UNIVERSITY OF CALIFORNIA, SAN DIEGO

Preliminary development of zebrafish platform for screening of *MECP2* therapeutics

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by

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The thesis of Amreeta Jammu is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

I dedicate this thesis to my family for their unconditional love and support.

To Amreen Homes for being the constant motivation in my life.

And finally, for the Rett syndrome community and families for their struggle and resilience to carry on through life with hope.

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ABSTRACT OF THE THESIS

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Amreeta Jammu
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Professor Jeffrey Neul, Chair
Professor Takaki Komiyama, Co-Chair

Rett syndrome is a severe neurodevelopmental disorder predominantly caused by mutations in the *Methyl-CpG-binding protein 2* (*MECP2*). Four out of eight common mutations in *MECP2* account for ~35% of RTT cases. A method for overcoming the phenotypes found in these nonsense mutations is treatment with nonsense suppression therapy. Nonsense suppression therapy are small molecules that suppress premature termination codons that would allow for full length readthrough of MeCP2. However, the ability screen for therapeutics has been limited due to the low throughput of RTT animal disease models.

Zebrafish have been shown to be a complementary model translatable for neuroscience research and for high throughput drug screenings. Here, I describe the applications and importance of zebrafish as a preliminary model for *MECP2* therapeutics. I re-capitulated the behavioral and molecular phenotypes characterized in zebrafish containing the *mecp2*^{Q63x} mutation by successfully showing expression of Mecp2 in the brain of zebrafish larvae. Using a custom video-based activity capture system, I demonstrated a decrease in total distance travelled, percentage of swimming activity, and decrease in anxiety-like behavior in the *mecp2*^{Q63x} fish. These findings reproduce previous reports demonstrating dysfunction in motor circuitry seen in RTT patients. I also found increased astrogenesis, as measured by expression of GFAP, in the *mecp2*^{Q63x} fish. Finally, I generated a novel *mecp2* zebrafish mutant with a complete excision of the *mecp2* locus using CRISPR/Cas9 system to demonstrate the ability to engineer this locus and pave the way for future generation of fish to contain human disease-causing nonsense mutations. In summary, this works provides the foundation and a platform to use genetically engineered zebrafish to perform high-throughput screening of nonsense suppression therapeutics

I.

Introduction

Rett syndrome (RTT) is a severe neurodevelopmental disorder that was first described in 1966 by Dr. Andreas Rett (Rett, 1966). Affecting 1 in 10,000 live female births, a vast majority (>95%) of people with RTT have mutations in the X-chromosome transcriptional regulator *Methyl CpG binding Protein 2* (*MECP2*) (Amir et al, 1999). The *Methyl-CpG binding protein* family contains several functional domains that include: a methyl-binding domain (MBD), intervening domain (ID), transcriptional repression domain (TRD), and C-terminal domain (CTD) (Linn, Nicholls 2016). MeCP2, in particular, is a nuclear protein that contains both the TRD and MBD and has been shown to be important in signal interpretation of methylated DNA (Hendrich & Bird 1998). MeCP2 is mostly expressed in the brain. (Skene, Illingworth 2010). It was discovered that the mutations in the gene encoding *MECP2* are associated with both familial and rare cases of Rett Syndrome (Amir 1999).

RTT almost exclusively affects female offspring with 99.5% of the cases being sporadic due to de novo mutations (Trappe, Lecone 2001). People with RTT develop normally until 6-18 months of life, then lose their acquired hand and spoken language skills with development of gait abnormalities and hand stereotypies (Neul 2010). Affected individuals also experience decreased somatic and brain growth and autonomic abnormalities such as breathing irregularities and cold, blue extremities (Neul, Fang 2008). Additional problems

involved in RTT include anxiety in response to novel stimuli, increased pain tolerance, as well as difficulty with communication. (Katz, Berger-Sweeny 2012).

There are at over 200 unique mutations involving *MECP2* that cause Rett syndrome, with eight common mutations (R106W, R133C, T158M, R168X, R255X, R270X, R294X, R306C) that are found in over 65% of the RTT patient population. Four out of eight common mutations are characterized as nonsense mutations that are represented in ~35% of the RTT population. Based on this finding, the opportunity to use mutation-specific therapy with compounds that suppress nonsense mutations, also called 'read through compounds' could prove to be an effective form of treatment for Rett syndrome (Pitcher et al 2015). Read through compounds bind ribosomal proteins and allow for insertion of a tRNA into a premature termination codon. This allows translational elongation to occur (Keeling, Wang, Conard, Bedwell 2012). Currently, aminoglycosides and non-aminoglycoside compounds have been used in animal models for cystic fibrosis and Duchenne muscular dystrophy to restore protein expression, but have not been fully explored for CNS diseases such as RTT. Studies in mouse models of RTT have shown that the disease may be reversible through re-expression of the *Mecp2* gene. Mammalian models have been eminent in the role of modeling human diseases due to the similar homology between mammalian genomes, as well as structural, cellular, and physiological similarities. MECP2, present in all vertebrates, has a high conservation of >95% protein sequence identity between humans and mice, as well as mimic some similar phenotypes found in the RTT

population due to high penetrance of *Mecp2* mutations in mice (Katz, Berger-Sweeny et al 2012). With the current advanced transgenic approaches, such as CRISPR/Cas9 system, to generate mouse models with specific allele modifications to mimic human diseases has made the mouse model a widely used form of human disease (Graham et al, 2007).

Given the emergence of promising therapeutic leads for nonsense suppression therapy, there is still some caution that must be taken with translating experimental results for neurological disorders in animal models to human disease therapies (Veeragan, Wan, et al 2016). There are many well-suited mouse models for studying the biology of RTT; however, they are not considered appropriate for high throughput screening of candidate therapeutics. Likewise, there are cellular models that are adaptable for high throughput screening, but have limited application for studying functional rescue. Zebrafish provide an intermediate model that could be utilized in a high throughput format with functional readouts for restoration of MeCP2 function. Currently, zebrafish have been utilized in therapeutics through the use of high-throughput drug screenings for small-molecule chemical modifiers in disease pathogenesis that has made them a suitable and beneficial model for pharmaceutical drug screenings to narrow compounds of therapeutic potential. One efficient tool to generate gene disruption (i.e. knock-out mutations) is through the CRISPR/Cas9 system in zebrafish, with a 75-99% frequency rate of gene knockouts (Uwe Irion, Jana Krauss, Christiane Nüsslein-Volhard 2014.)

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR- associated Cas9 systems are adaptive immune systems evolved in bacteria and Achaea that defend against intruding viruses and plasmid DNA (Hwang, Fu. et al 2013). It has been previously demonstrated that the Cas9 protein of type II CRISPR/Cas9 system from *Steptococcus pyogenes* is a dual RNA-guided endonuclease which relies on uptake of foreign DNA fragments into CRISPR loci. These fragments are then transcribed and processed into short CRISPR RNAs (crRNAs). These anneal and become trans-activating crRNA (tracrRNA), which mediate site-specific double stranded DNA cleavage. Various groups have reported to use the CRISPR/Cas9 system to successfully perform targeted genome editing in cultured human cells, bacteria, and mammalian model systems. It has only been of recent years that this alternative genome editing approach was demonstrated in zebrafish, showing efficiency in executing site-specific cleavage that allows for non-homologous end joining mediated indel mutations (Chang, Sun, Gao et al 2013).

Zebrafish has become a powerful tool to study CNS development, and recently been used to explore the role of MeCP2 in neuronal development (Gao, Bu, Wu, Wang, et al 2015). Synteny analysis has shown that *mecp2* is conserved within zebrafish on chromosomes 8 and 11. However, analysis has demonstrated that only one copy of *mecp2* on chromosome 8 is orthologous to the human MECP2 (Pietri, et al 2013). With a protein conservation of 48% between humans and zebrafish, *mecp2* has shown to be expressed throughout the body in various

organs, with particular enrichment in the nervous system. This has shown to be detected in early embryonic development. With similar structure and expression patterns of *mecp2* found in zebrafish and mammals, this model provides a complementary model system to understand the function of MeCP2 as well as a platform suitable for high throughput drug screening.

Currently, only two papers have been published regarding the functioning of *mecp2* in zebrafish. The first group used an antisense morpholino designed to knockdown *mecp2* in 48hpf zebrafish embryos, as well as CRISPR/Cas9 engineered fish with a nonsense mutation. This group was able to conclude that *mecp2* knockdown impairs neuronal differentiation and increased the expression of astrocytes. Additionally they showed that disrupting *mecp2* function leads to abnormal behaviors in later life (Gao et al, 2015).

A second paper generated a nonsense mutation in *mecp2* using ENU mutagenesis. Specifically, these fish contained a nonsense mutation in exon 2 upstream of the MBD and TRD domains, thus creating an early truncating mutation that should be functionally equivalent to a null mutation. With this model, the group showed that these mutant fish have shorter lifespans, as well as behavioral impairments with reduction in activity, decrease in anxiety-like behavior, as well as spontaneous motor activity at early embryonic stages (Pietri et al, 2015).

In order to develop a RTT model for potential drug screenings, the use of non-mammalian models, such as zebrafish, has great promise. Zebrafish can

serve as an intermediate model that provides complexity and robustness compared to human cells, as well as a faster and simpler approach to develop assays to narrow compounds that can be utilized for mammalian disease models. The development of zebrafish as a model for RTT has yet to be explored in detail, but yet raises interesting questions in terms of the functionality of *mecp2* in lieu of various mutations engineered, such as creating a full *mecp2* null. Another question is the possible recruitment of Mecp2 rescue to restore impairments observed in the mutants.

