LOYOLA UNIVERSITY CHICAGO

FINE-TUNING GENE EXPRESSION:
A NOVEL THERAPEUTIC APPROACH FOR RETT SYNDROME

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
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PROGRAM IN NEUROSCIENCE

BY
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>2’ MOE</td>
<td>2’-O-methoxyethyl</td>
</tr>
<tr>
<td>5hmC</td>
<td>Hydroxymethylated cytosine</td>
</tr>
<tr>
<td>ADAR2</td>
<td>Adenosine deaminase acting on RNA 2</td>
</tr>
<tr>
<td>AGO</td>
<td>Argonaute protein</td>
</tr>
<tr>
<td>ARE</td>
<td>A/U-rich element</td>
</tr>
<tr>
<td>AS</td>
<td>Alternative splicing</td>
</tr>
<tr>
<td>ASO</td>
<td>Antisense oligonucleotides</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor gene/transcript</td>
</tr>
<tr>
<td>CCR4-NOT</td>
<td>Carbon catabolite repression 4–negative on TATA-less</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>ChIP-seq</td>
<td>Chromatin immunoprecipitation followed by sequencing</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-guanine dinucleotide</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP response element binding protein</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>DDX6</td>
<td>DEAD-box helicase 6</td>
</tr>
<tr>
<td>DEM</td>
<td>Differentially expressed microRNA</td>
</tr>
<tr>
<td>DGCR8</td>
<td>DiGeorge syndrome critical region 8</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>H1</td>
<td>Histone H1 protein</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylases</td>
</tr>
<tr>
<td>HIF1α</td>
<td>Hypoxia inducible factor 1 α</td>
</tr>
<tr>
<td>HP1</td>
<td>Heterochromatin protein 1</td>
</tr>
<tr>
<td>ID</td>
<td>Interdomain</td>
</tr>
<tr>
<td>IDP</td>
<td>Intrinsically disordered protein</td>
</tr>
<tr>
<td>IDPR</td>
<td>Intrinsically disordered protein region</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
</tr>
<tr>
<td>IRSF</td>
<td>International Rett Syndrome Foundation</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LCA2</td>
<td>Leber congenital amaurosis 2</td>
</tr>
<tr>
<td>LNA</td>
<td>Locked nucleic acid</td>
</tr>
<tr>
<td>MBD</td>
<td>Methyl-CpG-binding domain</td>
</tr>
</tbody>
</table>
MBD1-6  Methyl-CpG-binding proteins 1-6
mCH    Methylated cytosine dinucleotide, in which H is any non-guanine nucleotide
mCpG   Methylated cytosine-guanine dinucleotide
MDS    MECP2 duplication syndrome
MeCP2  Human MeCP2 protein
MECP2  Human MECP2 gene/transcript
Mecp2  Murine Mecp2 protein
Mecp2  Murine Mecp2 gene/transcript
MEM NEAA Minimum essential medium non-essential amino acids
miRISC microRNA-induced silencing complex
miRNA  microRNA
MRE    microRNA response element
mRNA   Messenger RNA
mSin3A Paired amphipathic helix protein Sin3a
mTOR   Mammalian target of rapamycin
NCoR   Nuclear hormone receptor corepressor
ncRNA  Noncoding RNA
NDD    Neurodevelopmental disorder
NID    NCoR/SMRT interaction domain
NSC    Neural stem cell
nt     Nucleotide
NTD    N-terminal domain
PDE4D  Phosphodiesterase 4D
PEST   Proline, glutamate, serine, threonine enriched
PS     Phosphorothioate
PTM    Post-translational modification
qRT-PCR Quantitative real-time PCR
RBP    RNA-binding protein
RNA    Ribonucleic acid
RNAPII RNA polymerase II
RNA-seq RNA sequencing
RTT    Rett syndrome
sbASO  Steric/site-blocking antisense oligonucleotides
SIRT1  NAD-dependent deacetylase sirtuin-1
SMRT   Silencing mediator for retinoid or thyroid-hormone receptors
TRD    Transcriptional repression domain
TNRC6A Trinucleotide repeat-containing adaptor 6A protein
UTR    Untranslated region
WT     Wildtype
XCI    X-chromosome inactivation
Xi     Inactivated X-chromosome
CHAPTER 1

REVIEW OF LITERATURE

Rett Syndrome

Clinical Presentation

Rett syndrome (RTT) is a neurodevelopmental disorder (NDD) that is characterized by a myriad of debilitating symptoms impacting nearly every aspect of life. RTT is one of the leading causes of intellectual disability in women and affects approximately 1 in 10,000 live births [1]. Clinically, the disorder is characterized by an initial period of normal development until 6 to 18 months, followed by a brief stagnation period, then rapid developmental regression [2-4]. During the regression stage, patients experience a reduction or complete loss in acquired skills such as cognitive, social, and motor functions and begin to exhibit various symptoms including loss of speech, gait impairment, hand stereotypies, gastrointestinal problems, seizures, and breathing abnormalities [2, 4]. RTT patients remain in this developmentally regressed, symptomatic state for decades. The final stage of disease involves late motor deterioration, which is marked by chronic changes such as muscle dystonia and bradykinesia, and premature lethality associated with respiratory insufficiency and/or long QT syndrome [2, 4]. Despite its severity and prevalence, the widespread nature of RTT pathology has limited the development of effective therapeutics, leaving patients and families with little to no relief from this devastating disorder.
Genetic Origin

RTT is caused by X-linked de novo loss-of-function (LOF) mutations in the methyl-CpG binding protein 2 gene (MECP2) [5]. MeCP2 is an epigenetic modulator that is highly expressed in neurons and directs transcriptional events by mediating molecular interactions between methylated DNA or RNA sequences and regulatory binding partners [6-9]. Due to its integral role as a multifunctional hub and high steady-state expression levels in neurons, loss of MeCP2 results in an extensive disruption of gene expression patterns. This pervasive pathology is reflected in the symptomology of RTT which involves nearly every aspect of neuronal function [2].

To date, 863 RTT-pathogenic MECP2 variants have been reported across eight genetic databases [10]. Among these, the eight MECP2 “hotspot” mutations that account for 50 – 65% of all RTT cases (in order of frequency: T158M, R168X, R255X, R270X, R306C, R294X, R133C, R106W) have been well-characterized in both in vitro and in vivo models of the disease [3, 7, 8, 10-13]. Though seminal studies have revealed critical mechanisms underlying RTT pathology, the effort to translate these findings into therapeutic developments has been constrained by the high dosage sensitivity of MECP2, whereby even a modest 1-fold overexpression results in equally deleterious effects (discussed in subsequent sections) [14, 15].

MeCP2 is Critical for Neurodevelopment

The temporal progression of symptoms in RTT mirrors that of MeCP2 levels throughout development. MeCP2 expression begins low in embryonic stem cells (ESCs) and
increases with the initiation of neurogenesis, continuing to do so throughout postnatal neuronal maturation (Figure 1) [16]. Neuronal MeCP2 levels continue to rise until ~10 years of age in humans and remain high throughout adulthood at near-nucleosomal expression levels (~16 million molecules per nucleus), highlighting its role in both neurodevelopment and maintenance of mature neuronal function [9, 16-18].

![Stages of Neurodevelopment](image)

**Figure 1: MeCP2 Expression Throughout Neurodevelopment and Adulthood.** MeCP2 expression begins low in early gestation and increases concomitantly with neurogenesis and differentiation. Levels plateau during neuronal maturation at ~10 years of age and are constitutively maintained at these levels throughout adulthood. In RTT, MeCP2 expression fails to increase, leading to pathogenesis. Normal MeCP2 progression is depicted in blue, while progression in RTT is in red. The morphology of healthy and RTT neurons is shown at the bottom. Adapted from [16, 19].

In contrast, there is a stunted increase of MeCP2 expression during these critical developmental periods in RTT (Figure 1). Cells expressing the mutated X-chromosome undergo a loss of MeCP2 function that is exacerbated by compromised structural stability
of the mutant protein, which is degraded to the point of undetectability in severe mutations [20, 21]. This compounded reduction results in a premature plateau of MeCP2 at insufficient levels during neurodevelopment, causing abnormal neuronal morphology with impaired neural circuit formation in the RTT brain [6, 11, 16].

One of the most critical aspects of MeCP2 function is its ability to bind methylated DNA through its methyl-CpG-binding domain (MBD) [7, 17, 18, 22]. MeCP2 belongs to the MBD family of proteins, consisting of methyl-CpG-binding proteins 1-6 (MBD1-6); of these, only MeCP2, MBD1, MBD2, and MBD4 have been shown to preferentially bind methylated DNA as opposed MBD3, which nonspecifically binds both methylated and unmethylated DNA [18, 23, 24]. DNA methylation is an important epigenetic code that marks genes for regulation, allowing dynamic governance of gene expression during processes like neurodevelopment, while maintaining cell-type-specific neuronal transcription programs [25]. MeCP2 is the most abundant methyl-binding protein in mature neurons, whereas MBD1, 2, and 4 are expressed in ESCs and somatic tissue, indicating that MeCP2 is the principal reader of the neuronal epigenome [18, 23]. As such, MBD1, MBD2, and MBD4 knockout (KO) mice exhibit minimal phenotypes whereas Mecp2 deletion in mice led to severe neurologic symptoms and premature death around 9 weeks [18, 23, 24].

During normal development, MeCP2 directs early progenitor cells toward neuronal cell fate through the suppression of competing differentiation factors in a methylation-directed manner [16, 26]. The transcriptional refinement of neuronal cell identity is disrupted in RTT, wherein MeCP2-deficient neural stem cells (NSCs) exhibit aberrant
maturation patterns such as delayed cell-fate commitment and skewed differentiation [26, 27]. Furthermore, RTT NSCs are observed to preferentially differentiate into astrocytes in vitro due to deficits in MeCP2-mediated repression of GFAP (glial fibrillary acidic protein) expression, a marker of glial maturation [16, 26].

Neuronal maturation takes place following neurogenesis and includes processes such as dendritic arborization, spine morphogenesis, and neuronal circuit formation, which concomitantly occur with significant increases in MeCP2 levels, reflecting its high involvement in the maturation stage [16, 25]. MeCP2-deficient neurons display cellular defects such as smaller soma size, decreased dendritic complexity, and reduced spine density, leading to the formation of impaired circuitry (Figure 1) [9, 16]. Accordingly, RTT patients present with microcephaly and altered whole-brain connectivity, highlighting the necessity of MeCP2 regulation during neurodevelopment [4, 6, 7, 9].

In the adult brain, MeCP2 modulates activity-dependent transcription in dynamic processes such as synaptic plasticity and pruning for adaptive circuitry refinement [16, 17]. MeCP2 simultaneously maintains chromatin structure in mature neurons, remodeling chromatin for the proper execution of the neuronal transcription program [6, 16, 17]. In RTT, improper circuit formation during maturation stages leads to deficits in activity-dependent refinement, culminating in fundamental excitatory-inhibitory imbalances in neural circuitry which inadequately respond to activity-dependent feedback mechanisms [11].
MeCP2: Function and Structure

MeCP2 is an Epigenetic Multifunctional Hub

The field has progressed significantly in understanding the complex dynamics of MeCP2 regulation since the first report linking the gene to RTT in 1999 [5, 9]. Initially, MeCP2 was believed to be solely a transcriptional repressor due to its two primary functional domains: the methyl-CpG-binding domain (MBD) and the transcriptional repression domain (TRD) [5, 18]. The MBD preferentially binds methylated DNA sites, while the TRD primarily recruits the NCoR/SMRT (nuclear receptor corepressor/silencing mediator or retinoic acid and thyroid hormone receptor) complex, allowing MeCP2 to function as a “molecular bridge” that serves as a mediator between target genes and corepressor complexes to ultimately downregulate gene expression [5, 6]. However, subsequent transcriptomic studies in the brains of Mecp2-null mice revealed that over half of the affected genes were actually downregulated in the absence of Mecp2, suggesting that it also functions as a transcriptional activator [7, 28]. Analogous studies in mice overexpressing Mecp2 further corroborated this conclusion, as the same set of genes were upregulated upon excess of Mecp2, corroborating that MeCP2 is capable of both transcriptional repression and activation, potentially in a cell-type-specific and activity-dependent manner [28]. In the same study, MeCP2 was found to bind at gene promoters and recruit the transcription factor CREB1 (cAMP response element binding protein 1), mediating activation of gene expression despite low levels of methylation in promoters of upregulated genes [28]. These findings
introduced the notion that MeCP2 interactions were not restricted to methylated DNA and corepressor complexes through the initially characterized MBD and TRD.

The vital methyl-reading function of MeCP2 is facilitated by the MBD, which interacts with all three types of DNA methylation enriched in neurons: methylated CpG (mCpG), methylated CH (mCH; in which H is any non-G nucleotide), and hydroxymethylated cytosine (5hmC) [25, 29]. While MeCP2 readily binds to each of these, it has higher affinities for mCpG and mCH sites (particularly mCA). These methylation signatures serve as repressive cues and are enriched at intergenic regions, silenced genes, and repetitive regions of DNA while depleted at enhancers, promoters, and active gene bodies [25]. In contrast, 5hmC sites are associated with gene activation and are enriched at enhancers, transcriptional end sites, and gene bodies [25]. mCpG sites are more prevalent during neurodevelopment, whereas mCH sites accumulate during maturation and become the predominant form (~53%) of methylation in adult neurons [25, 30]. Moreover, a transgenic mouse model expressing an exclusively-mCpG-binding chimeric MeCP2 allele still developed severe RTT phenotypes, indicating that the genes regulated by MeCP2-mCH interactions are the most relevant in pathogenesis [31].

