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Therapeutic Strategies for Autism: Targeting Three Levels of the Central Dogma

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by

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The thesis of Derek Hong is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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TABLE OF CONTENTS

LIST OF SUPPLEMENTARY TABLES

1. Supplementary Table 1: hong_01_sequences_ASO.xlsx
2. Supplementary Table 2: hong_02_sequences_Cas13d.xlsx
3. Supplementary Table 3: hong_03_qRTPCR_sense.xlsx
4. Supplementary Table 4: hong_04_qRTPCR_antisense.xlsx

LIST OF FIGURES

LIST OF TABLES

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ABSTRACT OF THE THESIS

Therapeutic Strategies for Autism: Targeting Three Levels of the Central Dogma

by

Derek Hong

Master of Science in Biology

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Professor Lilia Iakoucheva, Chair
Professor Yishi Jin, Co-Chair

The past decade of research has yielded much success in the identification of risk genes for Autism Spectrum Disorder (ASD), with many studies implicating loss-of-function (LoF) mutations within these genes. Despite these successes, no significant clinical advances have been made so far in the development of therapeutics for ASD. Given the role of LoF mutations in ASD etiology, many of the therapeutics in development are designed to rescue the haploinsufficient effect of genes at the transcriptional, translational, and protein levels.

The first half of this thesis will begin by reviewing the various therapeutic techniques being developed from each level of the central dogma with examples including: CRISPR activation (CRISPRa) and gene delivery at the genetic level, antisense oligonucleotides (ASOs) at the mRNA level, and small-molecule drugs at the protein level, followed by a review of current delivery methods for the aforementioned therapeutics. The second half of this thesis will detail our own lab's experimental results using mRNA-level therapeutics to target natural antisense transcripts (NATs) that are complimentary to mRNA transcripts for ASD-associated, haploinsufficient genes with the following goals: 1. Delineating relationships between NATs and their respective sense genes and 2. Increasing the expression of the sense gene through degradation of the respective NAT. Specifically, the two methods we have utilized are the previously mentioned ASOs, as well as a new system known as CAS13d—a method of mRNA knockdown through a CRISPR/CAS construct. Given the bidirectional effect of NATs on the translation of genes, many of our experiments elucidated the relationships that NATs had on their respective sense genes. Additionally, although some ASOs effectively degraded the NAT mRNA as well as increased the expression of the sense gene, the CAS13d system showed no such success. Thus, further experiments are needed to optimize the CAS13d system in this therapeutic context.

Chapter 1. Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder **(NDD)** that is characterized by three core symptoms: the deficits in social interaction and communication, language development, and restrictive and repetitive behaviors [1]. A large proportion of children suffering from ASD manifest additional symptoms including cognitive deficits, developmental delay, anxiety and other medical comorbidities with mood and psychiatric disorders [2, 3]. As of 2020, the CDC has approximated that **1 in 54, or 1.85%**, of children in the United States have been diagnosed with ASD [4]**.**

Relationships between those diagnosed with ASD and their family members can potentially be strained as 85% of individuals with ASD have limited ability to live independently [5, 6]. This lifelong dependency on caregivers as well as ASD-associated social, cognitive, and behavioral deficits can contribute to parental stress—which then further strains marital relationships, leading to increased divorce rates in parents of ASD-diagnosed children [6-12]. At a financial level, the resources needed to care for individuals with ASD are approximately 3 to 5 million dollars more than that of neurotypical children due special education costs, productivity loss due to informal caretaking, and increased use of healthcare services [6, 13-15]. Therefore, given the prevalence of diagnosis, familial stress, and financial costs, it is imperative to develop and refine techniques to alleviate the social, cognitive, and behavioral symptoms in ASD.

There is a strong genetic basis for ASD. Earlier studies demonstrated that monozygotic twins have significantly greater concordance for ASD than dizygotic twins, and ASD heritability is estimated to be 83% [16]. While there is a monogenetic etiology for some forms of NDDs, such as Angelman Syndrome **(AS)**, Fragile X Syndrome **(FXS)**, and Rett Syndrome **(RTT)** [17-19], the etiology for ASD as a whole is significantly more heterogeneous [20-22]. Previous

studies of the genetic causes of ASD have identified rare *de novo* and inherited copy number variants (CNV) as major contributors to the increased risk for ASD [23-28]. Subsequently, whole exome sequencing studies of simplex families with one affected child demonstrated strong association of rare *de novo* exonic single nucleotide variants (SNV) with ASD [29-33], with more recent analyses highlighting over 100 high risk ASD genes [34]. For some of the genes, such as KMT2E, ANKRD11, ARID1B, DYRK1A, CHD8, SHANK3, and PTEN, animal models demonstrated association of the loss-of-function **(LoF)** mutations with ASD-related phenotypes [35-40]. In addition to rare *de novo* variants, a recent genome-wide association study **(GWAS)** has identified 5 genome-wide-significant loci, providing further evidence to the genetic heterogeneity of ASD [41]. Given such an extreme ASD genetic heterogeneity, and an unequivocal role of haploinsufficient genes in ASD etiology, it will be invaluable to shift from identification-based

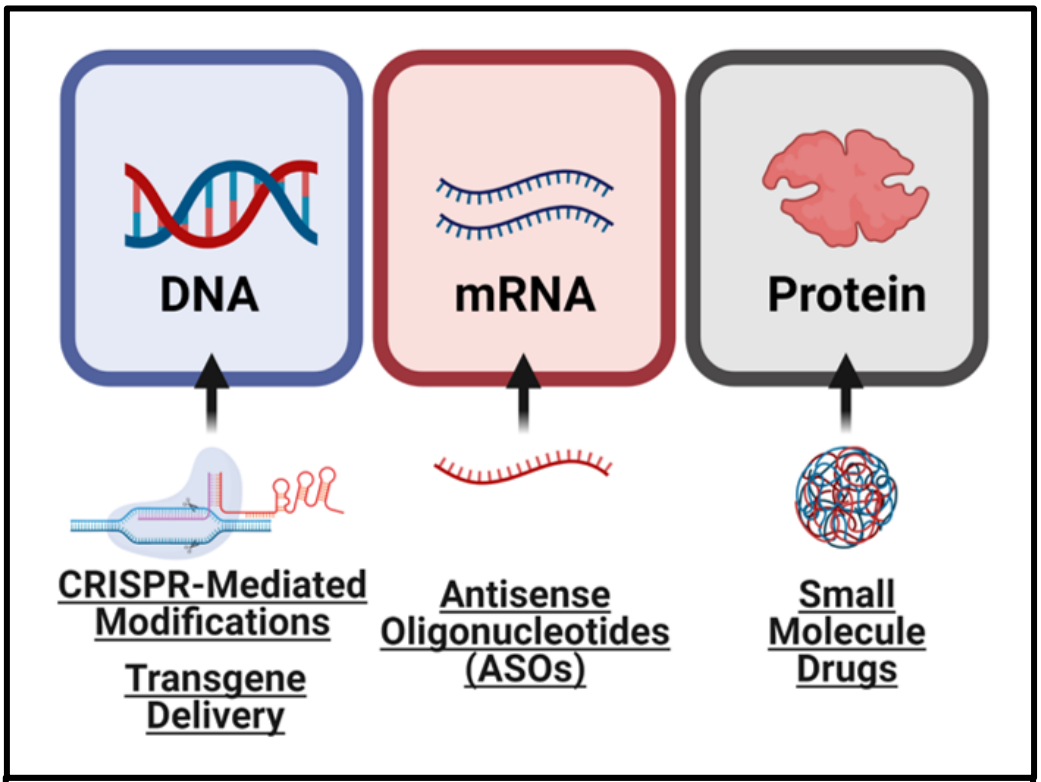

Figure 1: Overview of Therapeutics for ASD at Different Levels of the Central Dogma.

research and proceed to investigate therapeutic techniques that could increase the expression of ASD-associated LoF-impacted genes.