In this thesis, the objective of recapitulating some of the molecular and behavioral effects found in these two papers are demonstrated in order to validate phenotypes related to *mecp2* dysfunction in zebrafish. This also paves the way for establishing these preliminary assays for future MeCP2 therapeutic screenings. Here, I present similar behavioral finding to Pietri *et al.* in *mecp2* deficient zebrafish. These prominent behavioral abnormalities could be applied as functional metrics for restoration of mecp2. Likewise, I demonstrate molecular abnormalities identified in *mecp2* morphant zebrafish that are recapitulated in a mecp2 mutant zebrafish model. Finally, I show in a proof of concept investigation that CRISPR/Cas9 mediated genetic engineering can be applied to the zebrafish *mecp2* locus. This provides the groundwork for future introduction of patient specific mutations into zebrafish mecp2 with CRISPR. Together, this work will contribute to development of a highthroughput, zebrafish-based platform for screening *mecp2* targeted therapeutics.

II:

Results

Novel *mecp2* knockout zebrafish model is generated through CRISPR/Cas9

In order to demonstrate the ability to use CRISPR/Cas9 to genetically engineer the *mecp2* locus in zebrafish, I designed guide RNAs (gRNAs) flanking the entire *mecp2* locus on chromosome 8 (Figure 3). In order to genotype the putative engineered embryos, I used two PCR primers designed to recognized upstream and downstream genomic sequences. Exon 1 forward primer (located 158bp upstream of the Exon 1 gRNA) and Exon 3 reverse primer (located 533bp downstream of Exon 3 gRNA) were used in a polymerase chain reaction (PCR) to amplify the non-homologous end-joining product from the injected CRISPR injected embryos. If successful *mecp2* excision occurred, the resulting PCR product size was expected to be 691bp. PCR based genotyping of embryos 48 hours following microinjection of guides and Cas9 mRNA produced a 691 bp product (Figure 2). This indicated successful excision of *mecp2* in 60% of embryos that were screened in total. The wild-type lanes displayed no evident product, which suggested that the products seen in the KO lanes were indeed true bands from the 20kb excision of *mecp2*. This 20kb deletion removes the bulk of the coding sequence, as verified by Sanger sequencing (Figure 1). This demonstrated a proof of concept of successfully developing a genetically mutated *mecp2* model through CRISPR/Cas9.

Identification of *M*ecp2 protein using western blotting

Since restoration of *MECP2* proves to be a therapeutic rescue for RTT phenotypes in mice, the ability to detect Mecp2 protein in zebrafish is important for establishing in pre-clinical drug screening assays. To address this, I sought to use western blotting as a technique to detect full length Mecp2.

Previous studies have shown MeCP2 to be resolved at the 75kD band compared to the *mecp2* null *(mecp2*^{Q63x}*)* at 48hpf. To first determine if there was enough zebrafish protein to be detected, I made a dot blot using lysed 48hpf zebrafish samples. These samples were probed with an anti-H3 antibody, which detects Histone 3 that is shown to be highly conserved between species. My blot showed positive H3 detection in the zebrafish samples; therefore, I proceeded to western blotting for Mecp2 detection.

The first method that I used for protein isolation was the standard RIPA extraction buffer with protease inhibitor for homogenizing the whole zebrafish (deyolked and dechorionated). Following the methods for protein extraction that were previously published with the chicken anti-MeCP2 antibody (Gao et al, 2015), I was unable to successfully detect Mecp2 through western blotting.

I tried other protein isolation techniques, such as sonication and trituration, in order to see if this would enhance Mecp2 detection. Unfortunately, these methods did not provide sufficient amount of protein for Mecp2 detection. I then used a modified Nuclear Fractionation protocol to prepare the nuclear and

cytoplasmic extracts from the zebrafish tissue. Homogenization using a high-salt extraction method provided sufficient purification of nuclear proteins that could be detectable through western blot analysis (Baker & Zoghbi, 2013). From this protocol, I used lysed 48hpf embryos and lysed wild type and mutant R255X mouse. Since these mouse samples showed successful Mecp2 detection with this antibody, they served as primary antibody controls. My blot displayed multiple bands in the 48hpf zebrafish samples, including the reported 75kD band. In order to further determine which specific bands correlated with Mecp2, a *mecp2* ^{Q63x} null sample served as the negative control. I obtained a western blot that showed disappearance of band 150kD in the mutant 48hpf fish compared to the wild type (Figure 4). Interestingly, the 75kD band that was reported as Mecp2 was seen in both the wild type and mutant samples.

By successfully detecting Mecp2 with western blotting in zebrafish, this demonstrated the ability to detect functional Mecp2 that could be further applied to pre-clinical drug screenings.

Mecp2 expression and other neuronal markers detected by IHC in zebrafish embryos/larvae

In order to successfully utilize zebrafish to screen for read through compounds, it is essential to identify and quantify the amount of full-length MeCP2 protein in zebrafish. After successful results of Mecp2 detection through western blotting, I sought to use immunofluorescence histochemistry to visualize Mecp2 protein in zebrafish embryos.

As previously reported, Mecp2 expression has been found in hindbrain, midbrain, and forebrain in whole mount staining of 48hpf zebrafish (Gao et al, 2015). I attempted to recapitulate detection of Mecp2 protein found in whole-mounted 48hpf embryos by using the commercially available chicken anti-MeCP2 polyclonal antibody (ABE171) reported with the western blot analysis. There appeared to be no nuclear signal using a Donkey anti-chicken Alexa Fluor 594 secondary antibody. The sample displayed excessive autofluorescence within this spectrum, which raised the question if this was due to fixation duration.

To address the potential problem of fixation-duration induction of autofluorescence, small batches of embryos were fixed under different time points (30 minutes, 45 minutes, 60 minutes, and 3 hours) and were processed identically. The results demonstrated a decrease in autofluourescence with decreased fixation times. However, no clear nuclear expression of Mecp2 was observed with this antibody. I then sought to see if the ability to visual Mecp2 would be improved by sectioning the 48hpf embryos. Cryosectioned heads of 48hpf were fixed in short intervals (30 minutes, 60 minutes) before sectioning, or with on slide fixation. All sections were probed for Mecp2 and DAPI (fluorescent marker that binds to A-T rich regions in DNA). The on-slide fixation produced better resolution versus the short fixed slides, but still did not detect Mecp2 (Figure 5).

Based on these results, I then addressed the issue of various fixative methods that were potentially inhibiting detection of this antibody. Since

paraformaldehyde can potentially obstruct antibody binding, I tried using methanol, acetone, or a combination of both. Each type of fixation solution made the embryos soft and difficult to keep intact, therefore indicating that paraformaldehyde was the best fixative method to easily manipulate the embryos for staining. I also tested the secondary antibodies that emit fluorescence in different wavelengths (Alexa Fluor 488 and Alexa Fluor 594) to control for possible issues with tissue autofluorescence. I observed no apparent difference in autofluorescence in either channel using this fixative; therefore, I decided to test different protocols for processing the embryos after fixation

One protocol in particular (adapted from Adam McPherson) that I used required more detergents to be used in the blocking solution (2% Triton-X, 1% DMSO) as well an extra permeabilization step using 2% PBS +Triton-X with 0.5U dispase. This protocol showed better resolution and signal for other antibodies that were probed (HuC/D, GFAP). However, no Mecp2 nuclear signal was detected.

In order to test if high background was due to non-specific binding of the secondary antibody, embryos with the 30-minute fixation were stained with and without primary antibody. There appeared to be high background detected in the batch of embryos treated with the primary antibody. Therefore, these results suggested that the observed background was due to the anti-MeCP2 chicken polyclonal antibody and was not suitable to use for zebrafish experiments

Next, I attempted to try several other antibodies with epitopes relatively conserved in zebrafish Mecp2. I tested Cell Signaling 3456S, Genetex 22829, Genetex 13607, ProSci XW-7368, and Genetex 113230 antibodies with stock concentrations of 1mg/mL (Figure 6). After testing each antibody with the same dilution factor (1:200) and processing methods, I found that the Genetex 113230 antibody displayed what looked to be nuclear signal and co-registration of DAPI (figure 7). These results suggested that only the Genetex 113230 correctly identified nuclear Mecp2 signal in zebrafish. In order to further validate that this signal was truly Mecp2, I stained with a *mecp2* null 48hpf embryo (*mecp2* ^{Q63x}) under the same conditions to use as a negative control (Figure 7). The comparison of both wild type and mutant stained embryos demonstrated a similarity in what was thought as Mecp2 signal, with co-localization of DAPI present. Therefore, this suggested that this antibody was not suitable for zebrafish Mecp2 detection through immunostaining.

In a previous study, Mecp2 expression was found by immunostaining with a custom made antibody with zebrafish specificity (Gao et al, 2015). After receiving an aliquot of this antibody, courtesy of Dr. Keping, I tested the antibody with the same conditions as previously done with the Genetex 113230 antibody. In the wild-type embryos, there appeared to be no nuclear signal, but more so cytoplasmic architecture with evident gaps where DAPI was localized. I then tried dissecting the zebrafish brain at 6dpf to see if perhaps insufficient

permeabilization was preventing the antibody from diffusing into the brain. This approach also did not result in successful Mecp2 detection.