To date, over 40 putative binding partners have been identified in the literature, implicating MeCP2 in several critical pathways in addition to transcriptional repression and activation [18]. MeCP2 is now generally accepted as a crucial epigenetic modulator in neurons, serving as a multifunctional hub that directly and indirectly regulates essential cellular processes, some of which are discussed below [3, 6, 8, 9, 17].
De novo DNA methylation. The methylation code of the neuronal genome is “written” by three DNA methyltransferases (DNMTs). DNMT1 preserves the existing methylome by copying methylation patterns in hemi-methylated during DNA replication, while DNMT3A and DNMT3B are the de novo methyl-writers of mCpG and mCH [25]. The hmC mark is generated by ten-eleven translation methylcytosine dioxygenase proteins (TET1, 2, 3), which oxidize or “erase” mC nucleotides [18]. MeCP2 interacts with DNMT1 and DNMT3A, suggesting its regulatory role in de novo DNA methylation [29]. MeCP2 binds DNMT3A and inhibits its activity in a manner reversed by DNMT3A binding of the N-terminal tail of lysine-4-unmodified histone 3 (H3K4) [29]. It has been hypothesized that MeCP2 may be preventing inappropriate DNA methylation by inhibiting DNMT3A activity or acting as a recruiter to target sites with unmodified H3K4 for directed DNA methylation [29].

Chromatin remodeling. MeCP2 displaces histone H1 at mCpG sites on nucleosomal linker DNA and alters chromatin structure, modulating global transcription [7, 17]. MeCP2 has multiple DNA-binding domains outside of the MBD that are methylation-independent; in particular, it contains three AT-hooks (adenosine-thymine) that bind A/T-rich sequences and binary chromatin-binding sites in the CTD [17, 32, 33]. Though it primarily binds heterochromatin regions, MeCP2 has also been observed to associate with euchromatin regions by binding 5hmC sites at active genes [25, 34]. This function enables MeCP2 to condense methylated and unmethylated DNA over long ranges, mediating the formation of chromatin loops and DNA bridges to form higher-order structures [3]. MeCP2-MeCP2 homodimerization is suggested to function in chromocenter compaction and overall
architecture, producing an alternative structure to H1-linked chromatin [7, 34]. In most cell types, H1 is expressed at an approximate 1:1 molecular ratio with nucleosomes, whereas in neurons, its expression is halved into a 1:2 ratio, equal to that of MeCP2; moreover, H1 expression increased approximately 2-fold in a compensatory manner in Mecp2-null neurons, suggesting that MeCP2 is critical in maintaining neuronal chromatin structure [35].

In addition to NCoR/SMRT, MeCP2 TRD interacts with many other corepressors such as histone deacetylases (HDAC) 1 and 2, SIN3A, and c-Ski, all of which form corepressor complexes that remodel chromatin structure via histone modification [17, 34]. Further, MeCP2 modulates dynamic changes to chromatin architecture by recruiting numerous chromatin-remodeling factors such as heterochromatin protein 1 (HP1), ATRX, Brahma, and BAF57 [3, 8, 18].

**Transcriptional modulation.** As reviewed above, MeCP2 functions as both a transcriptional repressor and activator by binding methylated, hydroxymethylated, or unmethylated DNA and recruiting corepressors or coactivators to regulate gene expression. MeCP2-mediated transcriptional modulation is complex and dynamic, varying across brain regions, neuronal cell types, and molecular contexts [3, 17, 28, 36, 37]. It is also heavily influenced by the methylation profile in neurons, which changes with neuronal maturation and activity-dependent epigenetic modification in adulthood [25, 34]. For example, MeCP2 represses brain-derived neurotrophic factor (BDNF) by binding at the gene promoter, but releases upon depolarization-induced phosphorylation, thus activating transcription [3, 19].

Cell-type-specific MeCP2 regulation of BDNF modulates aspects such as synapse
formation and transmission [3, 17, 34]. Recently, MeCP2 was recognized as an RNA-binding protein (RBP) with a non-canonical RNA-binding motif within the TRD region, primarily interacting with noncoding RNAs (ncRNA) such as microRNAs (miRNA) and long noncoding RNAs (IncRNA), further implicating MeCP2 in the intricate epigenetic regulatory network of neurons [3, 6, 19].

**Alternative splicing (AS).** MeCP2 interacts with RNA and splicing factors in a context-dependent manner, most notably, Y box binding protein 1 (YB-1) [17]. MeCP2 binds YB-1 in the presence of RNA primarily through the TRD and promotes alternatively spliced exon (ASE) inclusion during splicing events; this regulatory interaction is enhanced by activity-induced dephosphorylation of MeCP2 pSer80 [38]. MeCP2 also interacts with pre-mRNA processing factor 3 (PRPF3) and serologically defined colon cancer antigen gene 1 (SDCCAG1) in the presence of pre-mRNA and mature mRNA, suggesting that it regulates the neuronal spliceosome [6]. Alternatively, MeCP2 can enhance exon recognition at AS sites by binding to methylated DNA in intragenic regions and recruiting HDACs to co-transcriptionally modulate histone acetylation while stalling RNA Polymerase II (RNAPII) elongation, which increases the chances of alternatively spliced exon inclusion [6, 22].

**miRNA biogenesis.** miRNAs are small ncRNAs (~22 nt) that direct fine-tuned post-transcriptional repression of gene transcripts [39]. They are transcribed by RNAPII as longer RNA hairpins called primary miRNA (pri-miRNA), which are co-transcriptionally processed into precursor miRNA (pre-miRNA) [39]. Pre-miRNA is then further refined by endonucleases into miRNA duplexes before being loaded into Argonaute (AGO) proteins as mature miRNA,
forming the miRNA-induced silencing complex (miRISC) [39]. Pri-miRNAs are cleaved into pre-miRNA by the Microprocessor complex, composed of endonuclease Drosha and DiGeorge syndrome critical region 8 proteins (DGCR8) [39]. RNA-sequencing data from the brains of MeCP2 KO and overexpressing mice showed a substantial overlap in differentially expressed miRNA, suggesting its capacity to bi-directionally regulate miRNA biogenesis [40]. Further studies demonstrated that MeCP2 directly interacts with DGCR8 in a phosphorylation-dependent manner, wherein depolarization-induced dephosphorylation of MeCP2 at Ser80 significantly reduced its binding of DGCR8 [40]. These findings indicated that MeCP2 regulation of miRNA processing is activity-dependent, such that when Ser80 is phosphorylated at a resting state, MeCP2 CTD directly binds and sequesters DGCR8, preventing Microprocessor assembly and inhibiting maturation of miRNAs (Figure 2) [40].

**Figure 2. MeCP2 Repression of miRNA Biogenesis.** *Left:* at resting state, MeCP2 is phosphorylated at Ser80 and competes with Drosha to bind DGCR8 at pri-miRNA transcripts, inhibiting Microprocessor assembly. *Right:* neural activity-dependent dephosphorylation of Ser80 causes MeCP2 to release DGCR8, allowing Drosha to bind DGCR8 and pri-miRNA processing. Adapted from [41].

Alternatively, MeCP2 can also promote miRNA processing by acting as a DGCR8 recruiter to methylated pri-miRNA gene boundaries and stalling RNAPII elongation in a
mechanism similar to AS regulation; this facilitates the assembly of the Microprocessor complex at the pri-miRNA transcription site, enhancing the likelihood of mature miRNA synthesis (Figure 3) [6, 42]. In addition, MeCP2 can associate with the Microprocessor complex and directly facilitate miRNA processing for a subset of target miRNAs to direct post-transcriptional regulation [43]. For example, MeCP2 indirectly regulates the mTOR signaling pathway via miR-199a biogenesis. miR-199a is specifically generated by a MeCP2-Microprocessor hybrid complex and downregulates the expression of mTOR inhibitors such as PDE4D, SIRT1, and HIF1α, increasing mTOR signaling [43]. Though this mechanism is not fully understood, a miR-199a KO in mice was sufficient to decrease mTOR activity in the brain and recapitulate select RTT phenotypes, illustrating a potential link between mTOR signaling and RTT pathophysiology [43]. MeCP2 may be involved in a feedback regulation in this pathway, as SIRT1 deacetylates MeCP2 while the mTOR-kinase ERK phosphorylates DGCR8 and increases its stability [6].

Figure 3. MeCP2 Promotion of miRNA Processing. Left: MeCP2 binds DGCR8 at methylated pri-miRNA gene boundaries and slows RNAPII elongation, allowing Microprocessor assembly and co-transcriptional pri-miRNA cleavage. Right: In the absence of DNA methylation and MeCP2, the elongation rate is rapid and Drosha fails to bind nascent pri-miRNAs, inhibiting miRNA biogenesis. Adapted from [42].
Intrinsic Disorder of MeCP2

**Conformational flexibility.** As a multifunctional hub, MeCP2 interacts with dozens of binding partners, each with its unique structure. Its ability to bind such structurally distinct molecules is attributable to its conformational flexibility as an intrinsically disordered protein (IDP) [17]. IDPs or disordered protein regions (IDPRs) are defined by their lack of a fixed three-dimensional structure due to strong electrostatic repulsions and low hydrophobicity in amino acid composition [44]. IDPs generally remain in unfolded states, existing as a dynamic ensemble of transient structures capable of detecting various targets. The recognition of a binding partner triggers “induced folding” of the protein into ordered conformations that are specific to the interaction [44]. The inherent conformational flexibility of an unfolded polypeptide sequence confers functional diversity to IDPs, many of which (like MeCP2) serve as multifunctional hubs involved in several important regulatory mechanisms and signaling pathways [17, 44]. Post-translational modifications (PTMs) of IDPs generate altered functional conformations, expanding their dynamic structural ensemble [44].

The intrinsic disorder of MeCP2 was first formally characterized in 2007 by Adams and colleagues, who reported that approximately 60% of MeCP2’s protein sequence was unstructured [45]. The authors identified the MBD as the only domain with a significant proportion of secondary structures (~60% ordered), whereas the remainder of the MeCP2 protein, comprised of the N-terminal domain (NTD), intervening domain (ID), TRD, and C-terminal domains α and β (CTD-α and CTD-β), are all highly disordered (60 – 85%
Disordered protein regions cannot be structurally resolved and are highly prone to degradation in experimental conditions, limiting our understanding of the functional conformation ensemble of MeCP2. Thus, most structural investigations of MeCP2 are centered on the characteristics of MBD interactions given the spectroscopically detectable ordered structure and well-defined DNA-binding function.

Figure 4. MeCP2 Domains and Location of RTT-Pathogenic Mutations. Above: Domain structure of brain-dominant MeCP2-E1 isoform. CTD-α and CTD-β are denoted as a single C-terminal region. Below: Location of frequent RTT-pathogenic missense and truncating mutations. For the latter, the “breaking point” of essential MeCP2 function occurs between R270 and G273, wherein males expressing truncating or frameshift mutations at or before R270 exhibit neonatal encephalopathy and death, whereas of occurring after G273 survive [6]. Adapted from [7].
**Implications of MeCP2 intrinsic disorder in RTT.** The disordered nature of MeCP2 explains the range of dysfunction observed across RTT-inducing mutations, as changes to any single residue has the potential to rearrange the structural assembly of the entire protein. This concept is best demonstrated by missense mutations occurring in the MBD, wherein any one mutated residue can have distinct consequences on MeCP2 function, even when mutations are close in proximity as in the case of R106W and R133C. In the MBD, R133 is exposed in a loop region and is one of two residues forming direct hydrogen bonds with DNA while R106 is almost fully buried in a β-strand facing the other DNA-binding residue (R111), likely forming stabilizing contacts with other residues [48]. Despite the loss of direct contact with DNA, the R133C mutant retains more DNA-binding affinity than other MBD mutations and exhibited no significant difference in heterochromatin localization compared to WT *in vitro* [18, 48, 49]. Conversely, R106W MeCP2 has drastically reduced heterochromatin binding and an unstable MBD structure, which engenders a higher propensity of unfolding due to larger structural rearrangement of the surrounding region, interrupting the normative configuration of functional conformations [18, 48-50]. Accordingly, R133C results in mild cases of RTT while R106W is considered one of the most clinically severe MECP2 mutations [18, 33, 48]. The drastic impairments of R106W mutants have been ascribed to the pivotal role of the MBD as the “structural hub” of MeCP2 [51]. As the most ordered domain, it serves as the origin of MeCP2 conformational transitions by directing interdomain organization with its structured regions [50-52]. In accordance with this central role, over 68% of all RTT-causing missense mutations occur in the MBD,
highlighting the immanent dependence of MeCP2 function on the stability of its structural assembly [10].

The fluid structure-function dynamics of IDPs are driven by a myriad of interdomain interactions that facilitate disorder-to-order transitions and stabilize substrate-bound conformations [33, 50]. IDP structural kinetics explain how mutations in disordered regions outside the MBD and TRD (primary functional domains of MeCP2) broadly impact MeCP2 function. For instance, the NTD and ID stabilize the structure of the MBD during DNA binding and increase the affinity of MBD-DNA interaction, while the ID and TRD form further stabilizing trans-interactions with the MBD in methylated DNA-bound MeCP2 dimers [33, 52]. The significance of this stabilization effect becomes more apparent in RTT such that the structural plasticity granted by extensive interdomain interactions may allow for some degree of compensatory rearrangement in protein conformation, as demonstrated in the retained structure and DNA-binding ability of R133C [33]. Simultaneously, the profound disorder of MeCP2 structure implies that its functionality is fundamentally dependent on interdomain connectivity facilitated by key residues, wherein any single-residue difference has the potential to alter the conformational ensemble of MeCP2 in a functionally detrimental manner, as in the case of R106W [33, 48, 49].