The therapeutic interventions in ASD aimed at rescuing haploinsufficiency of individual genes could be developed to target all three levels of the central dogma, DNA, mRNA, and protein **(Fig. 1).** Examples of such interventions include CRISPR-mediated genomic modifications and transgene delivery at the genetic level, antisense oligonucleotides **(ASOs)** at both, the transcriptional and post-transcriptional level, and the use of small-molecule drugs to target molecular pathways at the translational, or protein, level. This review will analyze the advantages and disadvantages of the various techniques across the central dogma in order to rescue ASD-associated phenotypes.

Chapter 1.1: Rescue at the DNA Level

Gene therapy encompasses techniques that can alter the expression of an organism's genes at the DNA level either through direct modification in the genome or transgene delivery, with the goal of therapeutically restoring a pathologically expressed gene to normal expression levels [42, 43].

Transgene Delivery:

The delivery of a transgene is a method of gene therapy employed when a LoF mutation in a gene would lead to the progression of the pathological phenotype [44]. Rett Syndrome **(RTT)** is an NDD and lies within the classification of ASD [45-48]. While the LoF of the Methyl-CpG-binding Protein 2 **(MECP2)** gene is associated with the progression of the disorder,

there is also a bidirectional effect of pathological MECP2 expression. Specifically, a duplication of the MECP2 gene would result in MECP2 Duplication Syndrome **(MDS)** [47, 49, 50]. It has been demonstrated that it is possible to reduce the severity of RTT pathology at the DNA level through the delivery of the MECP2 transgene within an $MECP2^{-/-}$ mouse model of RTT [51]. However, given the bidirectional nature of MECP2-associated syndromes, proper dose determination is needed prior to clinical translation. A more recent study demonstrated that the delivery of instability-prone Mecp2 (iMecp2) transgene cassette using adeno-associated virus **(AAV)** vector in symptomatic Mecp2 mutant mice significantly improved locomotor activity, lifespan and gene expression normalization [52].

Additionally, Fragile X Syndrome **(FXS)**, another NDD, is characterized by intellectual disability **(ID)** that has high comorbidity with ASD and is a result of a CGG triplet repeat expansion mutation in the fragile X mental retardation 1 gene **(FMR1)** that silences the production of its encoded FMRP protein [53-56]. One study utilized transgene delivery of the FMR1 gene using an **AAV** vector directly injected into the brains of the $FMR1^{-/-}$ mouse models of FXS to successfully rescue repetitive behavioral, social and seizure pathological phenotypes [57]. Outside the context of NDDs, the FDA has approved Luxturna—a transgene therapy to deliver the RPE65 gene and effectively treat a rare inherited retinal disease that results in vision impairment and blindness [58, 59]. Given the approval of Luxturna and successes in transgene delivery for the RTT and FXS NDDs, this mechanism may be of value for other LoF genes implicated in ASD as a whole.

CRISPR-Mediated Modifications:

Within the past decade, the advent of CRISPR/CAS9 has revolutionized gene therapy, opening a new therapeutic avenue based on DNA-level modifications [60]. CRISPR/CAS9 is a

construct consisting of a guide RNA (gRNA) that targets the genetic loci of interest and the CAS9 endonuclease enzyme, which functions as a pair of nucleotide scissors that cleave the DNA at the target site—effectively generating a double-stranded DNA break [61]. After this double-stranded DNA break is introduced, the traditional CRISPR/CAS9 construct can either decrease the expression of an over-expressed gene by mediating non-homologous end-joining, or alternatively, increase the expression of a gene with LoF mutation via homology-directed repair [62].

To provide context for a therapeutic target using the previously described CRISPR/CAS9 as a tool, it is necessary to delve into the idea of natural antisense transcripts **(NATs)** [63]. NATs are endogenously expressed in both prokaryotic, and eukaryotic organisms [64-66]. In eukaryotic systems, NATs can have a bidirectional regulatory effect on the transcription of their target genes, either suppressing or enhancing the translation of the target gene's mRNA [64, 67]. Mechanistically, inhibition of the target gene expression can occur through various mechanisms such as RNA interference **(RNAi)** once the sense-antisense mRNA duplex has formed, transcriptional interference **(TI)** in which the NAT can act as a physical barrier for RNA polymerase activity, or epigenetic methylation of the sense gene DNA, thus inhibiting the transcription of the sense mRNA transcript [68-71]. Although many NATs have inhibitory control over the expression of their complimentary genes, there are some cases in which NATs can directly increase sense gene expression [72]. With enhancement of target gene expression, it is proposed that NATs can increase the expression of the target gene through increasing the stability of the sense mRNA or euchromatin-associated epigenetic modifications [67, 72, 73]. Putting this into a therapeutic context, it may be possible to restore the expression of ASD risk genes that have LoF mutations through targeted suppression of the respective inhibitory NATs.

This strategy has recently been applied to Angelman Syndrome **(AS),** an NDD that can be driven by a LoF mutation in the maternal copy of the UBE3A allele [74, 75]. Since the paternal copy of UBE3A is normally inactive, there is therapeutic value in investigating the inhibition of the UBE3A NAT. There has been success in rescuing haploinsufficiency of the UBE3A gene through CRISPR/CAS9-mediated transcriptional inhibition of the UBE3A NAT in mice—effectively restoring UBE3A expression through re-activation of the paternal copy [74, 76]. In the context of FXS, instead of targeting NATs of the FMR1 gene, there has been success in restoring FMR1 expression in induced pluripotent stem cells **(iPSCs)** through direct CRISPR/CAS9-mediated deletion of pathological repeat sequences of the sense gene [77].

Modified CRISPR Activation:

Using the traditional CRISPR/CAS9 complex as a basis, scientists have generated a modified version in which the CAS9 enzyme is inactive or "dead" **(dCAS9)** [78]. This dCAS9

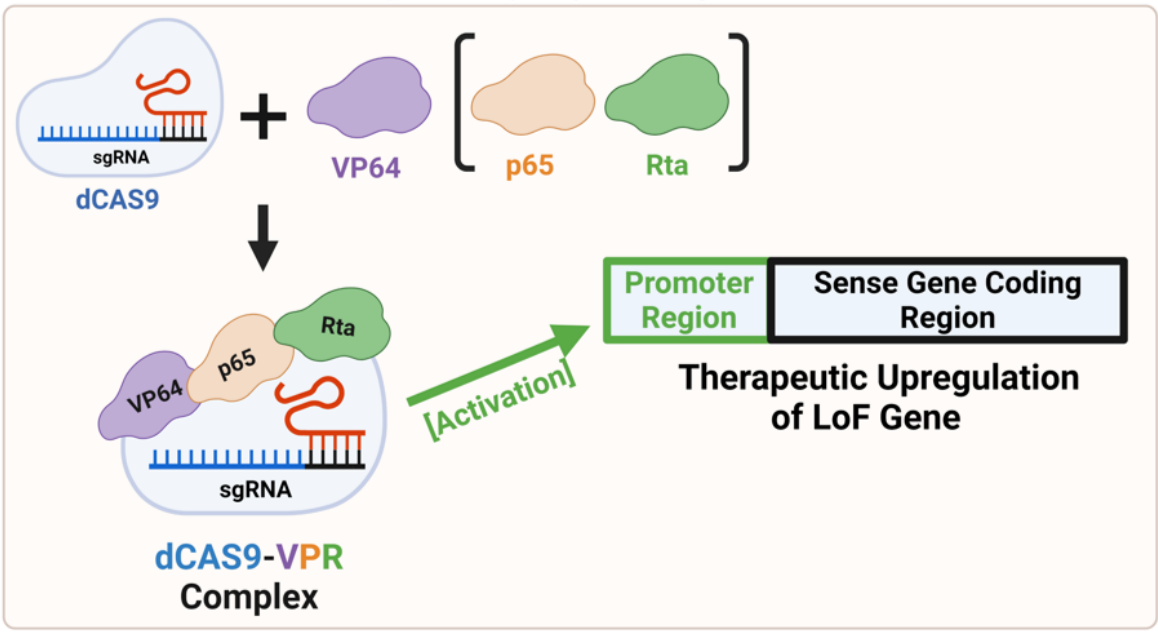

Figure 2: Schematic of CRISPR activation (CRISPRa) and its' role in ASD therapeutics.

system can be fused with activators of transcription in order to increase the expression of genes without inducing a double-stranded DNA break—a process known as CRISPR-mediated activation **(CRISPRa)** [79-81] **(Fig. 2)**.