In one section of the zebrafish, some of the yolk sac that was still attached to the embryo appeared to have some puncta that resembled Mecp2 signal. When observed with DAPI, there appeared to be some co-localization in the yolk sac, but with no apparent signal in the brain. This led to performing a heated sodium citrate antigen retrieval step with whole mount 48hpf fish to further investigate if the fixative process was inhibiting the detection of Mecp2. Unfortunately, no Mecp2 was found based on the extracellular structure found in the Alexa Fluor 647 far-red fluorophore channel (Figure 8), which also showed no co-localization with DAPI. I then proceeded to section the zebrafish heads with the same antigen retrieval protocol in order to investigate if the fixative was inhibiting Mecp2 detection. After antigen retrieval and cryosectioning of 6dpf zebrafish head, I was able to detect Mecp2 signal that co-localized with DAPI (Figure 9). This was expressed predominantly in the brain region, consistent with previous reporting. Therefore, whole mount staining using antigen retrieval was unsuccessful in obtaining nuclear MeCP2 signal in 48hpf zebrafish, but was successful in 6dpf zebrafish sections.

In addition to working towards the histological detection of Mecp2, I also investigated the difference in HuC, a neuronal cell marker, and GFAP, a radial glial marker. Previous reports have shown a significant decrease in *huc* in a CRISPR *mecp2* knock-down, as well as an increase in *gfap* signal (Gao et al,

2015). However, this phenotype has not been explored in the *mecp2*^{Q63x} fish. I attempted to stain both the wild type and *mecp2*^{Q63x} embryos at 48hpf to investigate if findings were comparable to previous reports.

Shown in Figure 10, it appeared that there was an observable increase in *gfap* staining in the spinal cord and hindbrain region in the *mecp2*^{Q63x*} fish compared to the wild type. However, the *huc* staining displayed similar expression in both the wild type and mutant embryos, suggesting that more optimization with the antibody is needed (Figure 8). Therefore, the results of the *gfap* signal were comparable to the in-situ data detected in CRISPR *mecp2* knockdown model.

mecp2^{Q63x} 6dpf fish show locomotor abnormalities.

The *mecp2*^{Q63x} mutant 6dpf fish previously published produced significant differences in terms of behavioral assays measuring spontaneous swimming activity, distance travelled, and thigmotaxis (Pietri et al, 2015). In order to reproduce these phenotypes, I recorded both wild type and *mecp2*^{Q63x} 6dpf larvae for 15 minutes in a muti-well plate using LSRtrack with custom Matlab scripts for computational analysis.

After recording with 51 wild type fish and 79 *mecp2*^{Q63x} mutant fish, it was shown that the movement traces of the *mecp2*^{Q63x} fish had increased movement compared to the wild type (figure 11). The wild type fish tended to spend the majority of the swimming paradigm in the periphery of the arena. The *mecp2*^{Q63x} fish showed a significant difference in movement path, with observed trajectories

more localized in the center of the wells. Density maps of both the wild type and mutant fish also demonstrated location preference of the wild type around the periphery of the arena compared to the mutants.

From previous findings, there was a reported difference in average velocity between 6dpf wild type and *mecp2*^{Q63x} larvae. I measured the total distance travelled as a comparison to the average velocity previously reported. Total distance travelled was measured as the total distance a fish moves within the well of interest between frames of the video. My results showed a difference of distance travelled ($p=0.016$) between the wild type and *mecp2*^{Q63x} larvae.

The percent of time active between the wild type and mutants was also reported to be significantly lower in the *mecp2*^{Q63x} fish versus the wild type (Gao et al, 2015). I also observed a difference of percent of time active ($p=0.0033$) between both wild type and *mecp2*^{Q63x}.

Based on these findings, I was able to successfully re-capitulate previous behavioral assays used for *mecp2* detection. These behavioral assays provide the ability to see functional phenotypes of Mecp2 that can be established for pre-clinical drug screenings.

III:

Discussion

In this work, I have recapitulated previous findings of molecular and behavioral abnormalities in *mecp2* mutant zebrafish, provided viable assays that allow for detection of full-length Mecp2 expression in zebrafish embryos and generated a novel *mecp2* deficient zebrafish line.

Development of novel *mecp2* null zebrafish through CRISPR/Cas9 mutagenesis

In order to develop zebrafish based assays for screening readthrough compounds for efficacy against premature termination codons (PTC) in the *mecp2* locus, it is necessary to generate zebrafish lines with nonsense mutations in *mecp2*. The *mecp2*^{Q63*} zebrafish used in these preliminary assays are one line that might be amenable to testing nonsense suppressing therapeutics; however, this model is not ideal due to this particular mutation being unrepresentative of the commonly occurring truncating mutations in the RTT population. Efficacy of nonsense suppression therapy has been reported to depend on the genetic context of pre-termination codons (Karijolich & Yu, 2014), therefore another zebrafish model with the mutations analogous to the patient population (R255X, R270X, R294X, and R168X) that are knocked into the endogenous *mecp2* locus would be ideal. CRISPR/Cas9 mediated engineering provides a highly efficient method to conduct this mutagenesis. Previous studies have shown that germline transmission of other genetically engineering methods, such as ZFN and TALEN, have ~2% somatic mutations in normally developed zebrafish embryos. (Hwang

et al, 2013). With the CRISPR/Cas9 system, these induced somatic mutation rates are at least 10% detected in embryos; therefore, this provides more efficient than other genetic editing methodologies (Wei et al, 2015).

To demonstrate that the zebrafish *mecp2* locus is amenable to CRISPR targeting, I designed gRNAs targeting the first and last exons of the zebrafish *mecp2* locus. When co-injected, the gRNAs direct Cas9 to create double stranded breaks near the beginning and end of the *mecp2* coding region. Upon repair of these double stranded breaks by non-homologous end joining. The beginning of exon 1 is fused to the terminus of exon 3 resulting in excision of 20 kb of the *mecp2* locus. I have demonstrated by PCR based genotyping and Sanger sequencing that this 20 kb deletion has successfully been introduced into zebrafish embryos by creating a fusion product of 691bp, with the major functional domains of *mecp2* eliminated. Additional embryos from these initial targeting experiments have matured to 3 months age, and will be used to establish a *mecp2* knockout zebrafish line.

In addition to providing evidence that our lab can successfully target the *mecp2* locus in zebrafish through CRISPR/Cas9 engineering, these knockout animals will provide a valuable tool for further investigation of molecular, cellular, and behavior abnormalities of *mecp2* dysfunction in zebrafish. One application of this *mecp2* knockout would be to mate it to existing transgenic zebrafish lines with fluorescent reporters, such as glial fibrillary acidic protein (GFAP), that would allow for expression of green fluorescent protein (GFP) in glial cells driven by GFAP in zebrafish (Bernardos & Raymond,2004). This would allow for in-vivo

characterization of molecular abnormalities we, and others, have identified in *mecp2* null fish. Additionally, this could provide a second, high-throughput, readout for *mecp2* functional restoration in drug screenings.

Creating this novel *mecp2* null zebrafish line is also significant in terms of creating a true *mecp2* knockout in zebrafish with a considerable deletion that has not yet been reported.

Re-capitulation and expression of *mecp2* and other neuronal markers in mutant and wild type zebrafish

As previously reported (Gao et al), the termination of primary neurogenesis occurs by ~48hpf; therefore expression of Mecp2 can be visualized from 48hpf to adult stages in zebrafish. At this stage, neurons and glia are differentiated and can be characterized by different neuronal markers.

HuC, a neuronal marker that labels nuclei of neuronal precursors, has shown high expression within the zebrafish CNS at 48hf. This marker was found to be downregulated in previous studies where *mecp2* was knocked down using morpholinos. This finding indicates that *mecp2* promotes neuronal fate. Through my experimentation, the expression of *huc* in both wild type and *mecp2*^{Q63x} zebrafish was seen through immunofluorescent staining; however, the results were inconclusive in differentiating expression between both wild type and mutant. Therefore, further investigation in optimizing *huc* staining or using other molecular methods may be needed to quantify the amount of *huc* detected to verify this reported phenotype (Gao et al, 2015).

Glial fibrillary acidic protein (GFAP), a marker for radial glia and astrocytes, is also reportedly expressed within the midbrain, forebrain, and hindbrain of 48hpf zebrafish. Conversely to the downregulation of *huc* in *mecp2* morphants, loss of *mecp2* results in upregulation of *gfap* expression (Gao et al, 2015). This phenotype was also expressed in the CRISPR engineered fish validating this to be a consistent phenotype found in fish with disrupted *mecp2*. Here, I demonstrated a similar misregulation of *gfap* expression in the forebrain, midbrain, and hindbrain *of mecp2*^{Q63x}. This also was observed in the spinal cord region similar to the reported decrease observed using *in situ* (Gao et al, 2015). Overall, these results suggest that the influence of *mecp2* down-regulation in neuronal fate causes an upregulation of astrogenesis.

This drastic phenotypic difference observed in *gfap* signal also raises the possibility of applying this marker either transgenically or through immunohistochemistry in the CRISPR engineered fish. This could be a fast and easy approach to verify downregulation of *mecp2* expression that can be incorporated as part of the zebrafish platform for high throughput drug screenings.

As reported by Gao *et al.*, *mecp2* is expression enriched in the CNS starting at 48hpf in zebrafish. Following extensive optimization of various immunohistochemistry protocols and testing multiple antibodies, I was able to successfully visualize Mecp2 in zebrafish via immunofluorescence. A crucial factor for detecting Mecp2 using the antibody reported by Gao et al. is implementation of heated sodium citrate antigen retrieval. Antigen retrieval

disrupts cross-links formed by formalin fixation that can potentially mask epitopes in a protein of interest. According to Song et al. 2014, antigen retrieval is a critical step for robust detection of Mecp2 via immunofluorescence in rodent tissue using numerous commercially available Mecp2 antibodies. Given these results, it is possible that other Mecp2 antibodies that were tested without an antigen retrieval step may, in fact, recognize zebrafish Mecp2 with incorporating this step in the staining protocol. Another approach that could be implemented to obtain Mecp2 detection is through an antibody cleanup, where commercial antibodies are pre-absorbed in solution lacking the antigen of interest. This method can be used as another technique for decreasing non-specificity of the antibodies of interest. It can also serve as a negative staining protocol to visualize the absence of the antigen when performing immunofluorescent histochemistry. However, these methods will need to be experimentally determined.