**Regulation of MECP2 Expression**

Due to its essential role and high dosage sensitivity, tight regulation of MECP2 expression is required for proper neurological function. Therefore, MeCP2 levels are controlled through multiple layers of post-transcriptional and post-translational regulation
Many of these regulatory interactions are repressive, serving as a safeguard against toxic MeCP2 accumulation. A description of these endogenous control mechanisms is outlined below.

**Post-transcriptional regulation of MECP2.** MECP2 has one of the longest 3’ untranslated regions (UTRs) in the mammalian transcriptome, with identified 3’UTR isoforms ranging from 1.8 to 10.2 kilobases (kb) compared to the average length of 0.8 kb [54]. In mature human neurons, the dominant 3’UTR isoform is the longest at 8.6 kb and is abundant in regulatory elements that bind endogenous repressors. RBPs recognize regulatory elements in mRNA and regulate the transcript’s cellular localization, splicing, stability, or translation efficiency [54]. Though the identification of RBP targets is notoriously difficult, pumilio RNA binding family member 1 (PUM1) and T-cell intracellular antigen 1 (TIA1) are known to bind to the 3’UTR and repress MECP2 expression during neurodevelopment [53]. PUM1 works in cooperation with miR-200a and miR-302c to actively degrade MECP2 transcripts, while TIA1 represses translation efficiency [53]. This RBP-induced repression of MECP2 expression is essential at early stages of neurodevelopment when low levels of MeCP2 are required for stem cell proliferation.

MECP2 is targeted by a multitude of miRNAs that bind sequence-complementary miRNA response elements (MRE) located on the 3’ UTR and mediate transcript degradation [54, 55]. Though an individual miRNA-MRE interaction generally exerts a modest repression of gene expression (usually around 50% and often less than 20%), miRNAs can simultaneously bind a 3’UTR to achieve combinatorial repression of the target transcript [39,
Case in point, the *MECP2* 3'UTR has been predicted to have 35 – 52 canonical MREs, each serving as the regulatory site of several repressive miRNAs (Figure 5) [34, 54].

**Figure 5.** Predicted Canonical MREs on *MECP2* 3'UTR. The blue rectangle represents the longest human *MECP2* 3'UTR isoform (8.6 kb). **Above:** Canonical MREs predicted by high stringency miRNA seed matches are labeled by their respective miRNA names. RBPs (labeled in pink) and miRNAs (labeled in blue) have been demonstrated to regulate mRNA stability and/or translation efficiency. **Below:** Locations of RTT-pathogenic 3'UTR mutations without concurrent changes in the coding sequence. Those labeled in red are associated with decreased levels of *MECP2* mRNA in RTT patients. Adapted from [54].

**Post-translational regulation of MeCP2.** As an IDP, MeCP2 is highly prone to PTMs such as phosphorylation, acetylation, methylation, O-GlcNAcylation, SUMOylation, poly-(ADP-ribosyl)-ation, and ubiquitination [17, 18]. The functional role of each PTM is not well understood, but activity-dependent phosphorylation of serine residues such as Ser86, Thr148/Ser149, and Ser164 have been observed to modulate the DNA-binding affinity of MeCP2 [18, 41]. MeCP2 is prone to proteasomal degradation by ubiquitination due to its 2 PEST (proline, glutamate, serine, threonine) motifs located in both N and C-terminal regions [6]. PEST motifs act as a proteolytic mark wherein sequential phosphorylation of serine residues in the motif is followed by ubiquitination of a nearby lysine; PEST ubiquitination is a signal for rapid degradation that is recognized by the ubiquitin/26S proteasome system.
This mechanism is likely involved in maintaining appropriate MeCP2 levels in the nucleus [6, 17].

**Therapeutic Developments in RTT**

**Challenges of Targeting MECP2**

Despite the vast cellular and systemic deficits observed in RTT, neuronal death does not occur, indicating that RTT is not a neurodegenerative disorder [56]. This lack of permanent change raised the question of whether the damage caused by pathogenic MeCP2 mutations during neurodevelopment could be repaired in adulthood. In 2007, the feasibility of this concept was substantiated through the utilization of a floxed conditional Mecp2 allele controlled by a Cre recombinase-estrogen receptor system (Cre-ER) [56]. Upon tamoxifen-induced gene activation, Mecp2-null mice displayed robust phenotypic rescue regardless of the stage in disease progression, establishing that restoration of normative MeCP2 expression may be a cure for RTT [56].

Unfortunately, targeting MECP2 is complicated by its high dosage sensitivity, as even a modest 1-fold overexpression results in a related NDD known as MECP2 duplication syndrome (MDS) [14, 15]. MDS shares some similarities with RTT symptomology, but primarily affects males due to the lack of protective mosaicism and often results in more severe symptomology and premature death [7, 57]. This narrow therapeutic window poses a considerable challenge for approaches directly targeting MECP2/MeCP2, as most traditional methods (gene therapy, protein replacement therapy, etc.) lack the fine-tuning mechanisms required to operate in such contexts [12, 58, 59].
Current State of RTT Therapeutics

Due to the profound effects of pathogenic MECP2 mutations and its dosage limitations, only minimal gains have been made in therapeutic development, which is primarily focused on downstream targets of MeCP2 as opposed to the gene itself [59]. Currently, Trofinetide, a synthetic analog of insulin-like growth factor 1 (IGF-1), is the only drug to be FDA-approved for the treatment of RTT (approval granted in March 2023); its mechanism of action is unclear, but likely ameliorates a disrupted downstream pathway involving the pathological overexpression of IGF-binding protein 3 (IGFBP3) [60]. Early clinical data for Trofinetide has indicated moderate improvement in breathing abnormalities, hand stereotypies, fear, and anxiety, as well as motor and gait impairments [60, 61]. While the approval of Trofinetide was a remarkable milestone in RTT therapeutics, not all patients are candidates for the drug and it remains that a cure requires targeting MECP2 itself. Modern advancements in gene-targeting strategies have invigorated ongoing studies in RTT and MECP2, culminating in potential therapeutics that are presently undergoing clinical trials.

**Novel gene therapies.** The last decade has overseen several advancements in gene replacement studies in mouse models of RTT, wherein optimization of Mecp2/MECP2 gene constructs, delivery of viral vectors, and regulation of transgene-MeCP2 levels have made considerable progress [12, 62]. In 2023, two gene therapies were approved for phase 1/2 clinical trials: TSHA-102 and NGN-401. Both approaches use modified gene constructs with auto-regulatory elements that control exogenous MeCP2 expression through synthetic
miRNA repression or intentional disruption of promoter efficiency [62-64]. Though promising, gene therapies can have unpredictable and/or adverse outcomes, particularly in contexts such as RTT, in which the pathogenic protein impacts thousands of downstream pathways. Further, the risk of pediatric treatment with AAV vectors is poorly understood, particularly in a setting where targeting the whole brain is required [8, 58]. As of 2024, three AAV-based gene therapies have been approved for the treatment of central nervous system (CNS) disorders: 1) Luxturna (AAV2-based), indicated for a rare inherited retinal disease (LCA2) in 2017, 2) Zolgensma (AAV9-based), indicated for spinal muscular atrophy in 2019, and 3) Elevidys (AAV9-based), which was indicated for Duchenne muscular dystrophy in 2023 [65]. The FDA approval of these viral gene therapies marked a significant milestone for treating genetic disorders of the nervous system, but these treatments caused high occurrences of side effects such as vomiting, hepatotoxicity, thrombocytopenia, and immunotoxicity [65, 66]. Further, all three of these CNS disorders occur outside of the brain, for which both CSF and intravenous AAV delivery have thus far failed to demonstrate complete transduction [67].

**RNA editing.** The most common forms of RTT arise from point mutations, making site-directed RNA editing an attractive strategy to repair the pathogenic mutation. Recently, this approach was successfully tested in a mouse model of RTT, wherein Mecp2<sup>G377A</sup> mice were injected with AAVs expressing a modified RNA-editing enzyme (ADAR2), resulting in 50% of edited Mecp2 mRNA [62, 68]. While this is an exciting development for RTT and other genetic neurological disorders, RNA editing approaches are challenged by the specificity of
**MECP2** targeting and the fact that ADAR2 primarily edits G to A mutations, which only account for a small fraction of RTT cases [8, 68].

**Xi-reactivation.** Another **MECP2**-targeted approach is to reactivate the silenced X chromosome (Xi) in cells expressing the mutated form. This strategy is speculated to be advantageous compared to gene therapies because the reactivated **MECP2** locus would be subject to endogenous regulatory mechanisms [13]. Therapeutic Xi-reactivation likely requires selective targeting of the **MECP2** locus due to the potential dosage constraints of the array of genes contained in the Xi, which total over a thousand [7, 13]. XCI is a selective process that occurs upon coating of the Xi by the ncRNA Xist, while the Xist-antisense ncRNA Tsix suppresses Xist expression on the activated X-chromosome (Xa) [69]. Given this inhibitory mechanism, a multimodal approach using Xist-targeted antisense oligonucleotides (ASOs) and 5-aza-2’-deoxynucleotide (Aza), a DNA methylation inhibitor, demonstrated the potential of therapeutic Xi-reactivation by significantly upregulating Xi-MeCP2 expression in vitro [70]. However, Aza is toxic when chronically administered due to its global inhibition of methylation, limiting the clinical applicability of this approach [13]. Further, RTT patients exhibit heterozygous mosaicism as a result of random XCI, meaning that about half of their cells express a WT **MECP2**. Reactivating the mutant allele in WT cells would result in an excess of MeCP2 levels, risking MDS-like deficits [12, 13].

**Alternative MECP2-Targeted Approaches**

An alternative gene-targeted approach is to upregulate endogenous MeCP2 expression. This approach is rooted in two key concepts: 1) common pathogenic MeCP2
mutants retain partial function, and 2) overexpression of the mutant protein partially rescues RTT phenotypes in mouse models. These principles were established by the Zhou lab in 2017 by overexpressing the RTT-pathogenic T158M MeCP2 mutant in MeCP2T158M/+ and MeCP2T158M/+ mice, both of which exhibited partial rescue of phenotypes without adverse effects [71]. T158M MeCP2 was shown to have decreased protein stability and retained partial DNA binding affinity, both of which increased in a protein-level-dependent manner [71]. Importantly, this study demonstrated that mutant MeCP2 expression had a wider therapeutic window compared to the strict dosage sensitivity of WT MeCP2 [71]. This protein-level-dependent functional rescue has been recapitulated in several studies characterizing RTT-causing mutations, including the most frequent MECP2 “hotspot” mutations [6, 33, 72]. Together, these findings demonstrated the viability and advantage of targeting mutant MeCP2 expression in RTT.

Antisense Oligonucleotide Therapeutics: Applicability to RTT

The potential for targeting the mutant MeCP2 allele is known; however, there are no published reports demonstrating how this can be achieved for clinical applications. The goal of this thesis is to show that MECP2-targeted antisense oligonucleotides (ASO) are one such approach that is capable of upregulating endogenous MeCP2 protein in a controlled and capped manner. ASOs are short, single-stranded oligonucleotide sequences that bind DNA or RNA and modify gene expression through several mechanisms; the two most common applications are ASO-induced RNaseH-mediated transcript degradation (gapmer) and
steric blockage of regulatory sequences (sbASO) such as start and stop codons, splicing sites, and various response elements [73, 74].

**Fine-Tuning MECP2: Disinhibition of miRNA-Mediated MECP2 Repression**

A practical implementation of ASOs in RTT could be to target endogenous repression of MECP2 transcripts. MECP2 expression is repressed through many post-transcriptional mechanisms, however, the most reasonable target would be miRNA for the following reasons. MECP2 transcripts are highly regulated by miRNAs in neurons while the MECP2 3'UTR contains at least 35 distinct MREs, providing a wide selection of potential miRNA-MRE interactions that could be inhibited with targeted sbASOs (Figure 5) [54]. Further, the regulatory effect of a singular miRNA does not exceed a two-fold change, allowing this approach to fine-tune MeCP2 expression and operate with its dosage restriction. Considering that one miRNA can bind and regulate hundreds of mRNAs, sbASOs specific to individual MREs on MECP2 3'UTR is favorable for this approach given it exclusively impacts the expression of the target gene. Taken together, this potential MECP2-based therapeutic approach for RTT consists of MRE-targeted sbASOs designed to disinhibit miRNA-mediated MeCP2 repression and marginally increase protein levels within a safe range.

**Chemical Modification of ASOs**

After the first reported *in vitro* application of ASOs in 1978, advancements in oligonucleotide chemistry have significantly improved pharmacological properties such as decreased toxicity and increased *in vitro* and *in vivo* stability, cellular uptake, and target binding affinity. Chemical modifications of ASOs can be grouped into three categories: 1)
phosphate backbone modifications, 2) ribose modifications, and 3) furanose ring modifications; ASOs are often designed with a combination of modifications based on application context [74, 75]. Belonging to the first category, the phosphorothioate (PS) is the most widely used overall and contains a sulfur group instead of a nonbridging oxygen in the phosphodiester bond. PS modifications significantly increase ASO nuclease resistance and thermal stability ($T_m$), improving the half-life from minutes to days [75, 76]. The second category broadly consists of alkyl additions to the 2’ oxygen of the ribose and includes 2’-O-methyl (2’OMe), 2’-O-methoxyethyl (2’MOE), and locked nucleic acids (LNA); these modifications confer higher target affinity and reduced toxicity, resulting in greater stability relative to a PS-modified backbone alone [76]. Most ASOs that entered clinical trials or are FDA-approved incorporate both a 2’MOE and PS modification, which provide synergistic enhancement of pharmacological properties [74-76]. The third category contains the latest and most radical ASO modifications, in which the furanose ring of the nucleotide is replaced by other moieties. The best characterized among these are the phosphoroamidate morpholino oligomer (PMO) and peptide nucleic acid (PNA), which improve upon previous generations of modified ASOs; these larger molecules require additional factors for cellular uptake, limiting their clinical utility [76].

