genes in an induced pluripotent stem cell model of the human telencephalon.* PLoS ONE, 2014. **9**(4): p. e94968.3988108
40. Stevens, H.E., et al., *Prenatal stress delays inhibitory neuron progenitor migration in the developing neocortex.* Psychoneuroendocrinology, 2013. **38**(4): p. 509-21.PMC3532962
41. Morrison, R.S., et al., *Neuronal survival and cell death signaling pathways.* Adv Exp Med Biol, 2002. **513**: p. 41-86,
42. Farley, M.M. and T.A. Watkins, *Intrinsic Neuronal Stress Response Pathways in Injury and Disease.* Annu Rev Pathol, 2018. **13**: p. 93-116,
43. Solleiro-Villavicencio, H. and S. Rivas-Arancibia, *Effect of Chronic Oxidative Stress on Neuroinflammatory Response Mediated by CD4(+)T Cells in Neurodegenerative Diseases.* Front Cell Neurosci, 2018. **12**: p. 114.PMC5934485
44. Cortelazzo, A., et al., *Proteomic analysis of the Rett syndrome experimental model mecp2(Q63X) mutant zebrafish.* J Proteomics, 2017. **154**: p. 128-133,
45. Ishikawa, F., *Cellular senescence as a stress response.* Cornea, 2006. **25**(10 Suppl 1): p. S3-6,
46. Kriaucionis, S., et al., *Gene expression analysis exposes mitochondrial abnormalities in a mouse model of Rett syndrome.* Mol Cell Biol, 2006. **26**(13): p. 5033-42.PMC1489175

47. Gibson, J.H., et al., *Downstream targets of methyl CpG binding protein 2 and their abnormal expression in the frontal cortex of the human Rett syndrome brain.* BMC Neurosci, 2010. **11**: p. 53.PMC2881102
48. Pecorelli, A., et al., *Genes related to mitochondrial functions, protein degradation, and chromatin folding are differentially expressed in lymphomonocytes of Rett syndrome patients.* Mediators Inflamm, 2013. **2013**: p. 137629.PMC3876710
49. Can, K., et al., *Neuronal Redox-Imbalance in Rett Syndrome Affects Mitochondria as Well as Cytosol, and Is Accompanied by Intensified Mitochondrial O 2 Consumption and ROS Release.* Front Physiol, 2019. **10**: p. 479.PMC6503037
50. Jagtap, S., et al., *Aberrant mitochondrial function in patient-derived neural cells from CDKL5 deficiency disorder and Rett syndrome.* Hum Mol Genet, 2019. **28**(21): p. 3625-3636.PMC6927463
51. Shulyakova, N., et al., *Mitochondrial Dysfunction in the Pathogenesis of Rett Syndrome: Implications for Mitochondria-Targeted Therapies.* Front Cell Neurosci, 2017. **11**: p. 58.PMC5348512
52. Chinnery, P.F., et al., *Point mutations of the mtDNA control region in normal and neurodegenerative human brains.* Am J Hum Genet, 2001. **68**(2): p. 529-32.PMC1235287
53. Chinnery, P.F. and D.M. Turnbull, *Mitochondrial DNA mutations in the pathogenesis of human disease.* Mol Med Today, 2000. **6**(11): p. 425-32,
54. Lee, K.L., et al., *Attenuating brain inflammation, ischemia, and oxidative damage by hyperbaric oxygen in diabetic rats after heat stroke.* J Formos Med Assoc, 2013. **112**(8): p. 454-62,
55. Haas, R.H., et al., *Oxidative metabolism in Rett syndrome: 1. Clinical studies.* Neuropediatrics, 1995. **26**(2): p. 90-4,
56. Sztainberg, Y., et al., *Reversal of phenotypes in MECP2 duplication mice using genetic rescue or antisense oligonucleotides.* Nature, 2015. **528**(7580): p. 123-6,
57. Matthaei, J., et al., *OCT1 Deficiency Affects Hepatocellular Concentrations and Pharmacokinetics of Cycloguanil, the Active Metabolite of the Antimalarial Drug Proguanil.* Clin Pharmacol Ther, 2019. **105**(1): p. 190-200,