Dravet Syndrome is an NDD caused by haploinsufficiency of the SCN1A voltage-gated Na^{2+} channel [82-84]. Through targeting long non-coding RNA [85] or the promoter region of the SCN1A gene with CRISPRa [86], researchers successfully increased SCN1A expression and restored dysfunctional neuron excitability and seizure phenotypes [86]. Outside the context of NDDs, CRISPRa has been used to target the KCNA1 voltage-gated potassium channel to rescue seizure frequency and cognitive dysfunctions in a mouse model of epilepsy [87]. Additionally, the haploinsufficiency of SIM1 and MC4R genes are implicated in obesity [88-91]. By using CRISPRa to increase the transcription of these genes, it was possible to successfully rescue the obesity phenotype in the respective haploinsufficient mouse models [92].

Chapter 1.2: Rescue at the mRNA Level:

As previously discussed, a strong target for LoF-impacted ASD risk genes is through the targeted inhibition of their respective inhibitory NATs. While we have discussed therapeutics at the DNA level, it is also possible to regulate the expression of NATs at the post-transcriptional level. When exploring this mRNA-level of regulation, it may be of value to assess the potential of using ASOs as they can increase the expression of genes through various mechanisms. These mechanisms can be classified under two main functional categories: **1.** upregulation of the sense gene through direct interactions with the sense gene mRNA transcript and **2.** upregulation of the sense gene through ASO-mediated inhibition of the NAT.

Direct Upregulation of Sense Gene:

When investigating the ability of ASOs to upregulate the sense gene through directly interacting with the sense gene mRNA, the first mechanism lies in ASOs that target the upstream open reading frames **(uORFs)** of the sense transcript [93] **(Fig. 3)**.

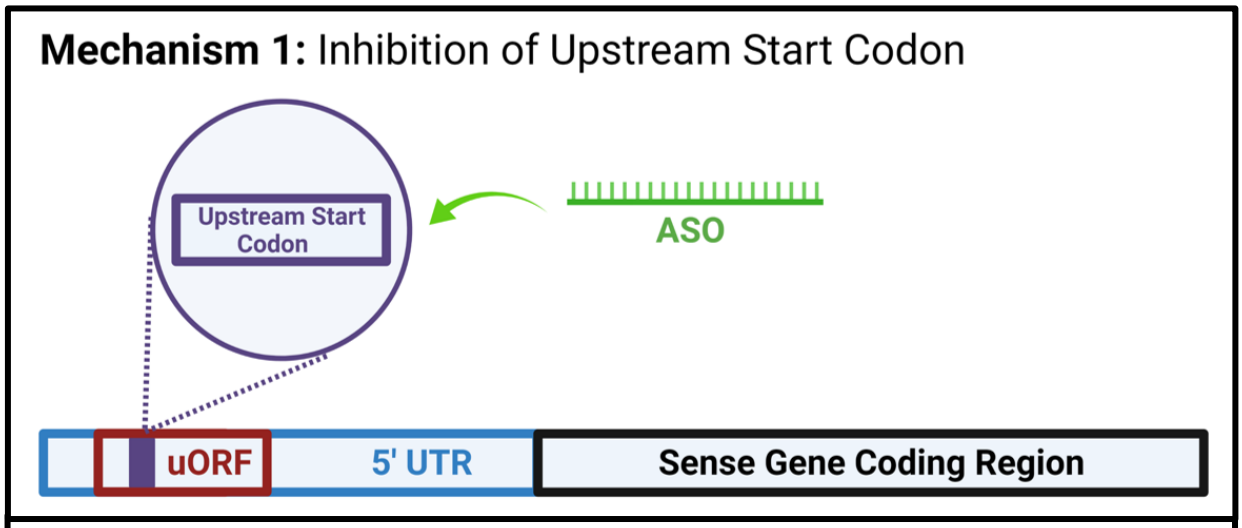

Figure 3: Diagram of ASO Mechanism 1, the inhibition of the upstream start codon within the upstream open reading frame (uORF) of an mRNA transcript.

The uORF is a region in the 5' untranslated region **(UTR)** of the mRNA transcript that often contains an additional start codon, amino acids, as well as an additional stop codon [94]. When translation is initiated at this locus, there can be a decrease in the efficiency of protein translation due to preferential translation beginning at the upstream start codon—producing a peptide that ultimately blocks ribosomal function [95, 96]. ASOs were designed to specifically target the uORF of the LRPPRC gene in mouse models, and this treatment was successful in increasing the LRPPRC protein expression, an experiment that provided evidence to the efficacy of this mechanism [95]. However, further studies are needed to investigate the success of uORF

targeting in disease models, as many of these studies have been proof of principle rather than demonstrating success in pathological models.

Second, ASOs can be designed to target inhibitory elements in the 5' UTR region of the mRNA transcript. To provide further context, there are translation-inhibiting secondary structures within the 5' UTR of mRNA transcripts, and by designing ASOs that are complementary to these regions, it is possible to relieve translational inhibition [93, 97] **(Fig. 4)**.

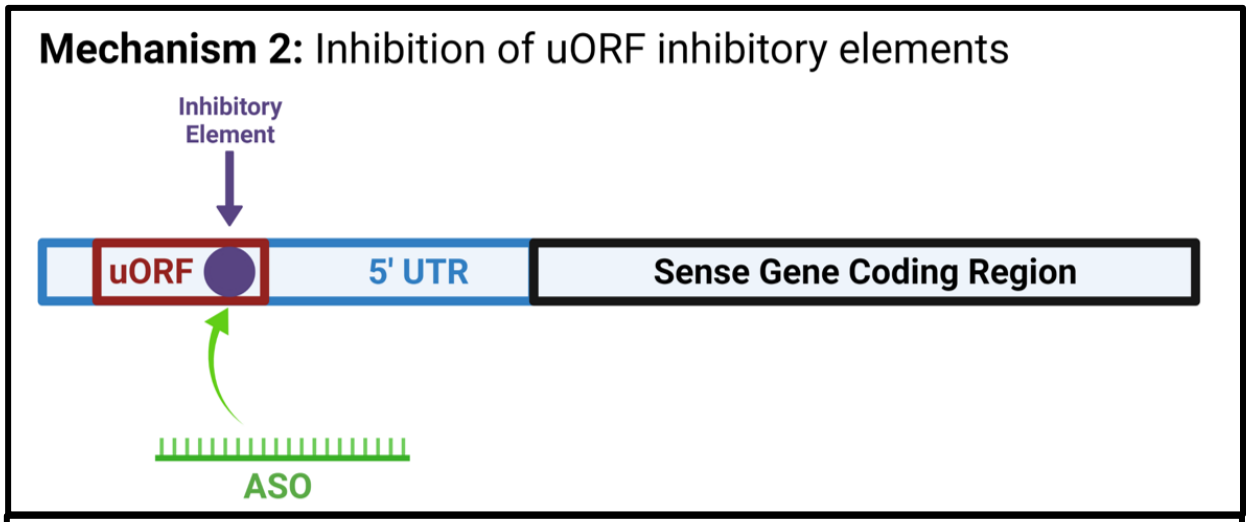

Figure 4: Diagram of ASO Mechanism 2, the inhibition of the inhibitory elements within the upstream open reading frame (uORF) of an mRNA transcript.

One study demonstrated that there was a hairpin structure in the 5' UTR region of the LDLR mRNA that was inhibitory for protein translation and administered an ASO that targeted this region—resulting in an increase of LDLR protein expression and LDL uptake in HEK293T cells [93]. Similar methods were used for cystic fibrosis **(CF)**, a disease characterized by significant pulmonary and pancreatic dysfunctions and is a result of LoF mutations in the CFTR gene, coding for a Cl^- channel [98]. In a cellular model of CF, ASOs were designed to target the inhibitory secondary mRNA structures in the uORF of the 5' UTR on the CFTR mRNA transcript—effectively increasing both the expression and function of CFTR [99]. However,

although success within the CF in-vitro model provides potential for clinical translation, further evidence of *in vivo* success with this specific method is needed.