**Advantages of ASO Therapeutics**

Therapeutic ASOs have several advantages compared to traditional approaches. Most notably, they can target molecules previously considered “undruggable” with sequence-specific complementarity, highlighting its customizability and lowered risk of
adverse off-target effects. ASOs have been FDA-approved for use in several CNS disorders, including Batten’s disease, Dravet syndrome, and spinal muscular atrophy (SMA) [73]. As of 2021, ASO-based approaches were undergoing clinical trials for ten genetic disorders, four of which were NDDs [73]. ASO therapy is a prime candidate for treating genetic NDDs due to its specificity and versatility, which enables personalized treatment of pathogenic mutations. PS and 2’MOE modified ASOs are highly stable in the CNS (half-life = ~100 days) and more efficiently internalized by cells, allowing safer dosage margins than pharmacological alternatives [77]. Further, any potential adverse effects of ASOs are reversible given their mechanism of action is to modulate endogenous regulatory events rather than alter the gene itself. The potential for individualized therapeutics and precision medicine for genetic disorders is furthered by FDA support of single-subject clinical trials (“N of 1” trials) for ASO treatments (FDA Draft Guidance for Individualized Investigational ASO Products).
CHAPTER 2
THESIS OVERVIEW AND EXPERIMENTAL DESIGN

Rationale

The two main obstacles in developing therapeutics for RTT are the extensive disruption of cellular processes caused by MeCP2 loss of function and the high dosage sensitivity of MECP2. The complex molecular mechanisms of MeCP2 have stymied the identification of viable downstream targets, leading to the scarcity of available treatments. Similarly, the development of MECP2-targeted therapies has been hindered by the strict dosage limitations that necessitate tight regulation of the gene product. To circumvent this dilemma, we have developed an alternative therapeutic strategy that targets endogenous post-transcriptional regulation of MECP2. This approach utilizes site-blocking antisense oligonucleotides (sbASOs) to target miRNA response elements (MREs) in the 3’ untranslated region (3’UTR), inhibiting repressive miRNAs from binding the transcript and thus increasing MeCP2 expression (Figure 6). This sbASO-mediated disinhibition approach is more advantageous than traditional gene therapies in that 1) it maintains the endogenous regulatory elements of MECP2, therefore avoiding the adverse effects associated with exogenous agents, and 2) the repressive effects of miRNAs on target transcripts are modest, allowing for fine-tuned upregulation of MeCP2 in adherence with its dosage restriction, and 3) it is not permanent, permitting potential adverse effects to resolve with time [39].
Figure 6. Schematic of sbASO-Mediated Disinhibition Approach. Top: miRNA-induced silencing complexes (miRISCs) bind complementary MREs on MECP2 3'UTR and mediate translational repression/transcript destabilization. Bottom: MRE-targeted sbASOs prevent miRISC binding to MREs, derepressing MeCP2 expression.

Preliminary data from sbASOs targeting the predicted MREs of three known MECP2-repressive miRNAs (miR-22-3p, miR-483-5p, and miR-132/212-3p) demonstrated the feasibility of this approach in vitro, wherein sbASO treatment increased MeCP2 levels in a dose-dependent manner safely within the therapeutic margins [2, 10, 19, 51]. Notably, there were additive effects when all three sbASOs were co-transfected at lower doses that were non-efficacious upon individual treatment. Evaluating the synergistic compatibility of MRE-targeted sbASOs may be critical for in vivo applications, particularly in contexts where target miRNA expression varies across cell types and/or MECP2 mutations (requires optimized therapeutic dosage of each sbASO for both individual and combinatorial treatment effects),
or in the case of severely destabilizing mutations requiring higher compensatory levels of MeCP2 (requires significant combinatorial effects maintained at steady levels).

The global disruption of RTT pathology extends to miRNA expression, which impacts the parameters of sbASO target selection criteria [78-80]. For instance, a miRNA of interest may be downregulated in RTT neurons, eliminating the de-repressive effects of a sbASO targeting the respective MRE. In contrast, an overexpressed miRNA may require higher dosages to competitively saturate the target MRE, and thereby increase the risk of off-target binding and adverse effects. Another contributing factor in target selection is the repressive capacity of each MRE. miRNAs recognize target transcripts through canonical MREs that are complementary to their seed region (nucleotide positions 2 – 8). The efficacy of each site is primarily determined by its pairing to the seed, wherein a perfect 8 nt match (8mer site) is the most effective in mediating repression. Given such considerations, an ideal MRE target would be a high-efficacy site on the MECP2 3'UTR with perfect sequence complementarity to the respective miRNA seed, whereas the corresponding miRNA should be stably expressed at steady-state levels in a MeCP2-independent manner. This thesis aims to further develop the sbASO-mediated disinhibition approach for clinical applications in RTT by 1) expanding the inventory of MRE-targeted sbASOs and assessing their therapeutic efficacy and its underlying mechanisms, and 2) quantifying the patterns of miRNA expression in the context of two pathogenic MECP2 mutations.
Hypothesis and Specific Aims

**Specific Aim 1:** To test the hypothesis that preventing hsa-miR-181a-5p, hsa-miR-422a, and hsa-let7-5p binding will increase MeCP2 expression.

1.1: To identify potential miRNA response elements on MECP2 3’UTR by locating sequences complementary to each miRNA’s seed, and design site-specific sbASOs to competitively block miRNA binding.

1.2: To establish the efficacy of developed sbASOs *in vitro* by conducting dose-response studies measured by MeCP2 levels in SH-SY5Y cells and RTT patient-derived fibroblasts expressing common MECP2 mutations.

1.3: To determine the mechanism by which effective target miRNAs mediate repression by examining MECP2 transcript levels in sbASO-treated SH-SY5Y cells.

**Specific Aim 2:** To test the hypothesis that miRNA expression differs across common RTT-pathogenic MECP2 mutations.

2.1: To quantify differences in miRNA expression in postmortem RTT patient samples (T158M, R255X) by using nCounter® miRNA Expression Panels (NanoString Technologies) with miRNA isolated from.

2.2: To measure and correlate the expression of target miRNAs in RTT patient-derived fibroblasts expressing common MECP2 mutations with *in vitro* sbASO efficacy profiles.
Experimental Design

To determine whether blocking miR-181a-5p, miR-422a, and let7-5p from binding their respective MREs would increase MECP2 expression (Specific Aim 1), potential high-efficacy sites on the long 3'UTR isoform (8.8 kb) were identified by locating complete sequence matches to each miRNA's seed region. Site-specific sbASOs were designed to block miRNAs from binding to MREs leading to the disinhibition of MeCP2 expression. The efficacy of each sbASO was determined by conducting dose-response studies and measuring changes in MeCP2 levels in SH-SY5Y cells. sbASOs demonstrating the desired effect were then tested following the same protocol in RTT patient-derived fibroblasts expressing the most common MECP2 mutations. Finally, the mechanism by which effective target miRNAs mediate MECP2 repression was determined by examining MECP2 transcript levels in sbASO-treated SH-SY5Y cells via qRT-PCR.

To examine miRNA expression patterns in distinct RTT-causing MECP2 mutations (Specific Aim 2), total miRNA was isolated from postmortem RTT patient temporal cortex samples with MBD (T158M) and TRD (R255X) mutations and profiled across 827 human miRNAs. To further characterize sbASO efficacy profiles, the expression of target miRNAs in RTT patient-derived fibroblast expressing five of the eight most common MECP2 mutations was determined by qRT-PCR.
Significance Statement

Rett Syndrome (RTT) is a devastating neurodevelopmental disorder characterized by a myriad of debilitating symptoms impacting nearly every aspect of life, including motor and gait impairment, social and cognitive impairments, as well as seizures and breathing abnormalities. RTT is caused by X-linked de novo loss-of-function (LOF) mutations in the methyl-CpG binding protein 2 gene (MECP2). MeCP2 is an essential epigenetic modulator that regulates transcription through molecular interactions between methylated DNA or RNA and co-repressor, co-activator, and modulatory complexes. Historically, most therapeutic developments for RTT have targeted downstream effectors due to the high dosage sensitivity of MeCP2, wherein a modest 1-fold overexpression above normative levels causes another deleterious neurodevelopmental disorder, MECP2 Duplication Syndrome (MDS). Though the indication of the IGF-1 mimetic Trofinetide, the only FDA-approved drug for RTT, has provided patients and families with much-needed relief, it serves to improve a subset of symptoms rather than address the root cause. It is widely accepted that any curative interventions for RTT must directly increase MeCP2 expression. Therapeutic approaches impacting MeCP2 dosage must be capable of fine-tuned control to safely operate within the narrow therapeutic index. This thesis aims to expand the current understanding of disrupted epigenetics in RTT and further develop a novel MECP2-targeted therapeutic approach using steric/site-blocking antisense oligonucleotides (sbASOs) to prevent miRNA-induced repression of MECP2. Here we report positive results of a novel sbASO that has efficacy across common pathogenic mutations in RTT and whose target
miRNA levels are not impacted by disease state. We contend that the strategy described herein could provide a curative measure for RTT that is safer and more personalized to each patient compared to traditional gene therapies.
CHAPTER 3

METHODOLOGY

Cell Culture

**SH-SY5Y Human Neuroblastoma Cells**

Immortalized human neuroblastoma cells were obtained from ATCC® (CRL-2266™), cultured in IMDM (1X) (Gibco™; Thermo Fisher Scientific; #12440053) supplemented with 10% HI FBS, 1% AA (100X), and 0.5% MEM NEAA (100X) (Gibco™; Thermo Fisher Scientific; #16140071, 15240062, 11140035), and maintained at 37°C and 5% CO₂. Cells were passaged using TrypLE™ Express (1x) (Gibco™; Thermo Fisher Scientific; #12605010).

**RTT Patient-Derived Primary Fibroblasts**

Primary fibroblasts derived from RTT patients were obtained from IRSF, cultured in DMEM (1X), high glucose, (Gibco™; Thermo Fisher Scientific; #11965092) supplemented with 15% HI FBS, 1% AA (100X), and 1% MEM NEAA (100X), and maintained at 37°C and 5% CO₂. Cells were passaged using TrypLE™ Express (1x).

**sbASO Design and Formulation**

MRE-specific sbASO sequences were designed using Primer3web version 4.1.0 ([https://primer3.ut.ee/](https://primer3.ut.ee/)) after identifying miRNA seed sequence matches in the 3’UTR of the longest MECP2-E2 transcript variant (10.4 kb; NIH GenBank). Resultant sbASO sequences were formulated with PS and 2’MOE modifications by Integrated DNA Technologies™ (Coralville, IA).
**Antisense Oligonucleotide Transfection**

Cells were plated in surface-treated 6-well sterile tissue culture plates and grown to approximately 80% confluency prior to assisted sbASO transfection with Lipofectamine™ 3000 Transfection Reagent (Invitrogen™; Thermo Fisher Scientific; #L3000150) in Opti-MEM reduced serum medium (Gibco™; Thermo Fisher Scientific; #31985070). Cells were treated with 3, 10, 55, 100, and 250 pmol sbASO per each transfection. For low-dose transfections, cells were treated with 0.1, 0.5, and 1 pmol sbASO per transfection. After 12-18 hours, transfected cells were washed with DPBS and maintained in growth medium for 24 hours, then harvested using TrypLE™ Express (1x) and stored at -80°C until processing.

**Subcellular Fractionation**

Transfected cells were collected and fractionated into nuclear and cytoplasmic fractions using the Lyse and Wash protocol by Senichkin et al. (2014) to isolate nuclear protein [43].

**RNA and miRNA Isolation**

RNA and miRNA were isolated from cells or tissue using PureLink™ miRNA Isolation Kit (Invitrogen™; Thermo Fisher Scientific; #K157001) per manufacturer instructions. Resultant RNA and miRNA were diluted in DEPC-treated water, tested for purity and concentration using NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific), and stored at -80°C until cDNA synthesis.

**Western Blot**

Nuclear protein concentration was estimated using Micro BCA™ Protein Assay Kit (Thermo Scientific™; Thermo Fisher Scientific; #23235). Samples were diluted into equal
concentrations before gel electrophoresis in 4-20% Criterion™ TGX™ precast polyacrylamide gels (Bio-Rad Laboratories; #5671095) and subsequent immunoblotting with Histone H3 (1B1B2) mouse mAb and MeCP2 (D4F3) rabbit mAb (Cell Signaling Technologies; #14269S, #3456S, respectively). Western blots were imaged using the Odyssey® Imaging system (LI-COR Biosciences; LICORbio™) and quantified using Empiria Studio® Software (LICORbio™).

mRNA Reverse Transcription and Quantitative RT-PCR

cDNA was synthesized using SuperScript™ IV VILO™ Master Mix (Invitrogen™; Thermo Fisher Scientific, #11756050). MECP2 transcript levels will be measured by qRT-PCR using TaqMan® Gene Expression Assay for MECP2 (Thermo Fisher Scientific; #4351372). qRT-PCR was performed in triplicate for each sample with the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories; #1855196), while using glucose-6-phosphate dehydrogenase (G6PD) levels measured by TaqMan® Gene Expression Assay (Invitrogen™; Thermo Fisher Scientific, #4331182) as the internal control for normalization, with negative controls in each plate.

miRNA Reverse Transcription and Quantitative RT-PCR

miR-cDNA was synthesized from isolated and purified miRNA using TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems™; #4366596) for target miRNAs (miR-22-3p, miR-132/212-3p, miR-181a-5p, miR-483-5p, U6 snRNA). miRNA qRT-PCR was performed in triplicate for each sample with the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, #1855196). Resultant quantification data was collected with CFX Maestro™ Software (Bio-Rad Laboratories, #12013758) and subsequently evaluated for fold changes via ΔΔCt method by normalizing to neurotypical expression levels in isogenic
wildtype HDFa human dermal fibroblasts isolated from heterozygous T158M RTT patient-derived fibroblasts.