58. Fan, W., et al., *BRCA1 regulates GADD45 through its interactions with the OCT-1 and CAAT motifs.* J Biol Chem, 2002. **277**(10): p. 8061-7,
59. Salvador, J.M., J.D. Brown-Clay, and A.J. Fornace, Jr., *Gadd45 in stress signaling, cell cycle control, and apoptosis.* Adv Exp Med Biol, 2013. **793**: p. 1-19,

Contributions:

EK contributed to the experimental design, cell culture, genomic analysis, analysis of RNA-seq data, evaluation of OCT1 expression upon various conditions, interpretation of the results and manuscript writing. The work presented was funded by EK's BSCRC training grant.

Chapter 4: Conclusion

Closing Remarks:

Understanding the Mechanisms of Stress Related Pathways in Rett Syndrome

The focus of this work was to elucidate pathways misregulated in Rett syndrome. To do so, we performed single-cell RNA sequencing of Rett patient brain samples and discovered a number of genes, both upregulated and downregulated, as well as numerous pathways affected by the lack of MECP2. We also identified OCT1 as an enriched regulator of these differentially expressed genes, which we hypothesize serves to mitigate neuronal stress experienced by mutant cells, a condition that arises from various causes such as toxicity, disease, age or injury and promotes a response cascade aimed to cope with the stress. Interestingly, neuronal stress has also been detected in various neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Activated pathways include autophagy, protein accumulation, neuroinflammation and oxidative/mitochondrial stress[1]. We detected neuronal stress in Rett patient brain samples as well as in our *in vitro* cultures, which we further used to understand the nature and mechanism of stress.

While the mechanisms leading to Rett disease remain unclear, lack of MECP2 does appear to be most damaging in the brain. Our previous work shows that neurons lacking MECP2 *in vitro* exhibit stress signatures consistent with upregulated P53 target genes, such as GADD45 and P21, and induction of senescence[2]. These findings have been supported by *in vivo* data. Rett patient brain samples exhibit higher levels of P53 target genes in both cerebellum and motor cortex, as detected by RT-PCR. P53 is a tumor suppressor protein that regulates a complex network of genes in order to elicit a cellular response to stress. Cellular stress can be intrinsic or extrinsic, occurring in various forms such as DNA damage, oxidative stress and starvation. Stress signals induce P53 to promote transcriptional activation of genes designed to respond and deal with stress in

various forms, such as DNA repair, apoptosis and senescence (Figure 1)[3]. This prompted us to further investigate potential causes of stress that Rett neurons experience. We have discovered that lack of MECP2 in our *in vitro* cultures results in higher levels of DNA damage in neurons. This points to DNA damage as being one of the potential causes of P53 pathway induction in neurons lacking MECP2. In addition to upregulated P53 target genes and senescence, our earlier work has demonstrated that Rett neurons display reduced dendritic branching complexity[2]. Interestingly, it has been previously reported that abnormal dendritic arborization is directly dependent on the P53 pathway[4]. In order to understand this connection, we turned to pifithrin, a potent P53 pathway inhibitor. Treating Rett neurons with pifithrin rescued dendritic complexity and neurite length.

Based on the work described above, we can propose the order of events leading to the Rett phenotype. Cellular stress causes induction of the P53 pathway, which in turn promotes senescence and a decrease in dendritic branching complexity. However, it remains unclear what other types of neuronal stress cells experience, and which types of stress, other than DNA damage, induce cellular adaptation. In an attempt to answer these questions and to improve our understanding of Rett at the mechanistic level, ultimately leading to a platform to identify potential strategies for the prevention and treatment of Rett syndrome, we turned to the analysis of the Rett patient brain samples by single-cell RNA sequencing. Subjecting mutant and control samples to single-cell RNA sequencing has revealed a number of changes due to lack of MECP2. We looked at genes that were significantly upregulated and downregulated. Gene ontology analysis revealed that lack of MECP2 causes deficiency in synaptic transmission and faulty metabolism.