Mecp2 expression validated and characterized through Western blot analysis

Pietri et al reported a 75kD western blot band for Mecp2 that is not present in *the mecp2*^{Q63x} samples. Using the same commercially available chicken anti-MeCP2 polyclonal antibody as Pietri et al, I did not detect a differentially expressed 75kD product between the wild type and *mecp2* ^{Q63x} fish. Instead, I discovered a distinct band at 150kD in the wild type that was not present in the mutant samples.

Beyond the paper mentioned, the assessment of Mecp2 protein expression in zebrafish has not been thoroughly characterized. It has been thought that molecular discrepancies may be indicated by the presence of post-translational modification in MeCP2 (Nagai & Miyake, 2007). For example, the amino acid sequence of MeCP2 is theoretically calculated at 53kDa, but has shown to be 75kDa in mice and 100 kDa in human cerebral cortex (Nagai & Miyake, 2007). Therefore, posttranslational modifications, such as phosphorylation, ubiquitination, and sumolyation, may contribute to the transcriptional activities of MeCP2 that produce these various protein products.

Further investigation validating these findings of the true protein size of Mecp2 is needed. These results could be interesting to compare to the CRISPR engineered fish that were designed to determine which bands are consistently absent versus the wild type. The ability to detect Mecp2 through western blot analysis can be a useful quantitative means for assessing full length Mecp2 protein production that is an important factor for determining readthrough compound efficacy.

Behavioral difference between wild type and *mecp2* mutant fish demonstrate impaired locomotion and anxiety-like behavior.

Behavioral assays are another important method in characterizing *MECP2* in RTT models. It has been shown that locomotion was decreased in *mecp2*^{Q63x} 6dpf fish versus wildtype by measuring spontaneous swimming activity. A decrease in thigmotaxis, or anxiety-like behavior, has also been shown in these mutant fish compared to the wild type. By using an LSRtrack with an open-

source Matlab script, I was able to obtain the same measurements in 6dpf *mecp2*^{Q63x} fish with accurate motion detection of positional preference, displacement, and frequency of movement and rest periods (Zhou et al, 2014).

From my experimental analysis, I observed decreased total distance travelled and percentage of swimming activity in the *mecp2*^{Q63x} compared to wild type fish. This suggests a dysfunction in the motor activity and locomotion in the *mecp2* mutant fish (Pietri et al 2015).

The time spent swimming on the edges of the well versus inside also significant differed between wild type and *mecp2*^{Q63x} fish. As previously reported, the mutants spent less time in the periphery of the arena versus wild type. This observation could suggest a decrease in anxiety-like behavior (Pietri et al, 2015). Evidence has shown that anxiety-like behaviors found in zebrafish are similar to environmental factors that are found in rodents (Champagne et al, 2010). This also can involve evolutionary circuits conserved in zebrafish that are responsible for aversive learning and emotion. (Jesuthasan, 2011; Lee et al, 2010). The observed *mecp2*^{Q63x} phenotype of increased distance covered within the inner portion of the arena implies a decrease in anxiety-like behavior (Pietri et al, 2015). This result proves to be a striking difference between what has been seen in RTT mouse models, as well as in the human RTT population. This raises the question as to what other mechanisms might be disrupted by loss of *mecp2* that result in this observed phenotype.

The dopaminergic system of zebrafish also plays a vital role in the motor and anxiety-like related behaviors (Pietri et al 2015). This could suggest that the

loss of *mecp2* causes a distortion of motor development in the fish that causes this decrease in locomotion in the mutant fish. This could be due to dysfunction in the reward circuitry pathway of the dopaminergic system that causes a loss of interest in exploration in the mutant fish. The decrease in locomotion could be due to reduced interest in exploring the environment, as well as increased thigmotaxis contributing to avoiding aversive stimuli by touching the edges of the well.

Previous reports have also shown an increased in hypersensitivity and arousal in autistic patients in response to sensory inputs (Belmonte et al, 2004). There is a possibility that this observed reduced anxiety in the *mecp2*^{Q63x} fish by decreased time spent along the periphery of the arena could actually be an artifact of an anxiety phenotype of avoiding tactile stimulation from the edges of the well (Pietri et al, 2015). Therefore, the interpretation of this phenotype may need further investigation to characterize anxiety in *mecp2* mutants.

Further analysis of other neuromodulatory systems are required in order to distinguish the phenotypes observed in the mutant fish as being truly congruent with the mutation itself versus other underlying factors. With an established behavioral assay that has been optimized and reproduced for testing a single phenotype, this can provide as an important model for characterizing deficits caused by *mecp2*, as well as incorporated in high throughput assays.

By using the LSRtrack, this allows for multiplexed behavioral analysis of zebrafish in a scalable format that is amenable for high throughput preclinical therapeutic screening. Through this system, various measurements involving

evident deficits in motor activity and anxiety-like behaviors could be used to assess functional rescue and drug efficacy when testing *mecp2* targeted therapeutics in zebrafish.

In order to establish zebrafish as an intermediate drug screening platform, this requires the ability to generate a nonsense mutant *mecp2* model to screen for nonsense suppression therapeutics. By engineering a CRISPR *mecp2* knockout zebrafish model, this demonstrates the potential to further introduce patient derived mutations that can be used in pre-clinical drug screenings. Another component to this platform is the ability to detect Mecp2 expression. I have shown successful detection of Mecp2 through immunohistochemistry and western blotting that provide valuable assays to be incorporated for high throughput drug screenings. Finally, the ability to detect the functional rescue of mecp2 is essential for testing the efficacy of these pre-clinical therapeutics. In my work, I established behavioral assays that showed significant phenotypic differences in a *mecp2* null versus wild type. This demonstrate the functional implications of *mecp2* that provide essential assays for testing nonsense suppression therapeutics in engineered *mecp2* mutant zebrafish. Therefore, these preliminary assays I have established can serve as a foundation for engineering patient specific zebrafish models for screening pre-clinical *mecp2* therapeutics.

IV.

Materials and Methods

Fish Maintenance

Wild-type AB, *mecp2*^{Q63x}, and CRISPR engineered zebrafish were raised and handled in accordance with the guidelines of the University of California, San Diego Animal Care and Use Committee, accredited by AAALAC.

Generation of CRISPR knockout zebrafish: Cas9/gRNA synthesis and microinjection

Zebrafish codon-optimized *Cas9* mRNA was *in vitro* transcribed from linearized pCS2-Cas9 (a gift from Alex Schier, Addgene plasmid #47322) via an mMessage Machine kit (Ambion) according to the manufacturer's protocol. Guide RNAs (gRNA) targeting exon 1 and exon 3 of the zebrafish *mecp2* locus (Table 1) were identified using Chop-Chop (https://chopchop.rc.fas.harvard.edu). DNA templates for the sgRNAs were generated through PCR amplification of the chimeric tracrRNA encoded in pX330-U6-Chimeric_BB-CBh-hSpCas9 (a gift from Feng Zhang, Addgene plasmid # 42230). Forward primers to the chimeric tracrRNA included a 5' tail encoding a minimal T7 promoter, the Chop-Chop identified guide. PCR amplification using these forward primers, a common reverse primer to the 3' region of the tracrRNA, and the pX330 template results in efficient formation of the expected ~120 bp sgRNA template product (Table 1).

sgRNAs for injection were *in vitro* transcribed from these templates using the MEGAshortscript Kit (Ambion) according to the manufacturer's instructions (Table 2). *Cas9* mRNA (200ng/ul) and *mecp2* targeting sgRNAs (50ng/ul) were injected into one-cell embryos. In all microinjection experiments, a volume of 1.5 nl was injected into one to two-cell stage embryos.

Western blot

Wild type AB strain zebrafish at 48hpf were lysed using a modified Nuclear Fractionation Protocol. Two buffers, Buffer A and Buffer B, were filtered and stored with pH 7.9 at 4°C. Buffer A consists of HEPES, 100mM MgCl2, 1M KCl, NP40, ddH20, DTT, and Sigma Protease Inhibitor Cocktail. Buffer B consists of HEPES, 100mM MgCl2, EDTA (pH 8), Glycerol, ddH20, DTT, NaCl, and Sigma Protease Inhibitor. All steps are performed on ice with dounce homogenizer using at least 50mg of embryo tissue (approximately 100 embryos) that are resuspended in 1x PBS, dried, and weighed. Samples were boiled in buffer, separated on an SDS-PAGE gel, and transferred to nitrocellulose membrane. The blot was probed with Millipore chicken anti-MeCP2 polyclonal antibody (ABE171) at a dilution of 0.5ug/ml and visualized with a goat anti-chicken IgY antibody at a dilution of 0.2ug/ml.