**nCounter® miRNA Expression Panels**

Total miRNA was isolated from postmortem temporal cortices of T158M and R255X RTT patients and neurotypical controls obtained from the NIH NeuroBioBank and the University of Maryland Brain and Tissue Bank, and sent to NUSeq Core at Northwestern University (Chicago, Illinois) for nCounter® miRNA expression analysis per manufacturer protocols. miRNA expression levels were normalized to total RNA levels and compared to neurotypical controls using nCounter® Advanced Analysis Software.

**Statistical Analysis**

All statistical analyses were performed using GraphPad Prism 10 (GraphPad Software). Protein quantification data from sbASO transfections was analyzed via unpaired t-tests with 4 to 8 replicates of each concentration of sbASO per cell line, while the total sbASO effect in each cell line was analyzed via ordinary one-way ANOVAs. MECP2 mRNA levels and miRNA expression levels were analyzed via Student’s t-tests. Outliers were removed via Grubb’s tests at $\alpha = 0.1$ for both protein and RNA analysis.
CHAPTER 4

SPECIFIC AIM 1

Rationale

The ability of sbASOs to disinhibit MeCP2 expression is driven by the repressive capacity of a target MRE and the consistency of its interactions with the respective miRNA. As post-transcriptional effectors, miRNAs downregulate target mRNA expression via translation inhibition and/or transcript destabilization [55, 81]. Their primary function is to fine-tune gene expression for optimal cell function across cell types and in response to molecular contexts [39]. As such, the regulatory effect of an individual miRNA is modest, rarely exceeding a 50% (and often less than 20%) reduction in protein levels [39]. However, miRNAs are termed “Sculptors of the Transcriptome” given their extensive epigenetic networks that direct 30 – 50% of human gene expression [39, 55]. This potent collaborative control of the transcriptome is facilitated by the ability of an individual miRNA to target numerous mRNAs through short complementary sequences (MREs) in the 3’UTR [39]. While multiple miRNAs can exhibit steady-state expression, most are expressed in response to cellular cues and execute combinatorial repression of target transcripts, providing a molecular buffer against harmful changes in protein levels or conducting gene expression programs for critical processes such as neurodevelopment and cellular stress response [39, 53, 55].
Canonical MREs are characterized by their complementarity to miRNA seed regions [39]. Since the primary target recognition mechanism of miRNAs is sequence pairing, higher complementarity between the 3'UTR MRE and miRNA seed is indicative of greater repressive efficacy (Figure 7) [39]. mRNAs with longer 3'UTRs are more susceptible to post-transcriptional regulation, as they contain numerous cis- and trans-acting elements recognized by regulatory components such as miRNAs and RBPs [53, 54]. MECP2 has one of the longest 3'UTRs in the mammalian transcriptome, with identified 3'UTR isoforms ranging from 1.8 to 10.2 kilobases (kb) compared to the average length of 0.8 kb [54]. A statistical model of canonical miRNA binding with high stringency parameters predicted at least 35 distinct MREs on the MECP2 3'UTR, each serving as the regulatory site of several repressive miRNAs (Figure 5; Chapter 1) [54].

![Figure 7. Classification of Canonical MREs and Relative Efficacies.](image)

Canonical MREs are classified by their complementarity to miRNA seed regions. Each site has a minimum 6 – 7 nt match with respective miRNAs. The most effective MREs (7mer-m8, 8mer) have a complete match to the miRNA seed region, while the 8mer contains an additional A at position 1. Relative efficacies are graphed in log scale. Adapted from [39].
By virtue of the small regulatory effects of an individual miRNA, MRE-targeted sbASOs can finely tune MeCP2 expression and allow for controlled de-repression of MeCP2 levels within the therapeutic limits. Further, the prevalence of predicted MREs on the MECP2 3′UTR and its susceptibility to post-transcriptional regulation presents an abundance of potential targets for our sbASO approach. Recent studies investigating MECP2-related noncoding RNAs (ncRNAs) have identified several miRNAs that directly regulate MECP2 expression and many others whose expression is correlated with MeCP2 levels [63, 82-84]. miRNAs that directly repress MECP2 are apparent candidates for our sbASO-mediated approach, but many confounds in RTT impact their clinical applicability. For instance, the selection of target miRNAs is complicated by cell-type-specific differences in miRNA expression patterns [83, 85]. Likewise, the expression of the target miRNA may change with development, such that its capacity to repress MeCP2 expression changes with time [53]. Alternatively, a target miRNA might play a critical role in regulating MeCP2 in response to activity-dependent molecular contexts, in which case sbASO-inhibition would result in adverse effects [39]. Unfortunately, many of these factors are presently unknown variables that must be defined empirically. As such, our sbASO target selection criteria were limited to miRNAs with observed repression of MeCP2 expression and their predicted MREs on the MECP2 3′UTR. The overarching goal of the first aim is to develop a series of sbASOs targeting new MREs to either 1) establish a target site unaffected by the aforementioned confounds, or 2) identify a sufficient number of MECP2-repressive MREs that can be targeted in aggregate, such that the additive effects dilute the impact of any one variable.
To achieve these goals, we identified three novel target miRNAs that have been reported to repress MECP2 expression in the literature: miR-181a-5p, miR-422a, and let-7c-5p. The first miRNA of interest, miR-181a-5p, is upregulated in the brains of RTT mouse models and was demonstrated to bind the MECP2 3'UTR and inhibit MeCP2 expression in vitro [63, 86]. Second, miR-422a represses MeCP2 in human bone marrow mesenchymal stem cells during adipogenesis and its expression is not affected by MECP2 silencing upon differentiation into adipocytes [83]. These studies used miR-422a mimics and inhibitors as well as mutated 3'UTR constructs to establish that miR-422a directly binds MECP2 transcripts and represses expression [83]. Third, let-7c-5p binds a known MRE on MECP2 3'UTR and inhibits translation in human induced neurons (iN), while overexpression of let-7c-5p in the iN results in MeCP2 deficiency and RTT-like neuronal morphology [84]. Potential target binding sites for each miRNA were mapped on the mRNA transcript of the dominant 3'UTR isoform in mature neurons (8.8 kb) by identifying sequences in the transcript that were complementary with miRNA seed regions. Seed sequences are located in nt positions 2 – 7 (seed ‘regions’ 2 – 8 nt) in miRNA and allow target element recognition and binding via complementarity (Figure 7) [39, 55]. Since all but one member of the lethal-7 (let-7) family of miRNA share the same seed sequence – which was the primary criteria for potential MRE selection – sbASOs aimed at let-7c-5p sites were more broadly corrected to “let-7-5p” sbASOs. There was only one perfect seed match for each miRNA despite the length of the MECP2 3'UTR.
Identification of Potential MREs and sbASO Design

Prospective MREs were identified by locating miRNA seed-complementary 3'UTR sequences using the human long MECP2 transcript from NIH GenBank. We searched for complete seed sequence matches (7mer-8, 8mer) and subsequently designed sbASOs that were 21 to 22 nucleotides (nt) in length, such that they entirely blocked the MRE (7 – 8 nt) and the surrounding sequence specific to the MECP2 3'UTR (Table 1). sbASOs were formulated with phosphorothioate (PS) backbones and 2’-O-methoxyethyl (2'MOE) nucleotide modifications to increase in vitro thermal stability, nuclease resistance, target binding affinity, and cellular uptake [75].

Table 1: sbASO Sequences and Respective MRE Classification and Position. Full 21 or 22 nt length of sbASO and the predicted MRE classification and location on MECP2 3'UTR (8.8 kb). Bolded and underlined regions are identical to respective miRNA seed sequences. sbASO sequences are fully complementary to target 3'UTR sites.

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sbASO-let-7 and sbASO-miR-181a Increase MeCP2 Expression in SH-SY5Y Cells

To establish their efficacy in increasing MeCP2 expression, each of the three new sbASOs was transfected with Lipofectamine™ 3000 for dose response in SH-SY5Y human neuroblastoma cells. Nuclear protein was isolated 24 hours post-transfection probed for
MeCP2 via immunoblotting (Figure 8). 4 – 5 passage-matched replicates were used for each tested concentration of sbASO tested (3 pmol, 10 pmol, 55 pmol, 100 pmol, 250 pmol) after outlier removal via Grubb’s tests performed at $\alpha=0.1$ (with the exception of 3 pmol sbASO-let-7, for which $n = 3$). MeCP2 levels at each sbASO concentration were normalized to levels of the internal control (histone H3) and resultant quantifications were normalized to vehicle-treated controls and individually analyzed via unpaired t-tests, while the total sbASO treatment effect across all concentrations was analyzed via ordinary one-way ANOVAs in each cell line.

**Figure 8. sbASO Dose Response Experimental Protocol.** Cells were transfected with sbASOs for 12 – 18 hours, then maintained in growth medium for 24 hours before collection for processing. Nuclear protein was isolated via lyse & wash fractionation [87] and separated by SDS-PAGE for immunoblotting. Created with [Biorender.com](http://www.biorender.com).

Western blot analysis demonstrated that sbASO-miR-181a and sbASO-let-7 significantly increased MeCP2 protein levels across multiple concentrations (Figure 9A-B); however, sbASO-let-7 did not show an overall treatment effect due to high variance ($p = 0.1047$, one-way ANOVA). sbASO-miR-422a had no effect on MeCP2 expression at any concentration (Figure 9C). Importantly, sbASO-miR-181a exhibited a broad treatment effect
across all concentrations and consistently increased MeCP2 expression by ~200%, demonstrating a stable treatment threshold within dosage restrictions (Figure 9A; p = 0.002, one-way ANOVA). Relative to the other sbASOs tested, sbASO-miR-181a had higher efficacy, wherein it achieved the same treatment effect at lower doses (Figure 9A). sbASO-let-7 had a more potent effect on MeCP2 levels, peaking at approximately a 300% increase in expression compared to vehicle-treated controls (Figure 9B).

**Figure 9. sbASO Dose Response in SH-SY5Y cells.** Cells were treated with sbASO-miR-181a (A), sbASO-let-7 (B), and sbASO-miR-422a (C) at 3 pmol, 10 pmol, 55 pmol, 100 pmol, and 250 pmol and assayed following the experimental protocol in Figure 8. **A)** sbASO-miR-181a significantly increased MeCP2 levels to ~200% of vehicle-treated controls at all doses except 250 pmol, indicating a low “ceiling effect” (p = 0.002, one-way ANOVA). **B)** sbASO-let-7 significantly increased MeCP2 levels at 3 pmol, 55 pmol, and 100 pmol; but there was extreme variance across samples, indicating unstable treatment effects (p = 0.1047, one-way ANOVA). **C)** sbASO-miR-422a had no treatment effect. MeCP2 protein levels in each concentration were normalized to H3 levels then normalized to vehicle control. All data are shown as mean ± SEM. Outliers were removed by Grubb’s tests at $\alpha = 0.1$, n = 4 – 5 per concentration with the exception of 3 pmol sbASO-let-7, n = 3. Statistical significance for individual concentrations was determined by unpaired t-tests and is denoted as follows: p < 0.5*, 0.01**, 0.001***, and 0.0001****.
sbASO-miR-181a Achieves Ceiling Effect at Lower Doses in SH-SY5Y Cells

SH-SY5Y cells treated with sbASO-miR-181a exhibited a peak in MeCP2 increase that reached a plateau even at the lowest concentration tested (3 pmol). To establish a dose-response relationship for sbASO-miR-181a, we treated SH-SY5Y cells with lower concentrations at 0.1 pmol, 0.5 pmol, and 1 pmol, then followed the same experimental protocol used in the initial sbASO dose response studies. When assessed by western blot, all three lower doses of sbASO-miR-181a increased MeCP2 expression to levels below the demonstrated ceiling effect at 3 pmol, confirming a dose-response relationship (Figure 10).