Third, there is evidence of successful restoration of aberrant mRNA splicing in non-NDD muscular conditions such as Spinal Muscular Atrophy **(SMA)** [100] and Duchenne Muscular Dystrophy **(DMD)** [101] through targeting splice junctions and cis-regulatory elements with ASOs [102, 103]. In SMA, individuals lack a working copy of the SMN1 gene so the ASOs are used to facilitate proper splicing of the SMN2 gene through inducing the inclusion of exon 7—ultimately rescuing the expression of the SMN protein within in-vitro mammalian cell models [102] **(Fig. 5)**.

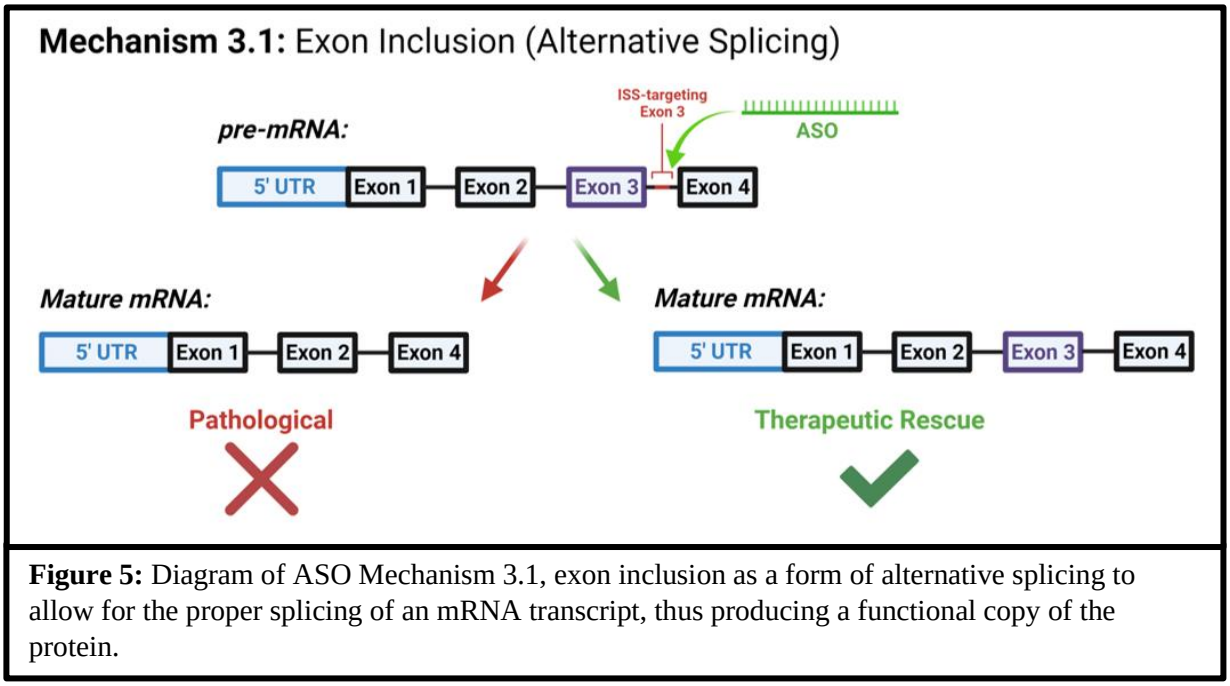

Figure 5: Diagram of ASO Mechanism 3.1, exon inclusion as a form of alternative splicing to allow for the proper splicing of an mRNA transcript, thus producing a functional copy of the protein.

In 2016, the FDA authorized the use of Spinraza, the first drug-based therapy for SMA [104]. Spinraza functions through this mechanism of targeted exon 7 inclusion in the SMN2 mRNA, effectively rescuing gross motor functions in patients[105, 106]. In DMD, mutations in the dystrophin gene induce a frameshift and end up producing a non-functional dystrophin protein [107]. DMD-targeting ASOs induce skipping of exons that are frequently implicated in the

frameshift mutations responsible for DMD, thus producing a partially functioning copy of the dystrophin protein. [103, 108] **(Fig. 6).**

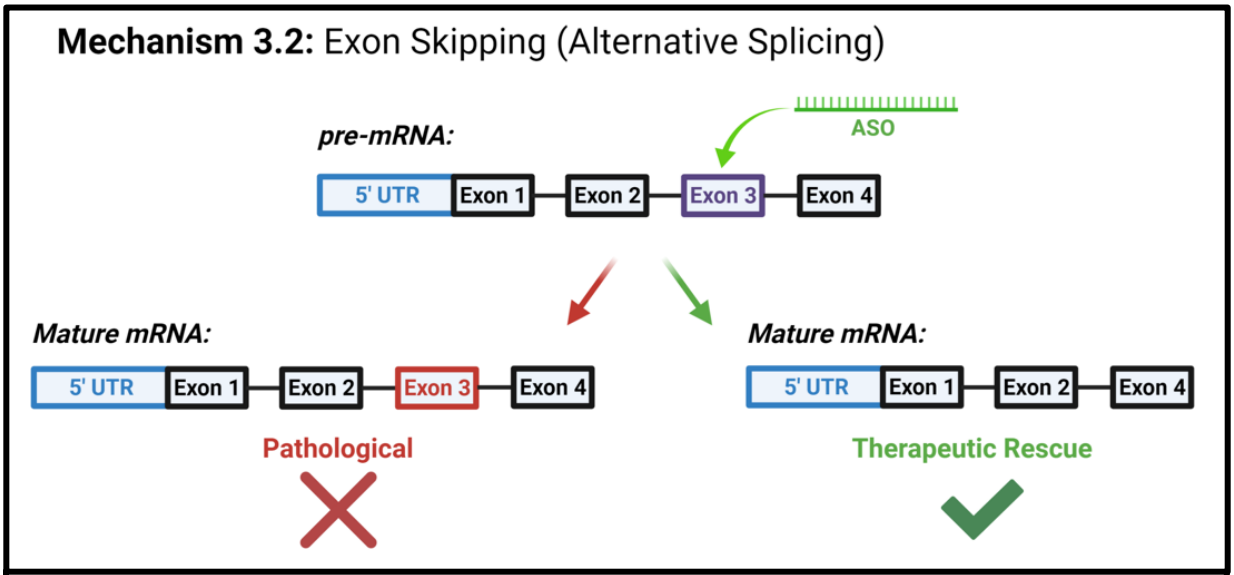

Figure 6: Diagram of ASO Mechanism 3.2, exon skipping as a form of alternative splicing to allow for the translation of a partially functional protein.

Although much promise was seen in early stages of clinical trials with the exon-skipping ASO Drisapersen for DMD, phase 3 trials failed to achieve clinical success [109-111]. However, hope was not lost as the FDA authorized the use of Eteplirsen, an ASO that induces exon-skipping to express partially functioning dystrophin, as the first drug-based therapy for DMD in 2016 [112]. Similarly, an ASO promoting alternative splicing by the name of Milasen was designed as a personalized drug to treat an individual with Batten disease, a neurodegenerative disease characterized by blindness, an increased susceptibility to seizures, and developmental delay [113-115]. The mutation in the MFSD8 (also known as CLN7) gene resulted in a truncated, and dysfunctional protein, and treatment with Milasen was able to effectively rescue seizure phenotypes and partially improve neurological scores [115].

Fourth, ASOs can suppress the nonsense-mediated decay **(NMD)** of mRNA transcripts by targeting the exon-junction complex **(EJC)** region located downstream of a transcript's premature termination codons **(PTC)** [116-118] **(Fig. 7)**.