In order to corroborate our findings, we performed similar analyses on additional datasets publicly available. We reanalyzed a published data set generated by the Greenberg group that also performed RNA sequencing of Rett patient brain samples[5]. In alignment with our findings, Greenberg's data showed that mutant neurons exhibit deficiencies in synaptic transmission. These findings were also supported by the analysis of the mouse brain lacking Mecp2 that fails to maintain healthy neuron migration, axon guidance and neuron projection regeneration[6]. Finally, we compared our data to a published data set generated by performing RNA sequencing of aging brain samples[7]. In accordance with signatures of these aging brains, neurons lacking MECP2 do display signs of premature aging, such as senescence and lack of dendritic branching complexity. This led us to wonder how similar gene expression profiles were between these brain samples. Similarities between Rett and the aging brain remained consistent when looking at the gene ontology analysis. The aging brain revealed that neurons fail to maintain high levels of synaptic transmission and exhibit aberrant metabolism. Overall, these comparisons gave us confidence in our data, but also raises interesting commonalities between neurological disorders.

To gain more insight into the changes in the Rett patient brain, we analyzed transcription factor binding sites enriched within the significantly differential gene sets generated from our RNA sequencing data. We discovered that OCT1 is one of the most enriched potential regulators of differentially expressed genes in the mutant brain. OCT1 is a ubiquitously expressed transcription factor that regulates a variety of genes. In mice, Oct1 is crucial for proper development, as lack of it leads to embryonic lethality[8]. Further, Oct1 deficient mouse fibroblasts exhibit higher levels of reactive oxygen species and are

more sensitive to toxic substances and harmful agents (Figure 2)[9]. Additionally, elevated levels of OCT1 have been detected in certain cancers, where increased expression of OCT1 leads to an onset of more aggressive tumor development[10]. Finally, OCT1 has been reported to be a stress sensor that regulates GADD45 activity in a P53 independent manner. The GADD45 promoter contains an OCT1 binding motif, and after inducing DNA damage in human carcinoma cells, OCT1 expression as well as its binding to GADD45 is increased, which in turns leads to an appropriate response to DNA damage[11]. We tested OCT1 induction by subjecting wild type iPSC-derived neurons to UV irradiation and also saw a significant upregulation of OCT1 upon DNA damage. Other types of stressors, such as metabolic stress, genotoxic stress and oxidative stress have been reported to induce OCT1 expression[10]. We discovered that treating wild type neurons with phenformin or rotenone, drugs that inhibit complex I of the mitochondrial respiratory chain, leads to OCT1 upregulation in a concentration dependent manner[12].

Although OCT1 has not been studied in the context of Rett syndrome, we did discover a significant upregulation of OCT1 protein levels in neurons lacking MECP2. This provided further evidence that Rett neurons are under various types of stress that leads to the induction of both P53 and OCT1. Given that OCT1 expression can also be promoted by metabolic and oxidative stress, we further analyzed Rett neurons *in vitro*. We measured metabolite incorporation into the citric acid cycle, and discovered that lack of MECP2 significantly decreases metabolite utilization leading to metabolic stress *in vitro*. This was consistent with our RNA sequencing data showing that gluconeogenesis is downregulated in Rett patient neurons. We also found that Rett neurons exhibit lower levels of mitochondrial respiration which leads to genotoxic stress *in vitro*. This supported

our RNA sequencing data showing that mitochondrion organization, respiratory electron transport and oxidative phosphorylation are all downregulated in Rett patient neurons lacking MECP2. In addition, it has been reported that fibroblasts lacking MECP2 have misshapen mitochondria that fail to undergo proper fusion/fission process[13]. Taken together, these findings provide an insight into the types of stress experienced by Rett neurons and propose an explanation for OCT1 induction.