Figure 10. sbASO-miR-181a Complete Dose Response in SH-SY5Y cells. Cells were treated with sbASO-miR-181a at 0.1, 0.5, and 1 pmol and assayed following the experimental protocol in Figure 8. sbASO-miR-181a significantly increased MeCP2 protein levels even at very low doses (p = 0.0031, one-way ANOVA) indicating high sbASO efficacy. All data are shown as mean ± SEM. Outliers were removed by Grubb’s tests at α = 0.1, n = 4 – 5 per concentration. Statistical significance for individual concentrations was determined by unpaired t-tests and is denoted as follows: p < 0.5*, 0.01**, 0.001***, and 0.0001****.
**sbASO-miR-181a Increases MeCP2 Expression in RTT Patient-Derived Fibroblasts**

Our initial series of sbASOs (sbASO-miR-22, sbASO-miR-132/212, sbASO-miR-483) showed mutation-specific patterns of efficacy (Figure 17). Since sbASO-miR-181a treatment demonstrated stable increases in MeCP2 expression within the therapeutic window in SH-SY5Y cells, it was further tested for dose response in five RTT patient-derived fibroblast lines representing common MECP2 mutations (R133C, T158M, R306C, R270X, R294X). Due to the instability of the R270X mutant, the truncated protein product was not detectable via western blot, whereas the R294X mutation produced a stable truncated protein (Figure 11D-F) [21]. The R294X mutant has a stronger binding affinity to chromatin compared to WT, which may also explain why the truncated protein product was more stable and readily detected in R294X fibroblasts (Figure 11E) [88]. In contrast to the observations made with the three initial sbASOs, sbASO-miR-181a consistently increased MeCP2 expression across the common MECP2 mutations in a manner capped at subtoxic levels (Figure 11). Significant treatment effects in both missense (R133C, T158M, R306C; Figure 11A-C) and truncating mutations (R270X, R294X; Figure 11D-F) were confirmed by one-way ANOVAs (p = 0.021, 0.0005, 0.0111, 0.0422, and 0.0366, for R133C, T158M, R306C, R270X, and R294X, respectively). As stated above, no other sbASO developed for this approach has exhibited this consistent pattern of efficacy across MECP2 mutations, suggesting that sbASO-miR-181a may hold greater therapeutic potential for clinical applications. The observed inverted-U dose response curve did not correspond with cell toxicity, implying that sbASO efficacy may be capped by endogenous regulatory mechanisms at higher concentrations.
Figure 11. sbASO-miR-181a Dose Response in RTT Patient-Derived Fibroblasts. R133C (A), T158M (B), R306C (C), R270X (D), and R294X (E-F) mutant fibroblasts were treated with sbASO-miR-181a at 3 pmol, 10 pmol, 55 pmol, 100 pmol, and 250 pmol and assayed following the experimental protocol in Figure 8 and measured for MeCP2 protein levels. One-way ANOVA results indicate sbASO-miR-181a has significant treatment effects in all mutations (A-E: p = 0.021, 0.0005, 0.0111, 0.0422, and 0.0366). All data are shown as mean ± SEM. Outliers were removed by Grubb’s tests at α = 0.1, n = 4 – 8 per concentration per mutant fibroblast line. Statistical significance for individual concentrations was determined by unpaired t-tests and are denoted as follows: p < 0.5*, 0.01**, 0.001***, and 0.0001****.
sbASO Treatment Does Not Affect *MECP2* mRNA in SH-SY5Y Cells

We next examined *MECP2* mRNA levels to determine whether the endogenous target miRNAs being occluded by sbASOs were repressing MeCP2 expression through transcriptional or translational mechanisms. Total RNA was isolated from SH-SY5Y cells treated at 100 pmol sbASO (sbASO-miR-22, sbASO-miR-132/212, sbASO-miR181a, sbASO-miR-483) (Figure 8) and used for cDNA synthesis, and *MECP2* mRNA expression was quantified via qRT-PCR. 100 pmol was selected as a universal test dosage because all four sbASOs exhibited significant treatment effects on MeCP2 levels at this concentration. Analysis of all four sbASO treatments quantified no significant change in mRNA levels despite the increase in protein levels. These data suggest that the endogenous target miRNAs do not destabilize the *MECP2* transcript, but rather mediate their effects through translational repression (Figure 12).
Figure 12. MECP2 mRNA Levels in sbASO-treated SH-SY5Y Cells. Cells were treated with sbASO-miR-22, sbASO-miR-132/212, sbASO-miR-483, and sbASO-miR-181a at 100 pmol following the protocol in Figure 8. Collected cells were processed for mRNA analysis via subsequent qRT-PCR. There were no changes in MECP2 mRNA levels in response to sbASO treatment. All data are shown as mean ± SEM, n = 4 – 6 per sbASO. Outliers were removed by Grubb’s tests at α = 0.1.
CHAPTER 5
SPECIFIC AIM 2

Rationale

RTT patient brains present with genome-wide disruptions in miRNA expression, which is explained in part by the regulatory interactions between MeCP2 and miRNA [40, 78, 79]. MeCP2 binds the Microprocessor complex subunit DiGeorge syndrome critical region 8 (DGCR8) to suppress the maturation of miRNAs that regulate critical genes during neurodevelopment [40]. Example genes targeted by MeCP2-inhibited miRNAs include CREB (cAMP response element binding protein), LIMK1 (LIM domain kinase 1), and PUM2 (Pumilio 2) (Figure 2; Chapter 1) [40]. MeCP2 can also enhance miRNA biogenesis by binding directly to methylated miRNA gene boundaries to stall RNA polymerase II (RNAPII) elongation, enabling co-transcriptional assembly of the Microprocessor complex (Figure 3; Chapter 1) [42]. In turn, dozens of miRNAs bind MREs on the MECP2 3'UTR and repress MeCP2 levels – to the point of silencing in some contexts – by destabilizing the transcript, leading to its degradation (Figure 5; Chapter 1) [54, 55]. These activity-dependent miRNAs serve as a mechanism to titer MECP2 levels to satisfy changing spatiotemporal dosage requirements [55, 83].

Previous biophysical studies have identified structural and functional differences across common MECP2 mutations, including alterations in DNA and nucleosomal binding
affinity, nucleolar and chromatin localization, and structural stability to different extents [33, 48, 62, 72]. The functional consequences of these mutations are further influenced by intrinsic factors such as X-chromosome inactivation (XCI) and post-transcriptional or post-translational regulation of the mutant MeCP2 [6, 7, 62]. The culmination of these mutation-induced effects is manifested in the disease-severity gradient across RTT-causing MECP2 mutations. For example, the common missense mutation T158M results in a destabilized protein structure with a partially functional MBD (methyl-CpG-binding domain) [71]. The partial retention of function produces a milder phenotype than truncating mutations such as R255X in which the entire MBD is lost [71, 89]. The extent of systemic disruption caused by each mutation is the driving force behind the varying symptom severity of RTT and has genotype-specific implications for gene-targeted therapeutic approaches.

Considering the role of MeCP2 in miRNA biogenesis and processing, and the known structure-function differences of mutant MeCP2 protein, we hypothesized that RTT-pathogenic mutations have distinct effects on miRNA expression. Such differences would undoubtedly contribute to mutation-specific presentations of disease severity. Importantly, altered miRNA levels across mutations would directly impact the efficacy of sbASOs designed to sterically inhibit miRNA regulation of MECP2 expression. Preliminary data in RTT patient-derived fibroblasts have demonstrated the initial three sbASOs do not perform consistently across MECP2 mutations. These efficacy patterns were highly unstable and, in some cases, even contradictory. One such instance is sbASO-miR-22, which readily increased R294X MeCP2 levels at all tested concentrations (Figure 13F) but elicited an observed significant decrease in T158M MeCP2 expression at 250 pmol (Figure 13B; p < 0.01,
unpaired t-test; significance not denoted); further, its dose response in R294X fibroblasts (WT and R294X MeCP2) was extremely variable (Figure 13E-F). sbASO-miR-132/212 had the strongest performance among the three when tested in mutant fibroblasts, but its efficacy was highly inconsistent, often presenting only a single effective concentration across the dose response (Figure 13). These data support the possibility of mutation-dependent miRNA expression and may suggest the need for genotype-specific refinement of sbASO treatments or, preferentially, the identification of target miRNAs whose expression is unaffected by MeCP2 dysfunction in RTT. The main objective of the second aim is to test the hypothesis that distinct RTT-pathogenic MECP2 mutations have unique effects on miRNA expression that contribute to mutation-specific patterns of sbASO efficacy.

To explore these potential mutation-dependent effects, we quantified target miRNA levels (miR-22-3p, miR-483-5p, and miR-132/212-3p, hsa-miR-181a-5p) in five RTT patient fibroblast lines to correlate in vitro efficacy data with a quantitative measure of the target miRNA. In parallel, we assessed global miRNA expression profiles in postmortem temporal cortex samples from T158M and R255X RTT patients using nCounter® miRNA Expression Panels. We hypothesized that such a correlation which enable us to improve our selection criteria for potential sbASO targets with greater clinical applicability.
**Figure 13. Mutation-Specific sbASO Efficacies in RTT Patient-Derived Fibroblasts.**

R133C (A), T158M (B), R306C (C), R270X (D), and R294X (E-F) mutant fibroblasts were treated with sbASO-miR-22 (red circle), sbASO-miR-132/212 (yellow square), and sbASO-miR-483 (blue triangle) at 3 pmol, 10 pmol, 55 pmol, 100 pmol, and 250 pmol and assayed following the experimental protocol in Figure 8 for MeCP2 protein quantification. sbASO efficacies were highly variable across fibroblast lines, suggesting mutation-specific differences influenced treatment effect. All data are shown as mean ± SEM (*upper SEM is curtailed in F). Outliers were removed by Grubbs’ tests at $\alpha = 0.1$, n = 3 – 5 per concentration per mutant fibroblast line. Statistical significance for increases in MeCP2 levels at individual concentrations was determined by unpaired t-tests and is denoted in sbASO-corresponding colors as follows: p < 0.5*, 0.01**, and 0.001***, 0.0001****. Figure key is displayed to the upper right.
miR-132/212-3p is Upregulated in RTT-Patient Derived Fibroblasts

Preliminary data from sbASO-miR-22, sbASO-miR-132/212, and sbASO-miR-483 showed mutation-specific patterns of efficacy in RTT patient-derived fibroblasts (Figure 13). We hypothesized that this variability was mediated by corresponding mutation-specific patterns of expression of the target miRNAs. To examine these potential differences, we isolated total miRNA from five different RTT patient-derived mosaic fibroblast lines expressing missense (R133C, T158M, R306C) and nonsense (R270X, R294X) mutations (n = 3 – 5 per mutation), and quantified target miRNA expression (miR-22-3p, miR-132/212-3p, miR-181a-5p, miR-483-5p) via qRT-PCR. Levels of each miRNA were normalized to U6 snRNA then compared to isogenic WT controls obtained from heterozygous T158M fibroblasts. There was insufficient expression of miR-483-5p across mutant fibroblasts for reproducible detection.

Despite high variance in miRNA levels across each sample (likely owing to the heterogeneity of fibroblast cultures), T158M and R270X (severe clinical phenotypes) had significantly elevated levels of miR-132/212-3p (Figure 14B). No significant difference was observed in miR-22-3p in both T158M and R270X mutant fibroblasts (Figure 14A). There were no significant changes in miR-181a-5p expression across RTT patient-derived fibroblasts with the exception of R294X, which exhibited a significant 0.29-fold decrease (Figure 14C). These results further support the speculation that miR-181a-5p is expressed in a MeCP2-independent manner. When analyzed in concert with the dose response data, no correlation could be established between endogenous miRNA levels and the variance of sbASO efficacy across cell lines. This suggests that factors external to miRNA abundance contribute to
sbASO-mediated derepression of MeCP2 which will need to be identified in future studies for optimization of sbASO design.

**Figure 14. Expression of Target miRNAs in RTT Patient-Derived Fibroblasts.** Total miRNA samples isolated from R133C, T158M, R270X, R294X, and R306C mutant fibroblasts were used to quantify levels of miR-22-3p (A), miR-132/212-3p (B), miR-181a-5p (C), and miR-483-5p (not shown) by qRT-PCR. All miRNA were normalized to U6 snRNA then compared to isogenic WT controls obtained from heterozygous T158M fibroblasts. **A)** No significant changes in miR-22-3p expression were detected, although an upward trend was observed in T158M and R270X. **B)** miR-132/212-3p was significantly increased in all mutations except R306C (p = 0.0155, 0.0033, 0.0067, 0.0178 for R133C, T158M, R270X, and R294X, respectively), and especially upregulated T158M (7.09-fold). **C)** There were no significant changes in miR-181a-5p expression in all mutations except R294X, in which a small significant change (0.29-fold) was detected (p = 0.05). All data are shown as mean + SEM, n = 3–5 per mutant fibroblast line. Statistical significance determined by Student’s t-tests is denoted as follows: p < 0.05*, 0.01**.
miRNAs are Differentially Expressed in RTT Patient Temporal Cortices

Age, sex, and PMI (postmortem interval) matched temporal cortex samples were obtained from neurotypical controls and RTT patients (T158M and R255X). Total miRNA was isolated using PureLink™ miRNA Isolation Kits, then profiled using nCounter® miRNA Expression Panels, which utilize an amplification-free quantification of miRNAs via molecular bar codes. The sample size for PMI-matched neurotypical controls, T158M, and R255X temporal cortices were 5, 3, and 4, respectively (Table 2). Quantification values from RTT patient samples were normalized to total RNA then compared to relative expression in neurotypical controls. When analyzed as an “all RTT” group that combined both mutations, we identified a total of 66 significantly downregulated and 74 significantly upregulated differentially expressed miRNAs (DEMs) compared to controls; the top 15 of each are shown in Figures 15 and 16.

Table 2. Postmortem Temporal Cortex Samples. T158M, R255X, and control samples obtained from IRSF were matched in age, sex, and PMI. Respectively, n = 3, 4, and 5. Average age and PMI of each sample group are listed.

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<tr>
<td></td>
<td>21.33</td>
<td>25.63</td>
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<table>
<thead>
<tr>
<th></th>
<th>R255X Average</th>
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<tr>
<td></td>
<td>13.75</td>
<td>11.28</td>
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<table>
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<tr>
<th></th>
<th>CTL Average</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>18.20</td>
<td>18.60</td>
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</table>
Among the 66 downregulated DEMs, 18 were also significantly decreased in the T158M subpopulation while 24 were significantly decreased in the R225X subpopulation. Only 9 DEMs were common to both mutations (Table 3). Within the 74 upregulated DEMs, 55 were significantly increased in the T158M subpopulation while 40 were significantly increased in the R255X subpopulation, with 35 common DEMs to both mutations (Table 3). While the overlap between genotypes was not complete, the majority of DEMs were conserved, and in contexts where they were not, the differential expression approached statistical significance. This suggests that both T158M and R255X mutations caused similar patterns of miRNA dysregulation.
Figure 15. Top 15 Upregulated DEMs in RTT Temporal Cortices. All 15 miRNAs were significantly increased when analyzed across both T158M and R255X mutations (purple). The range of changes in miRNA levels was 6.4-fold (miR-126-3p in R255X) to 396.4-fold (miR-29b-3p in T158M). All data are shown as mean ± SEM, n = 3, 4, and 5, for T158M, R255X, and control samples, respectively. Statistical significance of DEMs in combined RTT group was determined by Student’s t-tests, p < 0.05. Figure key is displayed to the right.
Figure 16. Top 15 Downregulated DEMs in RTT Temporal Cortices. All 15 miRNAs were significantly decreased when analyzed across both T158M and R255X mutations (purple). The range of changes in miRNA levels was -7.3-fold (miR-4516 in T158M) to -2.32-fold (miR-203a-5p in R255X). All data are shown as mean – SEM, n = 3, 4, and 5, for T158M, R255X, and control samples, respectively. Statistical significance of DEMs in combined RTT group was determined by Student’s t-tests, p < 0.05. Figure key is displayed to the upper right.
Interestingly, the magnitude and range of differential expression were far greater in upregulated DEMs compared to downregulated DEMs, wherein the average of fold changes was 16 for the former and -3 for the latter (Table 3). Within the 74 upregulated DEMs, the T158M subpopulation exhibited dramatically higher levels of expression compared to the R255X subpopulation (Table 3) such that the average difference between the two groups was 10-fold while the largest difference was 275-fold. These results suggest that the dominant regulatory role of MeCP2 in miRNA biogenesis is inhibition and that the T158M mutation is particularly deleterious for this function.