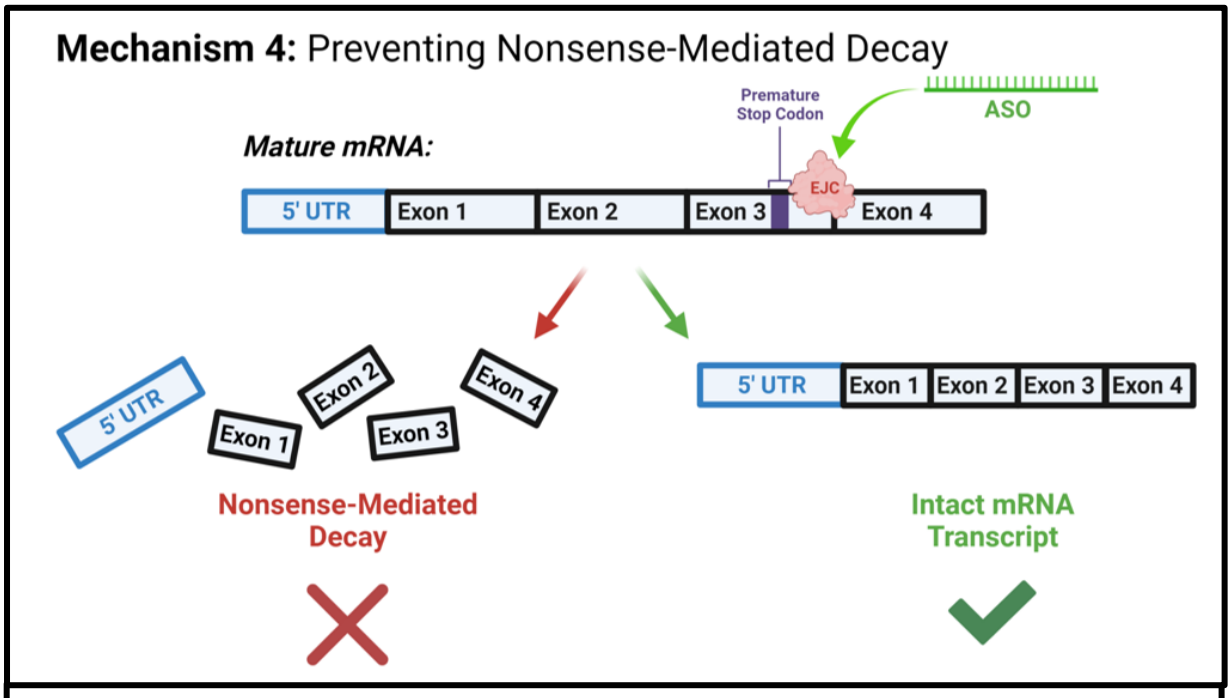

Figure 7: Diagram of ASO Mechanism 4, preventing the nonsense-mediated decay of an mRNA transcript.

Mechanistically, NMD is dependent on the presence of at least one EJC and targeting this pathway with ASOs led to the increase of MECP2 gene expression within in-vitro mammalian cell models [116]. This provides evidence towards potentially using ASOs to inhibit NMD of MECP2, thus opening a therapeutic avenue for Rett syndrome. However, it is again important to note the bidirectional pathologies associated with Rett syndrome. Additionally, further studies are needed to determine the ideal dose to prevent induction of MDS and more research is needed within in-vivo models prior to successful clinical translation.

Upregulation of Sense Gene via NAT Degradation:

While the previously mentioned mechanisms function to upregulate the sense gene by directly acting upon the sense gene mRNA transcript, one limitation lies in the fact that uORFs only exist in approximately 50% of mammalian mRNA and within this 50%, not all are inhibitory [119, 120]. Therefore, there is much value in the upregulation of ASD-associated sense genes through ASO-mediated NAT degradation. Mechanistically, once an ASO is bound to the NAT mRNA transcript, the RNAse H endonuclease enzyme will cleave the RNA duplex—effectively suppressing the inhibitory function of these NATs [75, 121] **(Fig. 8)**.

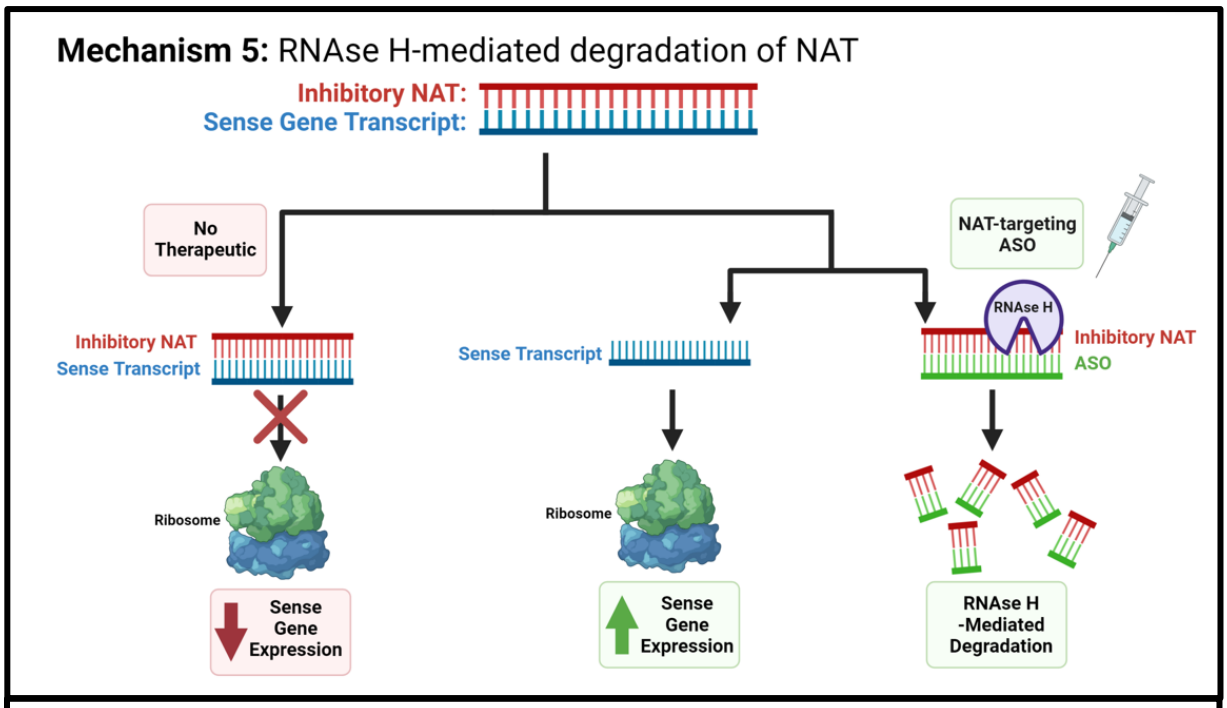

Figure 8: Diagram of ASO Mechanism 5, RNAse H-mediated degradation of a natural antisense transcript (NAT) in order to relieve inhibition of the sense mRNA transcript.

As previously discussed, Dravet Syndrome is an NDD caused by haploinsufficiency of the SCN1A voltage-gated Na^{2+} channel [84, 122]. With the use of the ASOs to target the SCN1A NAT, one study was able to rescue the sense SCN1A gene expression, ameliorate the pathological

decrease in neuronal excitability and the pathological seizure phenotypes in a mouse model of Dravet Syndrome—an effect that almost entirely recapitulates the rescued phenotypes observed via CRISPRa treatment [85, 86].

As previously mentioned, Angelman Syndrome is an NDD driven by haploinsufficiency of the UBE3A gene. While previous success was found in CRISPR-based rescue at the DNA level, it is also possible to target the UBE3A NAT mRNA transcript for degradation at the transcriptional level [76]. One study has shown that it was possible to decrease the expression of the UBE3A NAT, rescue the sense UBE3A expression, and ameliorate the pathological cognitive dysfunctions in a mouse model of AS through the RNAse-mediated degradation mechanism [75].