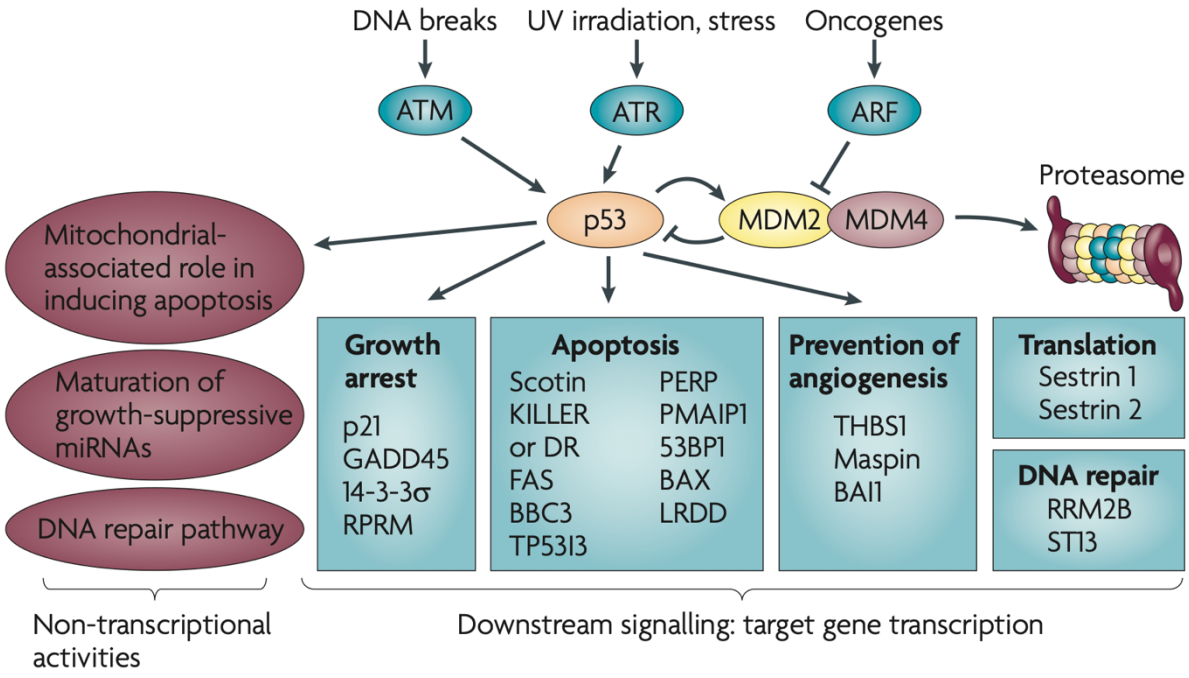
Our work has demonstrated that Rett neurons undergo neuronal stress caused by failed metabolism and high levels of DNA damage; as a result, stress pathways get activated. However, much remains to be investigated to understand the exact relationship between neuronal stress, induction of stress response pathways and the phenotype observed in Rett neurons. We will continue modeling Rett syndrome *in vitro* to further address the role of OCT1 in Rett syndrome pathology. We will start by focusing on OCT1 post-translational modifications that alter its ability to bind target genes. OCT1 is known to be phosphorylated, ubiquitinated and modified by O-linked β-N-acetylglucosamination. Interestingly, O-linked β-N-acetylglucosamination, specifically, has been implicated in the OCT1 stress response as well as its ability to interact with GADD45[14]. Thus, we will first establish OCT1 post-translational modification pattern in Rett neurons, and next we will monitor how this pattern changes upon various stress conditions such as DNA damage and metabolic stress. This will help us to establish a link between OCT1 induction and neuronal stress in Rett syndrome.