Immunostaining

Zebrafish embryos 48hpf were processed and stains using the following antibodies: rabbit anti-MeCP2 (courtesy of Gao et al, 2015), HuC (Thermofisher Scientific), and GFAP (Abcam ZRF-1). Emrbyos were fixed in 4%

Paraformaldehyde overnight at 4°C. Fixative consisted of 4% PFA, NaOH, ddH20, and 10X PBS. Embryos were washed with 0.5% PBS with Triton-X and permeablized with 0.5U dispase. The primary antibody was diluted 1:200 for anti-MeCP2, 1:500 for HuC/D, and 1:500 for GFAP in fresh aliquot of blocking solution. DAPI was also added at 0.1ulg/ml concentration to blocking solution (5% PBS, 2% PBS+ Triton-X, 20% Triton-X, 5% Donkey Serum). Embryos were incubated in primary antibody for 3 days at 4°C on a rocker, and washed for 3-5 hours with 0.5% PBSTx after at 4°C on rocker. Secondary antibody was diluted 1:500 in fresh aliquot of blocking buffer, and was placed on rocker at 4°C for 1-2 days. Embryos were washed for 3-5 hours with 0.5% PBS+ Triton-X at 4°C and mounted with Prolong Mounting Solution without DAPI mounting media and sealed with nail polish. Slides were stored at -20°C for further use. Images were taken on inverted and confocal microscopes with 10X, 20X, and 63X magnification. Z-stacks were taken using both confocal and inverted microscope for multi-dimensional analysis of any signal detection present.

Behavioral Assay

Wild type and *mecp2*^{Q63x} zebrafish were habituated in a 12hr light/dark cycle from 24hpf to 6dpf. The 6dpf fish were habituated at room temperature 20 minutes before testing. Larvae were randomly placed in a black wall 96 well plate with 500ul of embryo media at room temperature. Low backlighting was applied above of the multi-well plate to aid motion tracking and to reduce impact of ambient light changes on zebrafish movement. Fish were recorded with a high-

resolution CCD camera mounted beneath the multi-well plate. Motion tracking was performed post video capture with LSRtrack, an open-source zebrafish tracking Matlab application (Zhou et al, 2014).

Percent activity was measured by comparing total number of video frames where activity occurred with the total number of frames. Raw distance per frame taken from LSRtrack was converted to binary measures that were summed for all the video frames, and then divided by total number of frames to obtain percent of activity.

Two rounds of 15-minute free-swimming intervals were conducted before computational analysis to ensure there was movement in each well. Wells were subtracted within this preliminary round if movement was calculated below 2 standard deviations. Wells were also subtracted if poor centroiding was detected. Five 15-minute intervals of both wild type and Q63x 6dpf larvae were analyzed.

V.

Appendix

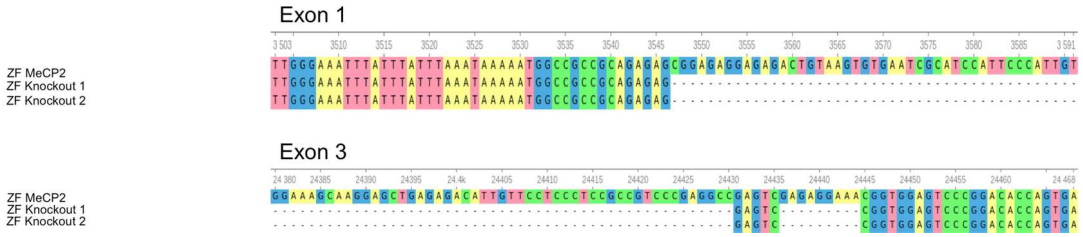

Figure 1: Sequence analysis to verify *mecp2* deletion

Graphic representation of the sequence analysis of CRISPR/Cas9 injected embryos using forward primers annealing upstream of Exon 1 (first block) targeting the guide RNA. The bottom block presents Exon 3 with a reverse primer annealing downstream the targeting guide RNA. The first row of each exon represents the wild type *mecp2* sequence for comparison. The columns below represent sequences from two isolated injected embryos with exon 1 forward and exon 3 reverse primers.

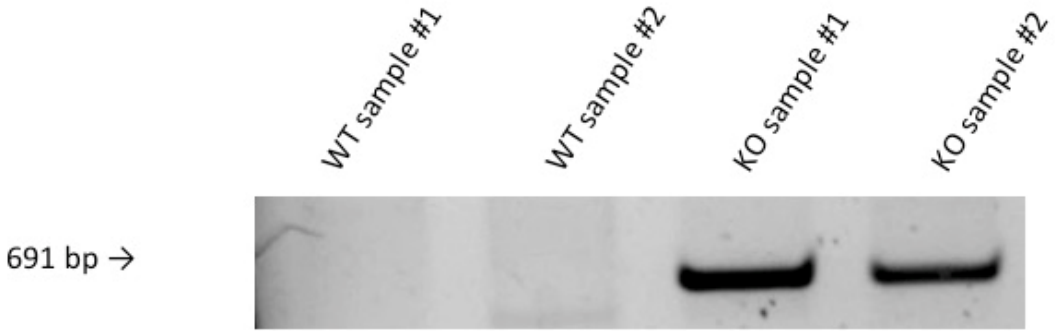

Figure 2: Verification of *mecp2* 20kb excision by genotyping analysis
In order to verify a complete excision of *mecp2* in the CRISPR engineered fish, individual injected embryos were collected at 48hpf and lysed for PCR amplification. The left two lanes contain 1ul of lysed DNA from wild type (WT) to serve as controls. The right two lanes contain 1ul of two different knockout (KO) embryo samples that were injected with the constructed exon 1 and exon 3 gRNA. Exon 1 forward primer (158bp) and exon 3 reverse primer (533bp) were used in the PCR reactions to produce a predicted fusion of 691 bp for successful CRISPR targeting. The expected product in the knockout fish was 691bp, which was found in both samples and not in the wild type.

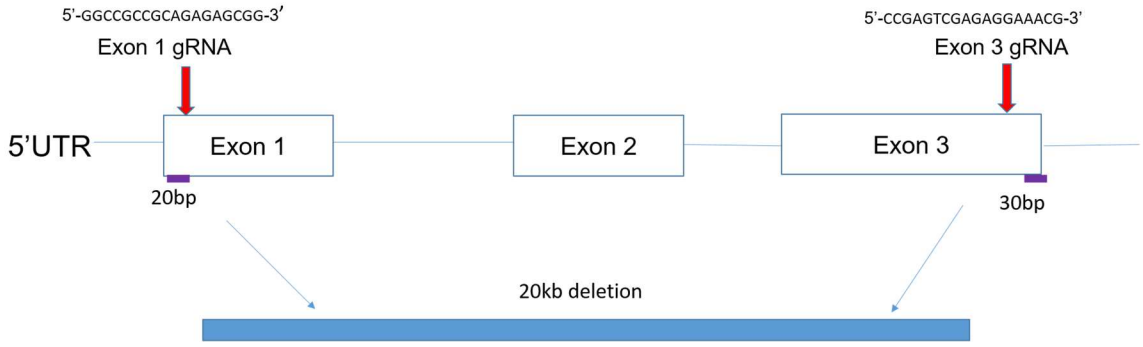

Figure 3. Schematic representation of Exon 1 and Exon 3 gRNA location of mecp2 locus

To create a 20kb excision of the *mecp2* locus, exon 1 gRNA (5'-GGCCGCCGCAGAGAGCGG-3') was targeted 20bp from the beginning of exon 1. The gRNA targeting exon 3 (5'-CCGAGTCGAGAGGAAACG-3') was targeted 30bp away from the end of the exon. This produced a cleavage product of 20kb the excised *mecp2* locus.

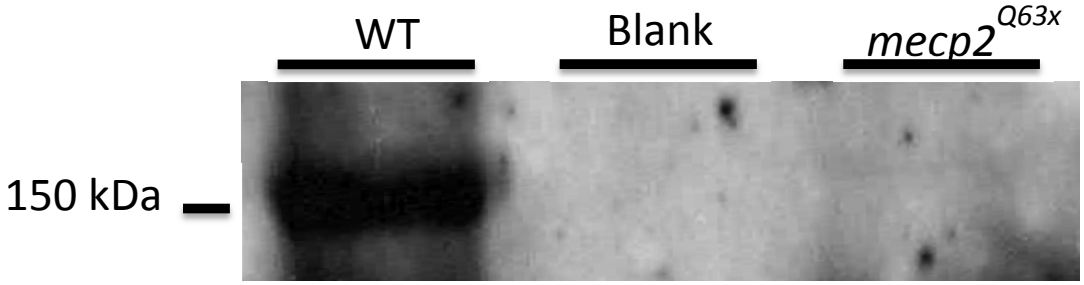

Figure 4: Western blot analysis of wild type and *mecp2* ^{Q63X}
Wild type and *mecp2*^{Q63x} 48hpf zebrafish were lysed and loaded in each lane with 20ug of protein. A major band around 150kD was detected in the wild type lane but not in the *mecp2*^{Q63x} zebrafish lane.