While growth factors such as neurotrophins do not have a single-gene etiology in NDD pathogenesis, it has suggested that neurotrophins such as brain derived neurotrophic factor **(BDNF)** can protect against synaptic plasticity, neurodegeneration and memory impairment in neurodegenerative diseases such as Alzheimer's Disease **(AD)** [123-125]. Therefore, it is of value to increase the expression BDNF. Within the context of mRNA therapeutics, one study showed that it was possible to increase the BDNF expression by targeting the NAT with ASOs—thus inducing NAT degradation via RNAse-H in mice [126]. However, further research using the NAT-targeting ASOs would be needed to ensure that there is a causative link between BDNF upregulation and the resulting protective effects on neurodegeneration, and the increase of synaptic plasticity in the proper in-vivo pathological models.

Chapter 1.3: Rescue at the Protein Level

Further downstream in the central dogma, a key therapeutic approach can lie in the activation or inhibition of molecular pathways implicated in NDDs. This would effectively shift focus from expression-based therapeutics to post-translational treatments.

Rescuing Inhibitory Signalling Pathways:

To provide context, it has been previously hypothesized that potential loss of balance in neuronal excitatory/inhibitory signals [127] within syndromic ASD subtypes such as FXS, RTT, AS, and in idiopathic ASD can be attributed to a decrease in the inhibitory $GABA_A$ receptor function [128, 129]. Therefore, upregulation or gain in $GABA_A$ receptor function could be a potential therapeutic target in rescuing ASD pathology. Arbaclofen, a $GABA_B$ agonist, has recently emerged as a potential therapeutic in targeting ASD pathology. Arbaclofen has been found to rescue memory deficits and male-female social interactions in the 16p11.2 deletion mouse model of autism [130]. In a small clinical trial of 25 adolescent, Arbaclofen has been found to rescue slow auditory sensory processing in males with idiopathic ASD [131]. However, it is important to consider that while some success was found in an idiopathic ASD study, phase III clinical trials in FXS patients proved to be unsuccessful [132]. Therefore, although stimulation of the $GABA_B$ receptor using Arbaclofen to increase inhibitory neuronal signals and rescue the abnormal excitatory/inhibitory balance in ASD may show promise, the heterogeneity of ASD-associated conditions makes it so that this drug is not universally applicable. Patient genetic stratification and better clinical outcome measures are needed for future clinical trials in ASD.

Rescuing Excitatory Signalling Pathways:

An alternative hypothesis is that an increase in the excitatory glutamate signalling can play a role in the dysregulation of neuronal excitatory/inhibitory signal balance. In FXS, it has been found that there was a causative increase in the metabotropic glutamate receptor 5 **(mGluR5)** that accompanies the loss of FMR1 expression [133]. Molecular inhibition at the mGluR5 loci has been found to be successful in multiple mouse models of ASD. In a BTBR mouse model, the use of the mGluR5 antagonist MPEP successfully rescued the repetitive grooming phenotype [134]. MPEP was also successful in rescuing not only the same repetitive grooming, but also other anxiety-associated behavioral phenotypes such as marble-burying and locomotion in a valproic acid **(VPA)** mouse model [135]. Furthermore, treatment using the mGluR5 negative allosteric modulator **(NAM)** CTEP successfully rescued the impaired memory formation pathological phenotype in the 16p11.2 copy number variant **(CNV)** microdeletion mouse model [136]. Clinical translation, however, has proven to be unsuccessful as preclinical trials for mGluR5 NAMs have not been able to rescue pathological phenotypes in human FXS patients [137]. Failures in the preclinical trials could possibly be attributed to improper dose extrapolation from mouse models, duration of treatments, or the fact that mGluR5 inhibition is preferentially effective in a younger population—parameters that can be adjusted in future trials [138, 139]. Early continuous inhibition of group 1 mGlu signaling partially rescues dendritic spine abnormalities in the Fmr1 knockout mouse model for fragile X syndrome [137].

Another recently discovered therapeutic strategy in ASD is targeting a small GTPase, RhoA, that is involved in cellular cytoskeleton structure and motility [140], and has found to be upregulated in some ASD models while being downregulated in others [141-144]. Cullin3 (Cul3) is an ASD risk gene in which haploinsufficiency contributes to a pathological decrease in neuronal

dendritic growth and a decrease in neuronal network activity [141]. Since a Cul3 haploinsufficient mouse model also showed an upregulation in RhoA expression, treatment using the RhoA inhibitor Rhosin was used to successfully rescue the deficiencies in dendritic growth and network activity *in vitro* in primary cortical neuron cultures derived from these mice [141]. In a social defeat mouse model, Rhosin has also been found to rescue behavioral phenotypes *in vivo* and neurite growth *in vitro* [145]. Similarly, in Kctd13 (a gene within 16p11.2 CNV) haploinsufficient and knockout mice, there was an increase in RhoA expression coupled with deficiencies in synaptic signalling—an effect that was ameliorated with Rhosin treatment [142]. These successes within mouse models provide evidence that Rhosin may be a valid therapeutic avenue for individuals with a deletion in the 16p11.2 or Cul3 loci.

Chapter 1.4: Delivery of Therapeutics

Viral delivery of CRISPR/CAS9, gene constructs, and ASOs have been a commonly used delivery method that uses vectors such as lentiviruses, adenoviruses, and adeno-associated viruses **(AAV)** [146-148]. Though widely used, it is important to consider the potential drawbacks such as a person's potential immunological response to these vectors, as well as limitations in packaging sizes [149]. Although an adenovirus vector may have a larger packaging limit of up to ~36 kb, there is a greater risk of an inflammatory immunological response [150]. Conversely, AAVs have a limited packaging size of ~5 kb with significantly milder inflammatory risk [151]. Another important viral vector is the lentivirus, which is a type of retrovirus that have a packaging size of ~9 kb—an intermediate between that of the adenovirus and AVV vectors [152]. One of the main advantages of the lentivirus is its ability to deliver transgenes and integrate them into the genome for longer lasting expression [153, 154]. However, this advantage contributes to the risk of long-

lasting off-target effects, known as insertion mutations, since lentiviruses do not have high specificity [155]. Studies have also shown that the lentivirus vector confers moderate inflammatory risk, but further studies in immunogenicity and prevention of recombination events are needed for optimized clinical translation [153, 156, 157]. Although the lentivirus seems to have more associated risks than AAVs, a potential advantage in using a lentivirus vector for NDD-associated pathologies would be the tissue specificity of lentiviruses—something that most AAV serotypes lack [147, 158-160]. However, even though lentiviruses can be tissue-specific, viral vectors as a whole do not penetrate the blood-brain barrier **(BBB)** with high efficiency and must be introduced via invasive direct injection or the potentially neurotoxic disruption of the BBB [161, 162].

Since many of the differentially expressed genes implicated in NDDs can be localized to brain tissue, the previously described viral vectors may appear to be sub-optimal delivery methods for NDD therapeutics. However, the AAV9 serotype has been found to have the ability to efficiently cross the BBB when intravenously delivered to neonatal and adult mouse and cat models [163-165]. Furthermore, unlike the adenovirus, lentivirus, and other AAV serotypes, the AAV9 vector appears to be able to effectively avoid two main issues: the need for invasive injection, and compromising the integrity of the BBB, although its transduction efficiency diminishes with increasing age [166]. In an RTT mouse model, it was found that AAV9-mediated intracranial delivery of the MECP2 transgene effectively increased survival and pathological behavioral phenotypes [51]. In a phase 3 clinical trial, this AAV9 vector has found success in intravenously delivering the SMA1 transgene—effectively restoring motor functions in SMA patients [167, 168]. Further studies are needed in specifically exploring the differences in transduction efficiency of therapeutics between intravenous vs intracranial/intrathecal injections in model organisms.