Next, we will continue investigating types of neuronal stress that cause OCT1 induction. We will start by subjecting our neurons to various DNA damaging agents and seeing whether OCT1 induction is specific to a certain type of DNA damage. We will use

ionizing radiation to induce double stranded DNA breaks, we will also keep subjecting the cells to UV irradiation to disturb the bonds between neighboring nucleotides, and induce reactive oxygen species production to modify the nucleotides. We will then treat the neurons with various metabolic agents inhibiting specific parts of the metabolic pathway, to induce metabolic stress, and monitor OCT1 expression. This will reveal whether OCT1 induction is triggered by misregulation of a specific metabolite. Finally, we will subject our neurons to ribosomal stress by using puromycin or neomycin to impair protein synthesis, since abnormal phosphorylation of ribosomal protein S6 and faulty protein synthesis have been reported in neurons lacking Mecp2[15]. These manipulations will help us pinpoint the types of neuronal stress that lead to OCT1 induction.

We will then investigate how manipulating OCT1 expression affects neurons, and whether lack of OCT1 can rescue Rett pathology. We will knock OCT1 down in Rett neurons using siRNA. We will then harvest these neurons and subject them to bulk RNA sequencing. This will reveal the genes changed due to lack of OCT1 and help us deduce the mechanism and the downstream targets of OCT1. We will also analyze the degree of dendritic complexity, and see whether lack of OCT1 can rescue the onset of senescence, abnormal metabolism and elevated levels of DNA damage. Finally, we will overexpress OCT1 in wild type neurons and see if it leads to abnormal neuronal morphology, senescence, faulty metabolism and accumulation of DNA damage. Manipulating OCT1 expression will ultimately reveal whether it is upstream or downstream of the phenotype observed in Rett neurons, and if it’s directly responsible for the disease phenotypes. Overall, this work will contribute to understanding neuronal stress in Rett syndrome and potentially take us one step closer to developing a treatment.

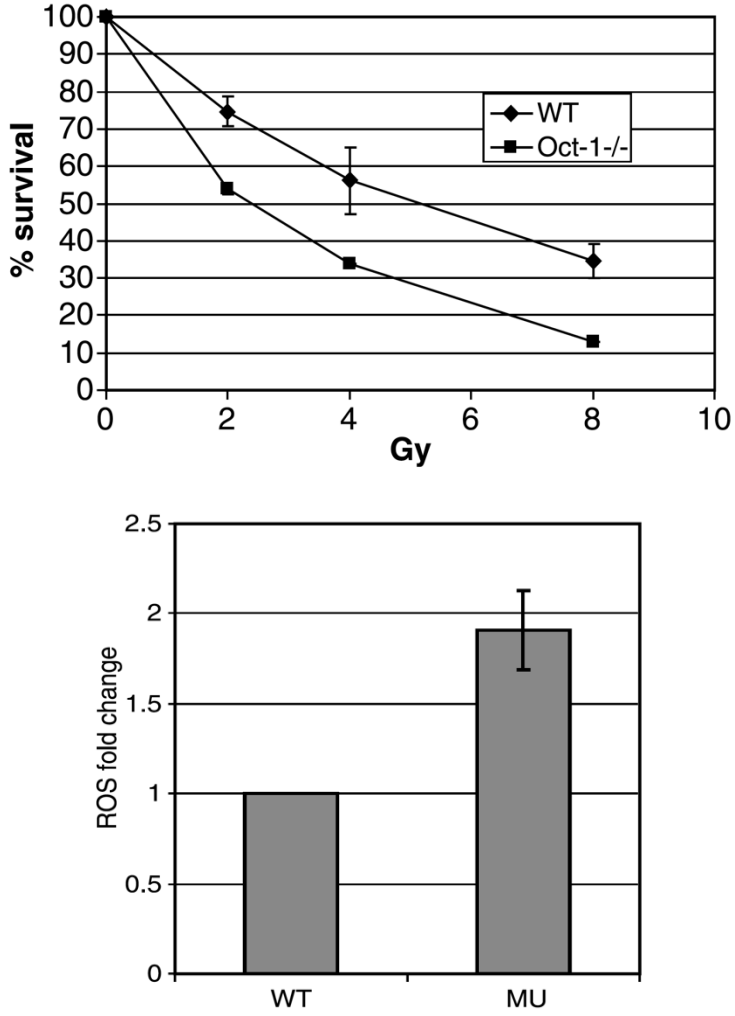
Figure 1



Adapted from Brown et al. 2009

Figure 1. P53 Pathway. Various extrinsic and intrinsic stress signals can induce P53 to promote transcriptional activation of genes designed to respond and deal with the stress in various forms, such as DNA repair, apoptosis and senescence. MDM2 serves to control P53 activity by ubiquitylating P53 for proteasomal degradation, once it has served its purpose.