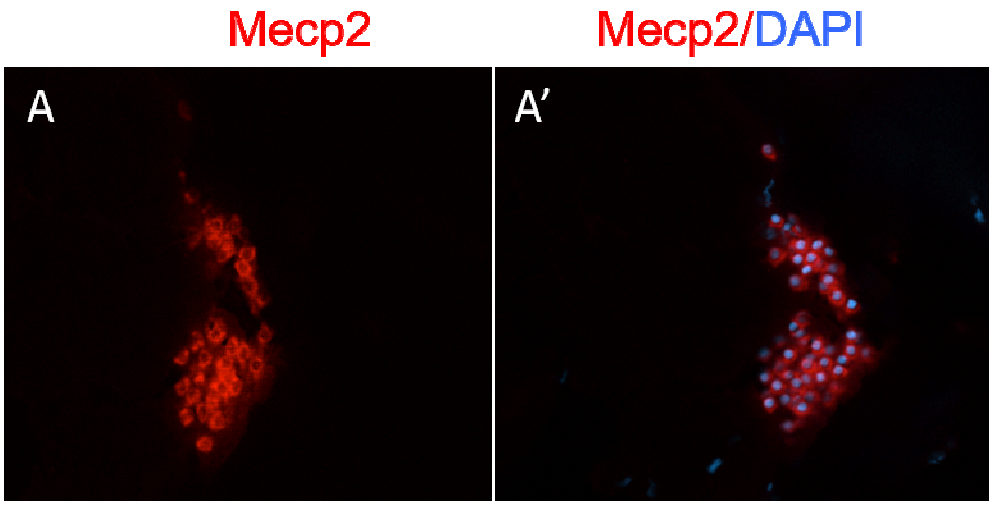

Figure 5: Anti-*MeCP2* chicken antibody staining in 48hpf sectioned zebrafish

(A-A') Coronally sectioned brain of the 48hpf fish probing for *mecp2*. The red signal (A) shows perinuclear staining inconsistent with expected *mecp2* localization, and is inconsistent with DAPI (A') co-localization.

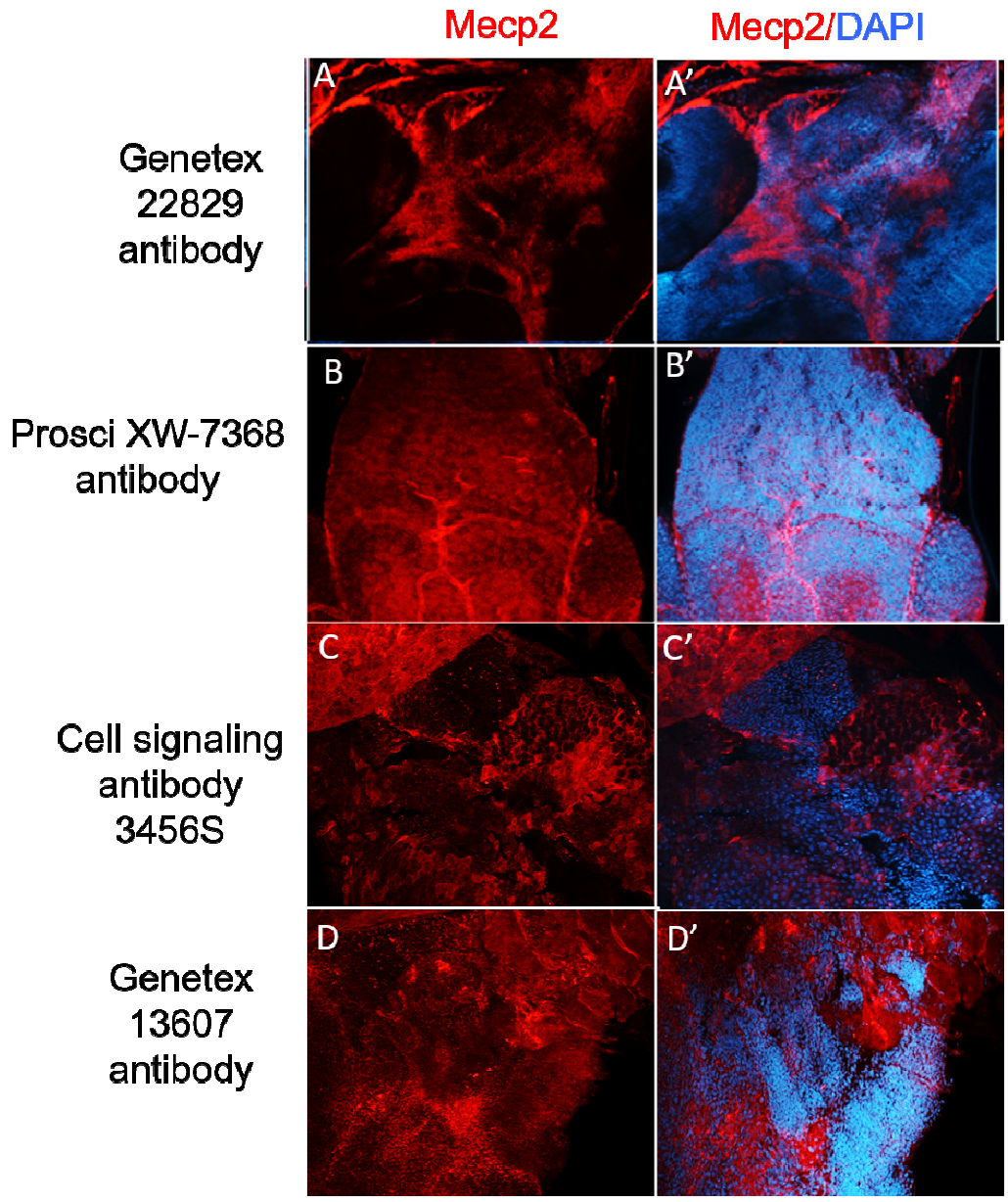

Figure 6: Primary antibody panel testing in whole mount 48hpf zebrafish produces unsuccessful Mecp2 signal.

(A-D') Whole mount immunostaining of 48hpf embryos tested with various antibodies with epitopes conserved in zebrafish *mecp2*. I did not see any nuclear signal (A,B,C,D) or DAPI colocalization (A',B',C',D') using Genetex 22829 (1:200 dilution, A, A'), Prosci XW-7368 (1:200, B,B') Cell Signaling (1:200, C, C') or Genetex 13607 (1:200, D, D'). In all cases MeCP2 is RED and DAPI is BLUE.

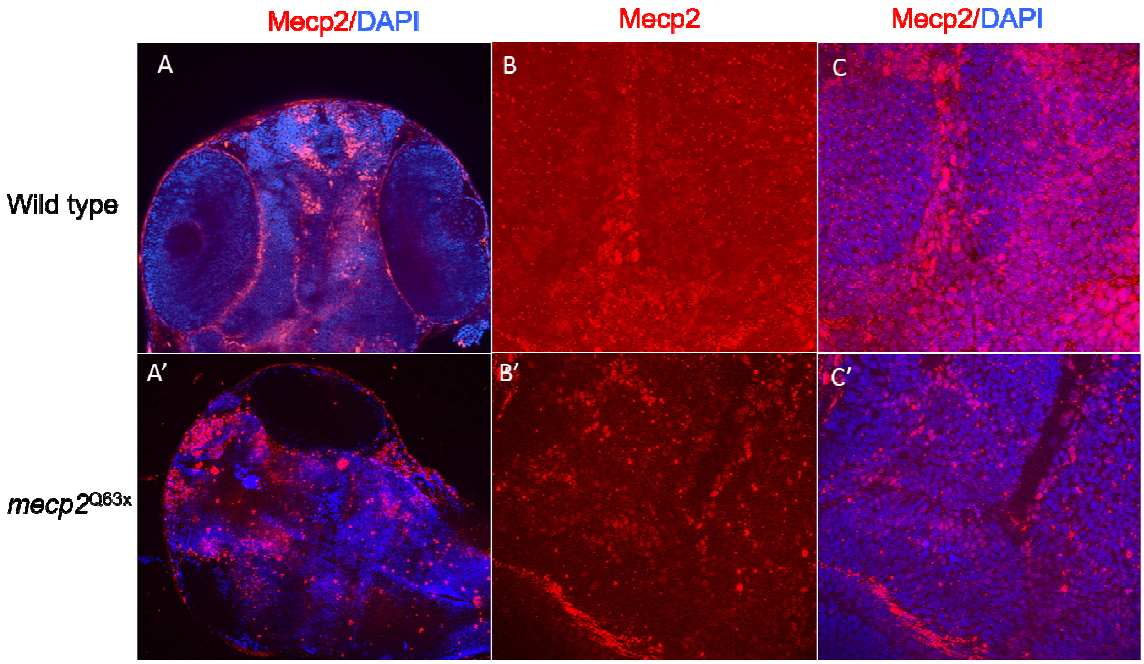

Figure 7: False detection of *mecp2* in whole-mount immunostaining with Genetex 11320 antibody.

(A-C') Whole mount immunotaining of 48hpf wild type and *mecp2*^{Q63x} embryos using Genetex 11320 primary antibody. Wild type embryo displayed several areas of signal that co-localizes with nuclear DAPI in the midbrain and forebrain area with 10X magnification (A) focused in the forebrain of the head. Nonspecific signal was detected in the red fluorophore channel (B) that demonstrated nuclear morphology that was consistent with co-localization of DAPI (C) signal in blue. To determine if this was a true signal, *mecp2* ^{Q63x} fish were stained under the same conditions and re-examined in the same forebrain region (A'). An overview of the head depicts similar morphology and location of red signal (B') found in the wild type. A focused view reveals co-localized signal in the red (C') and blue (B'') channel suggesting that this antibody produced non-specificity with false nuclear signal.