Although AAVs have traditionally been the main vector used to deliver CRISPR/CAS9 constructs, the relatively small packaging size (~4.5 kb) makes it so that researchers must deliver two separate vectors containing the CRISPR/CAS9 construct (~4.2 kb) and gRNA separate [146, 169]. To provide further context, this traditional CRISPR/CAS9 was derived from the Streptococcus pyogenes bacteria **(SpCAS9**). Recent techniques to overcome this size limitation involve utilizing a small Cas9 ortholog from Staphylococcus aureus **(SaCAS9)** (3.15 kb), which is ~1 kb shorter than the SpCAS9, so that the CRISPR/CAS9 construct and gRNA can fit within a single AAV vector [170, 171]. This technique was successful in restoring the expression of the UBE3A sense gene via NAT degradation in a mouse model of AS as well as rescuing the pathological phenotypes in a DMD mouse model [74, 172]. Similarly, CAS9 from Campylobacter jejuni **(CjCAS9)** (2.95 kb) has been used in single-AAV vectors to successfully decrease the pathological Choroidal Neovascularization **(CNV)** phenotype in a mouse model of age-associated macular degeneration **(AMD)** [173]. Further experiments should investigate the efficacy of CjCAS9 in rescuing pathological phenotypes associated with NDDs.

Since the BBB also prevents most small-molecule drugs in addition to the previously mentioned viral vectors from being able to enter the CNS, the recent development of AAV9 variants have been a potential key in solving this issue. Variants such as rAAV-PHP.B and a second generation rAAV-PHP.eB contain an engineered capsid that have an unprecedented efficiency in crossing the blood-brain barrier upon intravenous injection along with its high diffusion capacity into both neurons and glia [166, 174, 175]. Additionally, neurotransmitter-derived lipidoids (NT-lipidoids) are a promising delivery vector [176]. One study recently demonstrated that a NT-lipidoid vector successfully introduced a Tau-targeting ASO, small molecule drug, and a fusion protein into the brain of mice via intravenous injection [176].

Similarly, the use of lipid nanoparticles **(LPN)** can potentially be used to deliver therapeutics to the brain in a two-step process in which biotinylated antibodies are targeted to specific proteins on the brain endothelial cells, then the LPNs containing the therapeutic are directed towards the cells with the biotinylated antibodies [177]. Evidence from other studies have shown that ASOs, CRISPR/CAS9 constructs, and small molecule drugs can be delivered using a lipid nanoparticle vector [178-180]. Therefore, further investigation of this biotinylation-mediated mechanism of brain targeting may be valuable in advancing NDD-based therapies.

Chapter 1, in full is currently being prepared for submission for publication of the material as a review article. Hong, Derek; Tran, Stephen; Sebat, Jonathan; Iakoucheva, Lilia. The thesis author was the primary author of this material.

Chapter 2: Materials & Methods

To apply currently available techniques of mRNA therapeutics to ASD, we sought to utilize the ASOs to induce RNAse H-mediated degradation in NATs of LoF risk genes for ASD. Since not much information is known about the relationships between these risk genes and their associated NATs, a secondary objective is that these experiments will help elucidate whether there are activating or inhibitory relationships between the genes and their respective NATs. In addition to applying the previously established ASO technique, our lab also sought to establish, develop, and optimize the Cas13d technique to further advance mRNA therapeutics.

Design of ASOs: Gene targets were selected by using a gene database from the Simons Powering Autism Research **(SPARK**) foundation. Using 259 control brain samples from the PsychEncode-GVEX gene database sorting by the Spearman correlation between sense genes and their respective

natural antisense genes in RNA-seq data. A higher Spearman correlation would be indicative of coregulation between the sense and natural antisense genes. LoF mutations in many of the selected genes are implicated in other pathological phenotypes including DD, ID, and schizophrenia (SCZ) **(Table 1).**

Table 1: Table summarizing therapeutics for human disease at different levels of the central dogma.

Gene	Entrez Gene ID	Mutations	Location	Associated Disorders	PMID
KMT2E	55904	Stop-Gain	Exonic	ASD	25363768
KMT2E	55904	Frameshift	Exonic	ASD	22542183
SIN3A	25942	Nonsynonymous SNV	Exonic	ASD	25363760, 25363760
SIN3A	25942	Frameshift	Exonic	DD	25533962
SIN3A	25942	Synonymous SNV	Exonic	ID	27479843
HIVEP2	3097	Nonsynonymous SNV	Exonic	ID	24896178
HIVEP2	3097	Frameshift	Exonic	ID, DD	23020937, 25533962
IRF2BPL	64207	Nonsynonymous SNV	Exonic	ASD	22495309
IRF2BPL	64207	Frameshift	Exonic	ASD	25363768
CTBP1	1487	Nonsynonymous SNV	Exonic	DD	25533962
ARID1B	57492	Stop-Gain	Exonic	ASD, DD, ID	25363760, 28263302, 25533962, 27479843
ARID1B	57492	N/A	Splicing	DD	25533962
ARID1B	57492	Nonsynonymous SNV	Exonic	ASD, SCZ, DD	25363760, 25363768, 24463507, 25533962
ARID1B	57492	Frameshift	Exonic	ASD, DD	25363760, 23160955, 22495309, 25533962

First, by using the Integrated Genomics Viewer (**IGV**), we looked at the bulk RNA-seq database from the Gene Expression Omnibus (**GEO**) to determine what human immortal cell line best expresses both the sense gene, as well as the respective NAT.

We then designed two ASOs, each with a length of 20 nucleotides, to target the NAT at two different locations on their transcripts that had the greatest level of expression based on the bulk RNA-seq data from GEO. Once the ASO sequences were determined, modifications such as Phosphorothiolation of each bond, 2'methoxylation/2'methoxyethylation of the first five/final five nucleotides were made to increase the ASO's resistance to DNase-based degradation **(Supp. Table 1)**.

Cas13d and sgRNA plasmids: The Cas13d (EF1a-CasRx-2A-EGFP; **Addgene:** 109049) and sgRNA (CasRx gRNA cloning backbone; **Addgene:**109053) plasmids were purchased from Addgene. In order to clone target sequences into the backbone of the gRNA backbone, we began by performing PCR on two sections of the backbone. An agarose gel electrophoresis was then performed on the PCR products at 100 volts for 90 minutes on a 1% TAE gel to confirm that the PCR was successful. Using the QIAquick Gel Extraction Kit (Qiagen, Catalog: 28706), the fragments were then isolated and purified following the protocol from the manufacturer. Following this, we used the same 20 nucleotide sequences from the ASO design as a basis and extended them to 22 nucleotides for the gRNA target sequences. An NEB Hi-Fi DNA Assembly Kit (New England Biolabs, Catalog: E2621L) was then used to clone together the two backbone fragments, and the 22 nucleotide target sequence following the protocol from the manufacturer **(Supp. Table 2)**.

Cell Culture: HeLa cells and HEK293T cells obtained from the American Tissue Cell Culture (ATCC) organization and grown at 37 °C and 5% CO_2 on 10 cm cell culture plates in an incubator. The cells were kept for a maximum of 25 passages and subcultured at a 1:10 ratio every 4 and 3 days, respectively. During subculturing, the cells were washed with 5 mL of phosphate-buffered saline (PBS). To detach the cells, a mixture of 4 mL of PBS and 1 mL of TrypLE Express from ThermoFisher was added to each 10 cm plate and incubated at 37°C and 5% CO_2. Full detachment was observed after 10 minutes for HeLa cells and 4 minutes for HEK293T cells. Once detached, 5 mL of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) was added to each plate. Cells were then centrifuged down at 160 RCF for 4 minutes and the liquid was aspirated. The remaining pellet was resuspended in 1 mL

of DMEM with 10% FBS and 100μL of the resuspended cells were added to a fresh plate containing 10mL of DMEM with 10% FBS.

Transfection: 24 hours before transfection, $8x10^5$ cells were seeded into each well of a 6-well plate so that there are $1.60x10^6$ cells on the day of transfection. For each ASO transfection, two different ASOs were designed for each NAT target so every 6-well plate contained two replicates of three different conditions—**100nM** ASO1, **100nM** ASO2, and a mock transfection. These conditions were replicated across three total 6-well plates, with the fourth 6-well plate containing three replicates of a **100nM** positive control ASO targeting the PTEN mRNA transcript as well as two replicates of a **100nM** positive control Alexa Fluor dye to check for transfection efficiency. ASOs were all transfected using oligofectamine (ThermoFisher, Catalog: 12252011) as the transfection reagent, following the protocol from the manufacturer.