**Table 3. DEMs in T158M and R255X RTT Patient Temporal Cortices.** The number of upregulated (+) and downregulated (–) DEMs in postmortem RTT patient temporal cortices and the average fold change (FC) of significant DEMs in each of the combined All RTT group and the T158M and R255X subpopulations.

<table>
<thead>
<tr>
<th></th>
<th>+ DEMs (p &lt; 0.05)</th>
<th>Average FC of + DEM</th>
<th>– DEMs (p &lt; 0.05)</th>
<th>Average FC of – DEM</th>
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</thead>
<tbody>
<tr>
<td>All RTT</td>
<td>74</td>
<td>16.31</td>
<td>66</td>
<td>-3.05</td>
</tr>
<tr>
<td>T158M</td>
<td>55</td>
<td>28.92</td>
<td>18</td>
<td>-4.20</td>
</tr>
<tr>
<td>R255X</td>
<td>40</td>
<td>15.74</td>
<td>24</td>
<td>-3.65</td>
</tr>
</tbody>
</table>

**miR-22-3p and miR-132/212-3p are Upregulated in RTT Temporal Cortices**

Among our four sbASO-target miRNAs, miR-22-3p and miR-132/212-3p were significantly upregulated across both mutations, while miR-483-5p levels were too low for detection by the nCounter® assay (Figure 17). The primary miRNA of focus in this thesis (miR-181a-5p) exhibited no significant differential expression compared to neurotypical controls, suggesting that its expression is not reliant on MeCP2 functionality (Figure 17), which was in line with our observations in patient-derived fibroblasts. Notably, members of the let-7
family (let-7d-5p, ~7-fold; let-7e-5p, ~12-fold; let-7g-5p, ~6-fold; let-7b-5p, ~3.5-fold) were significantly increased in RTT samples while let-7c-5p was among the most upregulated DEMs (~60-fold). These data suggest that the pri-miRNA of let-7 (pri-let-7) is a direct target of MeCP2 inhibition during miRNA processing (Figure 18).

Figure 17. Expression of Target miRNAs in RTT Temporal Cortices. miR-132/212 and miR-22-3p were significantly upregulated across both T158M and R255X mutations, while miR-181a-5p showed no significant change in either mutation (though in R255X, there was an insignificant 2.6-fold increase). miR-483-5p (not shown) had undetectable expression in all samples. All data are shown as mean + SEM, n = 3, 4, and 5, for T158M, R255X, and control samples, respectively. Statistical significance determined by Student’s t-tests is denoted as follows: p < 0.05*, 0.01**, 0.001***. Figure key is displayed to the right.
Figure 18. Expression of hsa-let-7 miRNA Family in RTT Temporal Cortices. Members of the let-7 miRNA family were significantly upregulated across both mutations. In particular, let-7c-5p was dramatically increased (78.54-fold in T158M, 47.37-fold in R255X). All data are shown as mean + SEM, n = 3, 4, and 5, for T158M, R255X, and control samples, respectively. Statistical significance determined by Student’s t-tests is denoted as follows: p < 0.05*, 0.01**, 0.001***. Figure key is displayed to the right.
CHAPTER 6
DISCUSSION

Summary of Findings

Ongoing efforts to develop MECP2-targeted therapeutics for RTT are challenged by its high dosage sensitivity and wide involvement in essential cellular processes. As an alternative to traditional gene therapies, we have developed a sbASO-mediated (site-blocking antisense oligonucleotides) approach to disinhibit endogenous post-transcriptional regulation of MECP2. The goal of this strategy is to block repressive microRNAs (miRNAs) from binding to the MECP2 3'UTR (untranslated region) by using sbASOs designed with sequence-specificity to predicted miRNA response elements (MREs). The MRE-targeted sbASOs used in these experiments were chemically modified with a phosphorothioate (PS) backbone and 2'-O-methoxyethyl (2'MOE) bases to enhance binding affinity and stability for experimental and clinical applications. This thesis established the viability of this approach in vitro, wherein sbASO treatment successfully increased MeCP2 expression to subtoxic levels in both SH-SY5Y cells and RTT patient-derived fibroblasts. Importantly, the newly developed sbASO-miR-181a displayed consistent efficacy across five of the most common RTT-pathogenic MECP2 mutations, indicating strong clinical potential (Figure 11). Further, we show that expression of miR-181a-5p is unaffected by loss of MeCP2 in postmortem RTT patient brains, suggesting that it holds a repressive function that does not result in feedback inhibition. The results of this thesis not only demonstrate
the therapeutic feasibility of the sbASO-mediated disinhibition approach but also provide valuable insights into the epigenetic regulatory mechanisms underlying sbASO efficacy to further develop the strategy for clinical applications in RTT.

**miRNA-Mediated Repression of MECP2**

Mature miRNAs are bound to Argonaute (AGO) proteins and serve as guide strands of the miRISC to identify target mRNAs for repression [39]. Once bound to a target, the miRISC mediates post-transcriptional repression by destabilizing the transcript or deterring translation efficiency [39]. While miRISC-induced slicing of mRNA is more common in plants, mammalian miRNAs predominantly repress target transcripts through translation interference followed by transcript degradation via recruitment of TNRC6A (trinucleotide repeat-containing adaptor 6A protein), which is slower in comparison [39]. TNRC6A interacts with the poly-A tail of the mRNA and recruits deadenylase complexes (PAN2-PAN3 complex, CCR4-NOT complex) that destabilize the transcript and helicases (DDX6) that mediate translation inhibition [39].

In line with these mechanisms, sbASO treatment did not affect MECP2 mRNA expression in SH-SY5Y cells (Figure 12) despite significantly increasing MeCP2 protein levels (Figure 9). These results suggest that our target miRNAs mediate translational repression. Nonetheless, it is worth noting the inherent difficulty in isolating the specific effects of an individual miRNA, particularly in contexts where the target transcript remains subject to additional regulatory mechanisms. Provided the length of the MECP2 3’UTR and its known susceptibility to post-transcriptional regulation, sbASO effects at the transcript level could be diluted by other MECP2-targeted regulatory elements (such as miRNAs and RBPs),
contributing to the lack of detectable changes in mRNA. Further, due to the extreme dosage sensitivity of MECP2, even slight changes in its expression can trigger endogenous safeguards, wherein sbASO-induced MeCP2 levels could upregulate post-transcriptional repression. When these factors are taken into consideration, the absence of sbASO-mediated changes in mRNA levels may not fully reflect the miRNA’s mechanism of repression.

A single mRNA can simultaneously bind several ribosomes and undergo multiple rounds of translation, resulting in higher protein output per transcript [54, 90]. The long MECP2 3'UTR isoform is highly stable in mature neurons (barely detectable decay after 5 hours), and it is well-established that 3'UTR length is correlated with ribosome engagement [53, 54]. The MECP2 3'UTR is 8.6 kb compared to the average human 3'UTR of 0.8 kb, allowing for increased ribosome engagement in the coding sequence. Further, it has been demonstrated that active translation impedes miRISC interaction with target transcripts, indicating that an increase in protein expression is accompanied by a decreased chance of being targeted by other repressive miRNAs [90]. Based on the results of our studies, we can conclude that the sbASO-induced increase in MeCP2 expression is mediated by the enhanced translation efficiency of sbASO-bound mRNAs, which may simultaneously decrease its likelihood of being targeted by other MECP2-repressing miRNAs.

**Contributing Factors of Mutation-Specific sbASO Efficacy**

**Influence of Differential miRNA Expression**

The genome-wide disruption of miRNA expression in RTT is the result of both direct and indirect consequences of MeCP2 dysfunction [40, 41, 79, 80]. The primary effects of
MeCP2 mutations on gene expression patterns lead to compensatory changes in noncoding RNA (ncRNA), wherein levels of post-transcriptional regulators such as miRNA are adjusted to buffer against harmful gene dosages. MeCP2 also directly regulates miRNA biogenesis through several mechanisms; it can inhibit miRNA processing by sequestering DGCR8 or promote pri-miRNA transcription by stalling RNAPII at methylated miRNA gene boundaries to allow for Microprocessor assembly [40, 42]. The location of each MECP2 mutation has potentially significant implications on this regulatory function. For example, the R306C mutation is located in a non-canonical RNA-binding motif within the TRD, disrupting MeCP2 localization to pri-miRNA transcription sites for DGCR8 sequestration [6, 48]. The presence of mutation-specific expression of miRNA creates a scenario in which sbASOs, whose effects are dependent on miRNA-mediated repression, could also have mutation-specific patterns of efficacy.

As a rationale for this hypothesis, preliminary data from sbASO-miR-22, sbASO-miR-483, and sbASO-miR-132/212 revealed variable sbASO efficacy across RTT-causing MECP2 mutations (Figure 13). While all three sbASOs showed dose response relationships when tested in SH-SY5Y cells (Figure 19), their treatment effects were not maintained across mutant fibroblast lines. Of the three newly developed sbASOs in this thesis, sbASO-miR-181a exhibited consistent dose responses in T158M, R294X, R306C, R133C, and R270X mutant fibroblast lines, suggesting a mutation-independent mechanism of treatment efficacy (Figure 11).
**Figure 19. Preliminary Data of sbASO Dose Responses in SH-SY5Y Cells.** Dose response curves of sbASO-miR-22 (red circle), sbASO-miR-132/212 (yellow square), and sbASO-miR-483 (blue triangle) at 3 pmol, 10 pmol, 55 pmol, and 100 pmol. All three sbASOs demonstrated efficacy at individual concentrations. All data are shown as means, n = 3 – 5 per sbASO. Statistical significance determined by unpaired t-tests is denoted as follows: p < 0.05*, 0.01**, 0.001***.

Considering MeCP2’s role in miRNA biogenesis and regulation, we questioned whether miRNA expression was differentially impacted by each mutation. Though target miRNA expression was variable, potentially due to the heterozygous mosaicism of the fibroblasts, miR-132/212-3p was significantly upregulated about 3 to 7-fold in mutant cell lines (Figure 14A), whereas miR-181a-5p was more steadily expressed across mutations (Figure 14C). These results aligned with miRNA profiles of postmortem RTT patient temporal cortices, which indicated that miR-132/212-3p and miR-22-3p were upregulated approximately 6 to 10-fold across T158M and R255X mutations, whereas there was no significant difference in miR-181a-5p levels (Figure 17). Further, there was a negligible
expression of miR-483-5p in both RTT patient-derived fibroblasts and temporal cortices, in line with preliminary data showing a lack of stable sbASO-miR-483 effects in all mutant cell lines (Figure 13). The mutation-dependent overexpression of target miRNAs could explain the strong performance of sbASO-miR-132/212 in the T158M and R294X fibroblast lines, as it blocks the potent miR-132/212-mediated repression of MeCP2 expression. However, this creates a potential confound by which sbASOs targeting MeCP2-dependent miRNA may lack stable efficacy over time (discussed below).

In healthy brains, miR-132/212-3p is involved in a regulatory feedback loop with BDNF and MeCP2, wherein MeCP2 and miR-132/212-3p mutually exert negative feedback on the other in a BDNF-mediated manner (Figure 20) [19, 80, 91, 92]. As a result, miR-132/212-3p is overexpressed in RTT neurons, exacerbating the deficiency of MeCP2 through further post-transcriptional repression (Figure 20) [19, 80, 91, 92]. This signifies that miR-132/212-3p is expressed in a MeCP2-dependent manner, which impacts the potential treatment effect of the respective sbASO. It is possible that sustained sbASO-induced increases in MeCP2 levels may result in the downregulation of target miRNAs with MeCP2-dependent expression, ultimately negating the effects of the sbASO (Figure 20). In fact, sbASO-miR-132/212 had inconsistent results across MECP2 mutations and typically required higher average concentrations for treatment effect, evidencing the variability of miR-132/212-3p expression (Figure 13).
Figure 20. Schematic of Potential sbASO-Mediated Downregulation of Target miRNAs with MeCP2-Dependent Expression. **Top:** Mutual negative feedback relationship of MeCP2 and miR-132/212-3p in healthy neurons. **Middle:** Reduction of negative feedback on miR-132/212-3p expression due to MeCP2-deficiency in RTT. **Bottom:** sbASO-miR-132/212 may have significant efficacy by blocking the strong repression of MECP2 transcripts mediated by miR-132/212-3p. Over time, sbASO-induced increases in MeCP2 levels may downregulate miR-132/212-3p, negating treatment effects due to lack of miR-132/212-3p-mediated MECP2 repression.