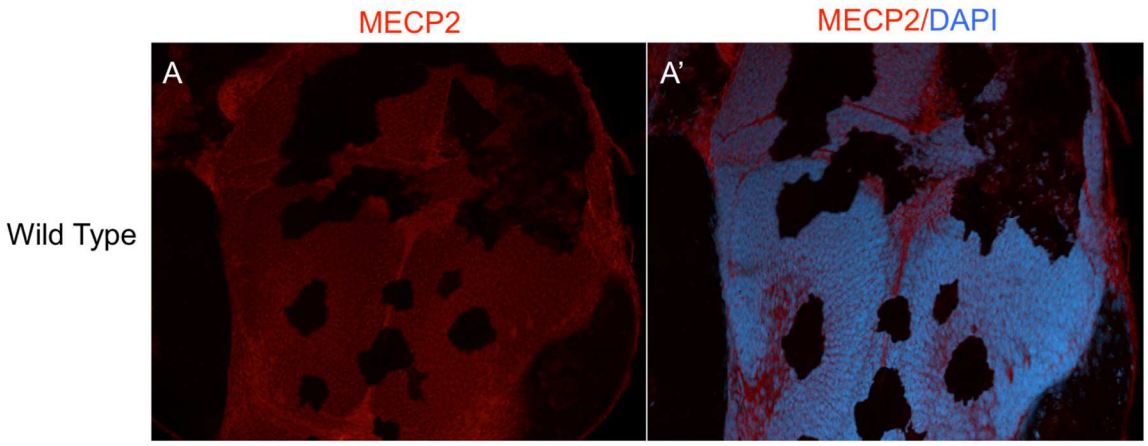

Figure 8:Inability to detect Mecp2 expression in 48hpf whole mount immunostaining.

(A-A')Whole mount 48hpf fish were probed for *mecp2* using rabbit anti-zebrafish MeCP2 polyclonal antibody. A 20X snapshot of the dorsal side of the head was taken. No evident nuclear signal was detected in the red fluorophore channel (A) and showed no co-localization of DAPI (A').

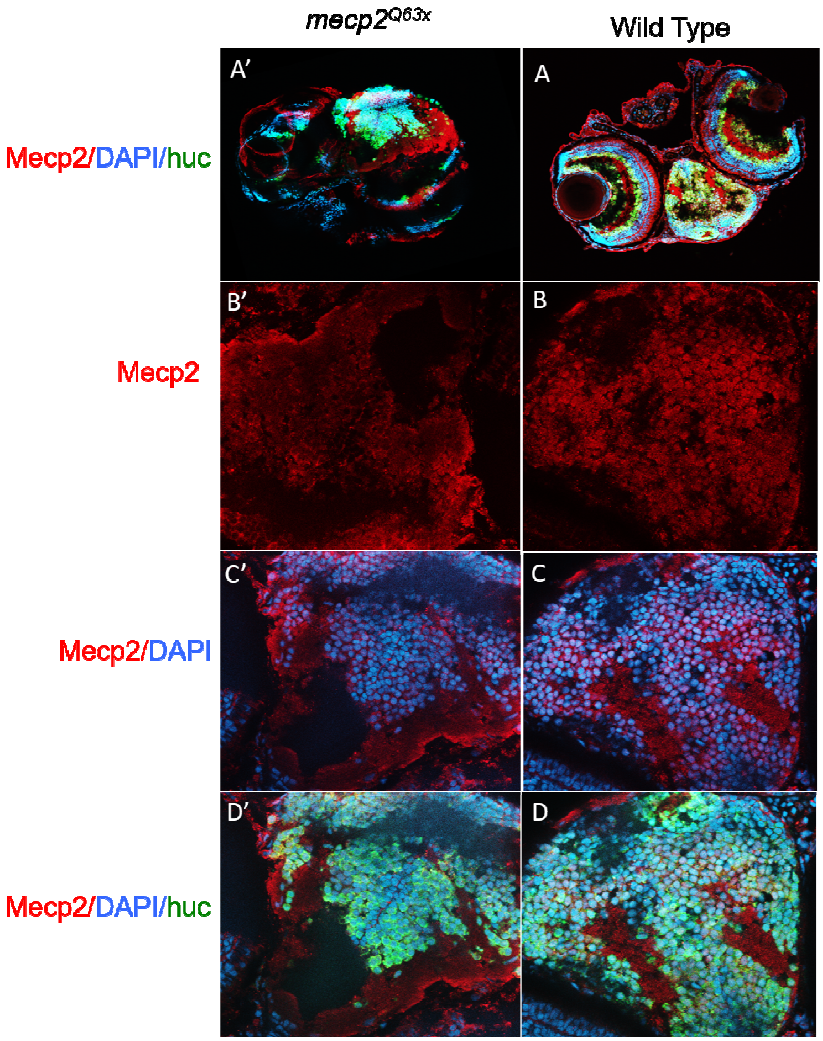

Figure 9: Successful detection of Mecp2 by immunofluorescence using antigen retrieval.

(A-D') Coronally sectioned 6dpf wild type and *mecp2*^{Q63x} heads were subjected to citrate-heat based antigen retrieval and then stained for *mecp2*. In the wild type (A) there appears to be nuclear signal in the brain and in the red channel (B) which was -localized with DAPI (C). HuC staining is diffusely expressed in the regions where DAPI and *mecp2* are co-localized(D). The *mecp2*^{Q63x*} mutants showed no apparent signal in the red channel (A') as well as no co-localization (B') with DAPI. The mutants also show comparable signal of HuC to the wild type (D').

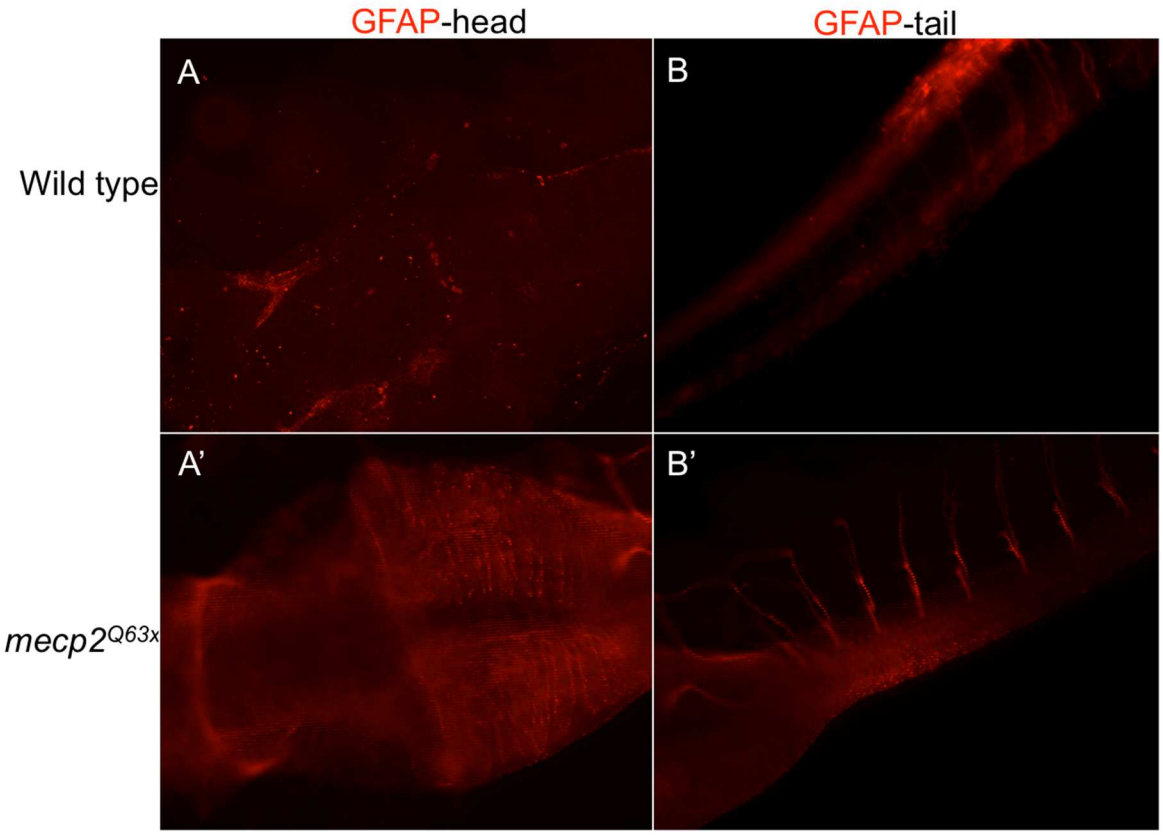

Figure 10: Increased expression of radial glial marker GFAP in *mecp2*^{Q63x} embryos versus wild type

(A-C') Whole mount zebrafish 48hpf wild type and *mecp2*^{Q63x} null embryos were stained with radial glial marker GFAP. Whole mount of the embryo head showed significant increased GFAP signal (A') of the mutant embryos compared to the wild type (A). The tails of the mutant embryos (B') also displayed higher signal of GFAP projections versus the wild type (B).