Figure 2



Adapted from Tantin et al. 2005

Figure 2. Lack of Oct1 is harmful to mouse fibroblasts. Top, Oct1 mutant mouse fibroblasts exposed to varying doses of ionizing radiation exhibit worse survival 4 days after exposure, as determined by trypan blue incorporation. The x-axis shows the degree of radiation in grays (Gy). Bottom, lack of Oct1 promotes the formation of reactive oxygen species in mouse fibroblasts, detected by CM-H2DCFDA staining.

References

1. M. Farley, “Intrinsic Neuronal Stress Response Pathways in Injury and Disease”, *Annual Review of Pathology: Mechanisms of Disease*, vol. 13, pp. 93-116, 2018.
2. M. Ohashi, “Loss of MECP2 Leads to Activation of P53 and Neuronal Senescence,” *Stem Cell Rep.*, vol. 40, pp. 1453–1463, 2018.
3. A. Levine, “The P53 pathway: what questions remain to be explored?”, *Cell Death & Differentiation*, vol. 13, pp. 1027–1036, 2006.
4. S. Ferron, “Telomere Shortening in Neural Stem Cells Disrupts Neuronal Differentiation and Neuritogenesis”, *The Journal of Neuroscience*, vol. 29, no. 46, pp. 14394 –14407, 2009.
5. W. Renthal, “Characterization of human mosaic Rett syndrome brain tissue by single-nucleus RNA sequencing”, *Nat Neuroscie*, vol. 21, no. 12, pp. 1670–1679, 2019.
6. H. Nakashima, “MeCP2 controls neural stem cell fate specification through miR199a-mediated inhibition of BMP-Smad signaling”, *Cell Reports*, vol. 35, 2021.
7. J. Zullo, “Regulation of lifespan by neural excitation and REST”, *Nature*, vol. 574, pp. 359–364, 2019.
8. V. Wang, “Embryonic Lethality, Decreased Erythropoiesis, and Defective Octamer-Dependent Promoter Activation in Oct-1-Deficient Mice”, *Molecular and Cellular Biology*, vol. 24, no. 3, pp. 1022-1032, 2004.
9. D. Tantin, “The Octamer Binding Transcription Factor Oct-1 Is a Stress Sensor”, *Cancer Res*, vol. 65, no. 23, pp. 10750-10758, 2005.
10. K. Vazquez-Arreguin, “Oct1/Pou2f1 is selectively required for colon regeneration and regulates colon malignancy”, *PLoS Genet*, vol. 15, no. 5, 20019.
11. S. Jin, “Transcription factors Oct-1 and NF-YA regulate the p53-independent induction of the GADD45 following DNA damage”, *Oncogene*, vol. 20, pp. 2683-2690, 2001.
12. H. Thomas, “Mitochondrial Complex I Activity Is Required for Maximal Autophagy”, *Cell Rep*, vol. 24, no. 9, pp. 2404–2417, 2018.
13. I. Crivellari, “Impaired mitochondrial quality control in Rett Syndrome”, *Archives* of Biochemistry and Biophysics, vol. 700, 2021.

14. J. Kang, “Regulation of Oct1/Pou2f1 transcription activity by O-GlcNAcylation”, The FASEB Journal, vol. 27, pp. 2807-2817, 2013.
15. S. Ricciardi, “Reduced AKT/mTOR signaling and protein synthesis dysregulation in a Rett syndrome animal model”, *Human Molecular Genetics*, vol. 20, no. 6, pp. 1182–1196, 2011.
16. C. Brown, “Awakening guardian angels: drugging the p53 pathway”, *Nature Reviews Cancer*, vol. 9, pp. 862–873, 2009