The miR-181 family is highly conserved across species and participates in essential functions including stem cell proliferation/differentiation, metabolism, and homeostasis, and their differential expression has been implicated in oncogenesis and hematological abnormalities [93-95]. While the regulatory mechanisms are unclear, the stability of miR-181a-5p levels across prevalent MECP2 mutations suggests that its expression is not subject to feedback loops as that of miR-132/212-3p. Stable levels of the target miRNA
would likely be indicative of mutation-independent efficacy, which is consistent with the results of our sbASO-miR-181a studies in RTT patient-derived fibroblasts (Figure 11). Previous studies in miRNA KO mice have shown that removal of other target miRNAs (miR-22-3p, miR-132/212-3p, let-7c-5p) results in moderate to severe phenotypes, whereas miR-181a-5p deletion is embryonically lethal, demonstrating that miR-181a-5p-directed gene repression is vital [39]. This suggests that targeted mRNA-3’UTRs may have conserved regulatory interactions with miR-181a-5p, which accounts for its stable repression of MECP2 transcripts and its MeCP2-independent expression, contrary to miR-132/212-3p.

In our analysis, members of the let-7 miRNA family were significantly upregulated in both T158M and R255X temporal cortices (Figure 18). The let-7 miRNAs show high levels of evolutionary conservation and have well-documented biological functions in critical processes such as differentiation and tumor suppression [96]. Consequently, dysregulation of let-7 expression results in pathological physiology, and hence its expression is tightly controlled through multifaceted regulatory mechanisms [96]. The significant upregulation of let-7 miRNAs, particularly with respect to let-7c-5p (~70-fold in all RTT samples), further corroborates the biological significance of MeCP2-mediated inhibition of miRNA and the contribution of the loss of this function in RTT pathology. These data indicate that 1) the expression of miR-22-3p, miR-132/212-3p, and miR-483-5p is altered by loss of MeCP2 function in RTT, and 2) their repression of MeCP2 is not a steady-state regulatory mechanism, and most importantly, 3) the mutation-specific patterns of sbASO efficacy did not correlate with expression of the target miRNA, indicating that there are other factors contributing to the observed discordance in treatment effects.
Influence of 3'UTR Context

Another variable contributing to sbASO efficacy is the physical capability of each MRE to bind a repressive miRNA. A perfect sequence match is not required for miRNA binding; however, higher 3'UTR-miRNA seed complementarity is correlated with more efficient repression of the target transcript [39]. As such, sbASOs for this approach were designed to target high-efficacy MREs to maximize their de-repression effect (7mer-m8, 8mers) (Table 4). Interestingly, the potency of each MRE did not directly correlate with sbASO efficacy, as sbASO-let-7 (targets predicted 7mer-m8 site) exhibited significantly more robust treatment effects compared to sbASO-miR-132/212 (targets predicted 8mer site) in SH-SY5Y cells.

Table 4. Classification and Position of sbASO-Targeted MREs. Target sequences were mapped out onto the longest MECP2 transcript variant from NIH GenBank. Positions reflect nucleotide (nt) on 8.8 kb long 3'UTR, which is defined as the transcript sequence immediately following the stop codon at the end of the coding sequence.

<table>
<thead>
<tr>
<th>sbASO ID</th>
<th>Predicted MRE</th>
<th>Target Site 3’UTR Position (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sbASO-miR-22</td>
<td>8mer</td>
<td>42 – 57</td>
</tr>
<tr>
<td>sbASO-miR-132/212</td>
<td>8mer</td>
<td>6882 – 6897</td>
</tr>
<tr>
<td>sbASO-miR-483</td>
<td>8mer</td>
<td>407 – 422</td>
</tr>
<tr>
<td>sbASO-miR-181a</td>
<td>8mer</td>
<td>8457 – 8478</td>
</tr>
<tr>
<td>sbASO-let-7</td>
<td>7mer-m8</td>
<td>3440 - 3461</td>
</tr>
</tbody>
</table>

These contradictory findings may be the result of the physical location of each MRE, as the repressive efficacy of miRNA-MRE interactions is known to be influenced by the positioning of the site in the 3’UTR, particularly in the context of long 3’UTRs as in the case of MECP2 [97]. For example, features found to enhance site efficacy include distance from
the center of long UTRs, structural accessibility, local A/U-rich elements (ARE), and proximity to other MREs for co-expressed miRNAs [97]. Such is the case of the sbASO-miR-181a-targeted 8mer site located at the tail end of the MECP2 3’UTR (8457 – 8478 nt); its positioning away from the center may increase the accessibility of the MRE, enhancing its ability to engage miRNA-induced silencing complexes (miRISCs). On the other hand, MREs located in the middle of the 3’UTR such as the 8mer site for miR-132/212, are likely less accessible due to secondary and tertiary mRNA conformations that are incompatible with miRISCs. More important than site positioning is the 3’UTR microenvironment surrounding the MRE [97]. The local 3’UTR context of the miR-181a-5p 8mer site is enriched in A/U content, wherein the regions immediately upstream and downstream (150 nt in either direction) of the MRE are composed of 70.26% A/U content and contain four AUUUA pentamers, which are canonical ARE motifs. AUUUA motifs are cis-acting elements that destabilize transcripts via the recruitment of ARE-binding factors such as miRNAs and RBPs and are responsible for the rapid turnover of short-lived mRNAs [98-100]. Altogether, this suggests that miR-181a-5p-mediated regulation of MECP2 is maintained through stabilized miRNA-binding of the MRE and strengthened by cooperative regulatory elements, allowing for reliable repression of the transcript. In a healthy brain, miR-181a-5p is likely responsible for steady-state regulation of MECP2 expression, which explains the consistency in sbASO-miR-181a-induced effects across RTT-causing mutations.

**Influence of MeCP2 Mutation**

Our investigation of miRNA expression patterns in RTT patient temporal cortices encompassed the T158M and R255X pathogenic mutations. The T158M mutation is located
in the MBD and significantly reduces methylated DNA binding affinity, while the R255X mutant occurs in the TRD and truncates the remaining C-terminal region, disrupting NCoR recruitment and heterochromatin clustering [6, 48]. The location of each mutation impacts the mode of perturbations in miRNA regulation, such that the reduced binding to methylated DNA in T158M can prevent the expression of MeCP2-promoted miRNA, whereas the elimination of the DGCR8 interaction site in the CTD could impede MeCP2 inhibition of miRNA processing [40, 42, 48]. In patient samples with either mutation, the upregulation of DEMs was dramatically higher than that of downregulated DEMs, suggesting that MeCP2’s contribution to miRNA expression was primarily inhibition. Further analysis of common upregulated DEMs in both subpopulations, however, showed that the magnitude of differential expression was significantly greater in T158M samples compared to that of R255X (Figure 2; \( p = 0.02352 \), Student’s t-test). These data indicate a more severe impairment of miRNA-inhibitory function in the T158M mutant protein, despite the loss of the DGCR8 interaction site in R255X MeCP2. This contradicting result may be mediated by the structural dynamics of MeCP2 as an IDP. The MBD behaves as a structural hub, directing the organization of MeCP2 domains for functional assembly [50-52]. The T158M mutation likely interferes with the disorder-to-order conformational transitions required for the sequestration of DGCR8, resulting in the drastic upregulation of normatively MeCP2-inhibited miRNA. Thus, these findings suggest that while miRNA expression may not have distinct patterns across RTT-pathogenic mutations, the extent of disruption may be significantly different based on mutation-dependent alterations in MeCP2 function. Such mutation-specific differences in the degree of differential miRNA expression would imply
potential genotype-specific dosage optimization of sbASOs, wherein certain mutations may require higher sbASO concentrations for treatment effect.

Figure 21. Magnitude of Upregulated DEMs in T158M and R255X. Overlapping upregulated DEMs in T158M and R255X subpopulations were analyzed for magnitude of differential expression. The average fold change of upregulated DEMs common to both mutations was significantly higher in T158M when compared to that of R255X, indicating greater disruption of MeCP2 regulatory function (p = 0.02352, Student’s t-test). All data are shown as mean ± SEM, n = 35. Statistical significance determined by Student’s t-test is denoted as p < 0.05*.
Limitations

The thesis work described herein represents an encouraging development for the sbASO-mediated disinhibition approach and establishes sbASO-miR-181a as a strong potential candidate for RTT therapeutics. In spite of these developments, there are several limitations and considerations that must be addressed. An inherent constraint in the interpretation of the findings is the heterozygous mosaicism of RTT patient-derived primary fibroblasts. Missense MeCP2 mutations are equivalent in size to the WT protein, meaning that the quantified levels of MeCP2 in these studies encompassed both the WT and the mutant forms. In the scenario that a mosaic fibroblast line was skewed toward WT expression, the in vitro results of sbASO treatment would not be fully reflective of its effects on the mutant allele. Even so, in view of the fact that treatment with sbASO-miR-22, sbASO-miR-132/212, and sbASO-miR-483 had no effects in the majority of mutant fibroblast lines, it is likely that the efficacy of sbASO-miR-181a in these cells is meaningful and relevant. In addition, these in vitro sbASO experiments were performed with the assistance of a transfection agent, which is not compatible with in vivo studies or clinical settings. Though PS and 2’MOE modified ASOs have well-documented pharmacokinetic/pharmacodynamic profiles, the sbASOs designed for this approach must target the entire brain to achieve robust therapeutic effects, thus necessitating further investigation of its cellular uptake over time as well as optimization of delivery methods [101, 102].

Another limitation of this study was the small sample size of postmortem temporal cortices used for miRNA profiling. The cohort of T158M and R255X RTT patient samples was 3 and 4, respectively. Though we were able to obtain conclusive data on disrupted miRNA
expression through these experiments, it is difficult to ascertain whether the results are representative of all RTT patients with these mutations. Further, these findings may be restricted to the temporal cortex, as miRNA expression is cell-type-specific and locally activated in a context-dependent manner [39, 83, 85]. Despite these constraints, the high correlation between T158M and R255X miRNA profiles suggests that other mutations may exhibit similar patterns of expression. To gain a more complete understanding of the miRNA expression patterns in RTT and improve the efficacy of this approach, further characterization of various brain regions across additional MECP2 mutations is needed.

**Future Directions and Concluding Remarks**

The goal of this thesis was to identify novel miRNA/MRE targets for the sbASO-mediated disinhibition approach and define the factors contributing to treatment efficacy in RTT. Overall, our findings demonstrate that sbASO-targeting of key sites within an enriched regulatory context can yield enhanced effects beyond that of the repressive capacity of individual miRNA-MRE interactions. These data provide a blueprint to instruct optimization of target selection criteria for future sbASO design such that the ideal target miRNA/MRE 1) has repressive effects that do not exceed 200% of WT levels when blocked by sbASOs, 2) has consistent patterns of repression across common mutations, 3) is expressed in a MeCP2-independent manner, and 4) is located in ARE-enriched regions near the distal end of the 3’UTR. We acknowledge that each of these factors may not always be translational and must be tested empirically; nevertheless, they represent parameters intended to guide the initial phase of target identification.
While this work did not investigate the implications of using a combination of sbASOs for our approach, preliminary data indicated that combinatorial treatment produced synergistic effects, wherein administration of sbASOs at individually subtherapeutic concentrations resulted in a significant increase of MeCP2 expression. Simultaneously targeting multiple lower efficacy MREs to achieve therapeutic MECP2 de-repression may be important for clinical development considering the potential confounds of targeting high efficacy MREs, which may be sites of essential regulation. Such an approach would require the identification of additional targets, for which this thesis has contributed an expansion of our inventory of sbASOs.

The next step in validating the clinical applicability of sbASO-miR-181a is to perform in vivo tests in mouse models of RTT to characterize imperative components of sbASO treatment such as the half-life of sbASO-bound MECP2 mRNAs, the pharmacokinetics of PS-2'MOE modified sbASOs in the brain, and the optimal dosage and delivery of sbASOs to reverse existing RTT phenotypes. In the heterozygous context of RTT, the therapeutic index of the mutant X-chromosome-expressing cells is significantly greater than that of the WT [65, 71]. While the ceiling effect of sbASOs is designed to be protective against dosage-related adverse effects, it is crucial to determine experimentally whether any such critical threshold of sbASO concentration exists. Further, our sbASO dose response studies were performed in RTT patient-derived fibroblasts while miRNA expression profiles were obtained from postmortem temporal cortex homogenates. Both MeCP2 and miRNAs have cell-type-specific expression, which necessitate further characterization of sbASO efficacy in the context of neuronal subtypes and glial cells [3, 17, 34].
The findings of this thesis validate the therapeutic feasibility of the sbASO-mediated disinhibition approach and provide compelling evidence in support of its clinical potential in RTT. sbASOs targeting predicted MREs on the MECP2 3'UTR were able to outcompete endogenous miRNA binding, and by doing so, finely de-repress MeCP2 expression without breaching the dosage restrictions. Given that sbASOs are designed to target specific sequences, this strategy is widely applicable for genetic disorders associated with loss of function in dosage-sensitive genes, as is the case for many neurodevelopmental disorders.
REFERENCE LIST


VITA

The author, Yewon Rhee, attended Loyola University Chicago where she earned a Bachelor of Science in Cellular and Molecular Neuroscience and a Bachelor of Arts in Sociology in May of 2021. Upon graduation, Yewon continued her undergraduate research in computational neuroaesthetics as a research specialist in the lab of Dr. Norberto M. Grzywacz. In the fall of 2022, Yewon matriculated into the Neuroscience Graduate Program at Loyola University Chicago Stritch School of Medicine. At Loyola, she joined the lab of Dr. Rocco G. Gogliotti, where her thesis work focused on the development of a novel therapeutic approach for Rett syndrome using antisense oligonucleotides. After the completion of her master’s degree, she will continue her graduate education at Northwestern University as a PhD student in the Interdepartmental Neuroscience Program.