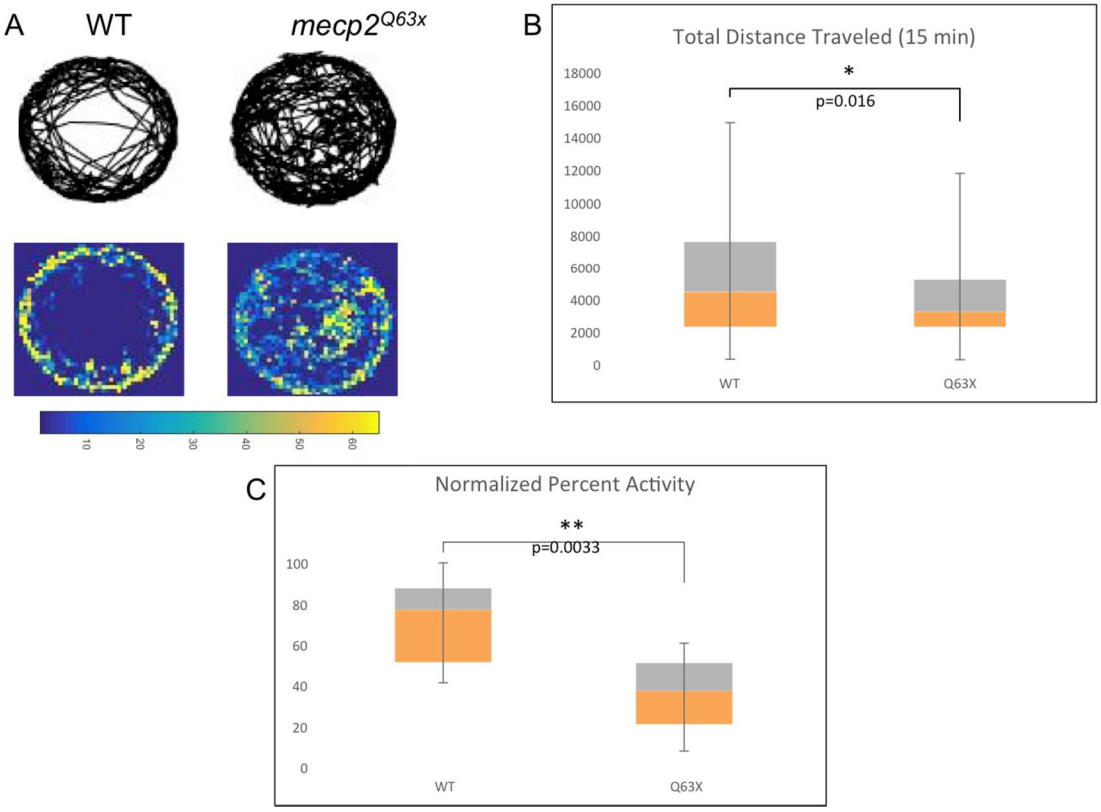

Figure 11: Spontaneous swimming activity shows decreased anxiety-like behavior and movement

(A-C) Behavioral analysis of 6dpf wild type and *mecp2*^{Q63x} mutants were recorded under LCR camera for spontaneous swimming activity in 96 well plates for 15 minutes. The wild type fish preferred to swim on the edges of the well versus the mutant fish that tended to explore the arena with no location preference (A). The total distance (B) was measured to have a *p-value* of the total distance travelled was 0.016 between mutants and wild type. (C) The percent activity was also analyzed to have a *p-value* of 0.0033. The mutants proved to be significantly different in both measurements.

Table 1: Sequences of designed primers for Exon 1 and Exon 3 on *mecp2* locus

Primers	Sequence
Exon 1 Forward primer	5’-ACTACCCTTCAACCCCACCT-3’
Exon 3 Forward primer	5’-TCGAGTAGGGACTCCCAGAA-3’
Exon 3 Reverse primer	5'- AAAAGCACCGACTCGGTGCC -3’

Table 2: Zebrafish Exon 1 and Exon3 gRNA sequences and location on *mecp2* locus

Guide RNAs	Sequence
Exon 1 gRNA	5’-GGCCGCCGCAGAGAGCGG-3
Exon 3 gRNA	5’-CCGAGTCGAGAGGAAACG-3’

REFERENCES

Amir, R. E., Sutton, V. R., & Van den Veyver, I. B. (2005). Newborn screening and prenatal diagnosis for Rett syndrome: implications for therapy. *Journal of Child Neurology*, *20*(9), 779–783.

Amir, R. E., Van den Veyver, I. B., Wan, M., Tran, C. Q., Francke, U., & Zoghbi, H. Y. (1999). Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nature Genetics*, *23*(2), 185–188. http://doi.org/10.1038/13810

Belmonte, M. K., Cook, E. H., Anderson, G. M., Rubenstein, J. L. R., Greenough, W. T., Beckel-Mitchener, A., … Tierney, E. (2004). Autism as a disorder of neural information processing: directions for research and targets for therapy*. *Molecular Psychiatry*, *9*(7), 646–663. http://doi.org/10.1038/sj.mp.4001499

Bernardos, R. L., & Raymond, P. A. (2006). GFAP transgenic zebrafish. *Gene Expression Patterns: GEP*, *6*(8), 1007–1013. http://doi.org/10.1016/j.modgep.2006.04.006

Chang, N., Sun, C., Gao, L., Zhu, D., Xu, X., Zhu, X., … Xi, J. J. (2013). Genome editing with RNA-guided Cas9 nuclease in Zebrafish embryos. *Cell Research*, *23*(4), 465–472. http://doi.org/10.1038/cr.2013.45

Cuddapah, V. A., Pillai, R. B., Shekar, K. V., Lane, J. B., Motil, K. J., Skinner, S. A., … Olsen, M. L. (2014). Methyl-CpG-binding protein 2 (MEPC2) mutation type is associated with disease severity in Rett Syndrome. *Journal of Medical Genetics*, *51*(3), 152–158. http://doi.org/10.1136/jmedgenet-2013-102113

Hagberg, B., Aicardi, J., Dias, K., & Ramos, O. (1983). A progressive syndrome of autism, dementia, ataxia, and loss of purposeful hand use in girls: Rett's syndrome: report of 35 cases. *Annals of Neurology*, *14*(4), 471–479. http://doi.org/10.1002/ana.410140412

Hendrich, B., & Bird, A. (1998). Identification and characterization of a family of mammalian methyl-CpG binding proteins. *Molecular and Cellular Biology*, *18*(11), 6538–6547.

Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Kaini, P., Sander, J. D., … Yeh, J.-R. J. (2013). Heritable and Precise Zebrafish Genome Editing Using a CRISPR-Cas System. *PLOS ONE*, *8*(7), e68708. http://doi.org/10.1371/journal.pone.0068708

KARIJOLICH, J., & YU, Y.-T. (2014b). Therapeutic suppression of premature termination codons: Mechanisms and clinical considerations (Review). *International Journal of Molecular Medicine*, *34*(2), 355–362. http://doi.org/10.3892/ijmm.2014.1809

Katz, D. M., Berger-Sweeney, J. E., Eubanks, J. H., Justice, M. J., Neul, J. L., Pozzo-Miller, L., … Mamounas, L. A. (2012b). Preclinical research in Rett syndrome: setting the foundation for translational success. *Disease Models & Mechanisms*, *5*(6), 733–745. http://doi.org/10.1242/dmm.011007

Kishi, N., & Macklis, J. D. (2004). MECP2 is progressively expressed in post-migratory neurons and is involved in neuronal maturation rather than cell fate decisions. *Molecular and Cellular Neurosciences*, *27*(3), 306–321. http://doi.org/10.1016/j.mcn.2004.07.006

Lin, P., Nicholls, L., Assareh, H., Fang, Z., Amos, T. G., Edwards, R. J., … Voineagu, I. (2016). Transcriptome analysis of human brain tissue identifies reduced expression of complement complex C1Q Genes in Rett syndrome. *BMC Genomics*, *17*. http://doi.org/10.1186/s12864-016-2746-7

Miyake, K., & Nagai, K. (2007). Phosphorylation of methyl-CpG binding protein 2 (MeCP2) regulates the intracellular localization during neuronal cell differentiation. *Neurochemistry International*, *50*(1), 264–270. http://doi.org/10.1016/j.neuint.2006.08.018

Neul, J. L., Kaufmann, W. E., Glaze, D. G., Christodoulou, J., Clarke, A. J., Bahi-Buisson, N., … Percy, A. K. (2010). Rett Syndrome: Revised Diagnostic Criteria and Nomenclature. *Annals of Neurology*, *68*(6), 944–950. http://doi.org/10.1002/ana.22124

Rett, A. (2016). On a remarkable syndrome of cerebral atrophy associated with hyperammonaemia in childhood. *Wiener Medizinische Wochenschrift*, *166*(11–12), 322–324. http://doi.org/10.1007/s10354-016-0492-8

Samaco, R. C., & Neul, J. L. (2011a). Complexities of Rett syndrome and MeCP2. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *31*(22), 7951–7959. http://doi.org/10.1523/JNEUROSCI.0169-11.2011

Skene, P. J., Illingworth, R. S., Webb, S., Kerr, A. R. W., James, K. D., Turner, D. J., … Bird, A. P. (2010). Neuronal MeCP2 is expressed at near histone-octamer levels and globally alters the chromatin state. *Molecular Cell*, *37*(4), 457–468. http://doi.org/10.1016/j.molcel.2010.01.030

Trappe, R., Laccone, F., Cobilanschi, J., Meins, M., Huppke, P., Hanefeld, F., & Engel, W. (2001). MECP2 mutations in sporadic cases of Rett syndrome are almost exclusively of paternal origin. *American Journal of Human Genetics*, *68*(5), 1093–1101. http://doi.org/10.1086/320109

Veeraragavan, S., Wan, Y.-W., Connolly, D. R., Hamilton, S. M., Ward, C. S., Soriano, S., … Samaco, R. C. (2016). Loss of MeCP2 in the rat models regression, impaired sociability and transcriptional deficits of Rett syndrome. *Human Molecular Genetics*. http://doi.org/10.1093/hmg/ddw178

Wei, J., Wagner, S., Lu, D., Maclean, P., Carlson, D. F., Fahrenkrug, S. C., & Laible, G. (2015). Efficient introgression of allelic variants by embryo-mediated editing of the bovine genome. *Scientific Reports*, *5*, 11735. http://doi.org/10.1038/srep11735

Zhou, Y., Cattley, R. T., Cario, C. L., Bai, Q., & Burton, E. A. (2014). Quantification of larval zebrafish motor function in multiwell plates using open-source MATLAB applications. *Nature Protocols*, *9*(7), 1533–1548. http://doi.org/10.1038/nprot.2014.094