Cas13d experiments employed lipofectamine (ThermoFisher, Catalog: L3000015) as a transfection reagent to co-transfect **2.5 ug** of the Cas13d plasmid as well as **2.5 ug** of the sgRNA plasmid. Like the layout of the ASO experiments, each 6-well plate had two replicates of three different sgRNA conditions—gRNA1, gRNA2, and a gRNA with a non-targeting vector obtained from [181]. The fourth plate contained three replicates of a positive control gRNA targeting STAT3 from [181]. Four hours after transfection for both ASO and Cas13d experiments, the cell media was changed with 10 mL of DMEM with 10% FBS.

RNA Extraction and qRT-PCR: 24 hours after the initial transfection for ASO experiments and 48 hours after the initial transfection for Cas13d experiments, an RNAeasy Plus Kit (Qiagen, Catalog: 28706) was used to extract the RNA from cells following the protocol from the manufacturer. Once extracted, the RNA was quantified using a Nanodrop 2000 Spectrophotometer (ThermoFisher).

To measure the transcriptional expression of the genes, reverse transcription was performed for the sense transcripts (Bio-Rad iScript cDNA Synthesis Kit, Catalog:1708890**)** while strand-specific reverse transcription was performed for the NATs (SuperScript III First-Strand Synthesis System for RT-PCR, ThermoFisher, Catalog: 18080-051) to produce the corresponding cDNA—both following the protocol from manufacturer. To perform the strand-specific reverse transcription of the NAT, we used a primer targeting the NAT with a specific tag sequence attached **(Supp. Table 4)**, followed by exonuclease digestion to remove any remaining antisense primers. Using this cDNA, qRT-PCR was performed using the SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (Bio-Rad, Catalog: 1725017) to measure the transcriptional expression of the sense transcripts and NATs from both ASO and Cas13d transfection experiments **(Supp. Table 3-4),** following the protocol from the manufacturer. For the qRT-PCR of the NAT, instead of using a standard set of primers, one primer will be targeting the NAT sequences while the other primer targets the specific tag sequence from the strand-specific reverse transcription step **(Supp. Table 4)**. The housekeeping gene used for all qRT-PCR experiments were GAPDH. T-tests were performed comparing the experimental conditions with the negative controls to determine statistical significance.

Chapter 2, in part will be prepared in the far future for submission for publication of the material. Hong, Derek; Tran, Stephen; Sebat, Jonathan; Iakoucheva, Lilia. The thesis author was the primary author of this material.

Chapter 3: Results

Gapmers targeting KMT2E, SIN3A, and HIVEP2 NATs had no effect on neither the sense transcript nor antisense transcript mRNA expression.

The ASOs, also known as Gapmers, were transfected into HeLa cells and results showed

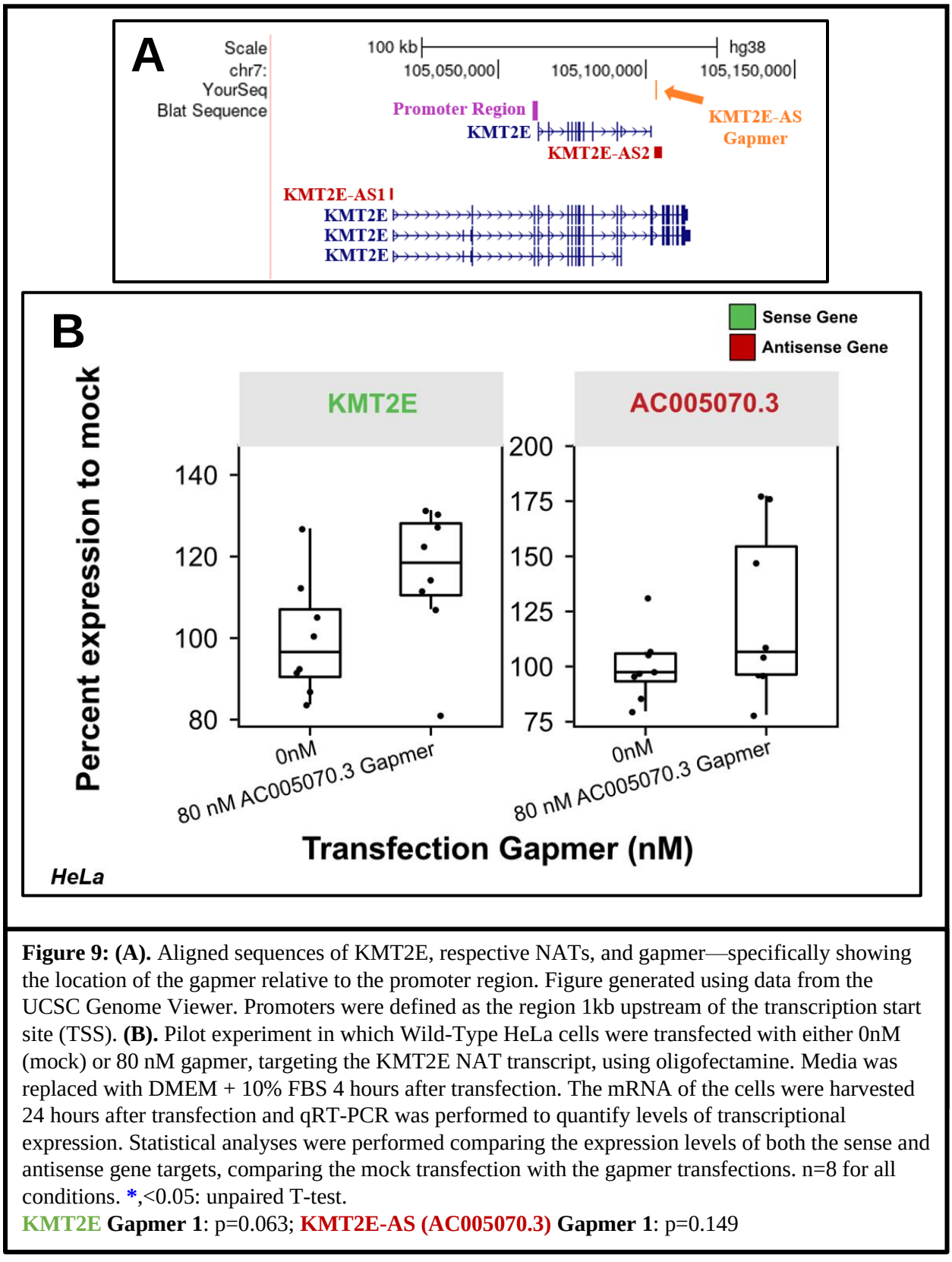

Figure 9: (A). Aligned sequences of KMT2E, respective NATs, and gapmer—specifically showing the location of the gapmer relative to the promoter region. Figure generated using data from the UCSC Genome Viewer. Promoters were defined as the region 1kb upstream of the transcription start site (TSS). **(B).** Pilot experiment in which Wild-Type HeLa cells were transfected with either 0nM (mock) or 80 nM gapmer, targeting the KMT2E NAT transcript, using oligofectamine. Media was replaced with DMEM + 10% FBS 4 hours after transfection. The mRNA of the cells were harvested 24 hours after transfection and qRT-PCR was performed to quantify levels of transcriptional expression. Statistical analyses were performed comparing the expression levels of both the sense and antisense gene targets, comparing the mock transfection with the gapmer transfections. n=8 for all conditions. *,<0.05: unpaired T-test.
KMT2E Gapmer 1: p=0.063; **KMT2E-AS (AC005070.3) Gapmer 1**: p=0.149

that the gapmers targeting NAT's of KMT2E (AC005070.3), SIN3A (SIN3A-AS), and HIVEP2 (AL355304.1) had no statistically significant effect on the mRNA levels of neither the sense transcript nor the respective antisense transcript when compared to the expression levels of cells

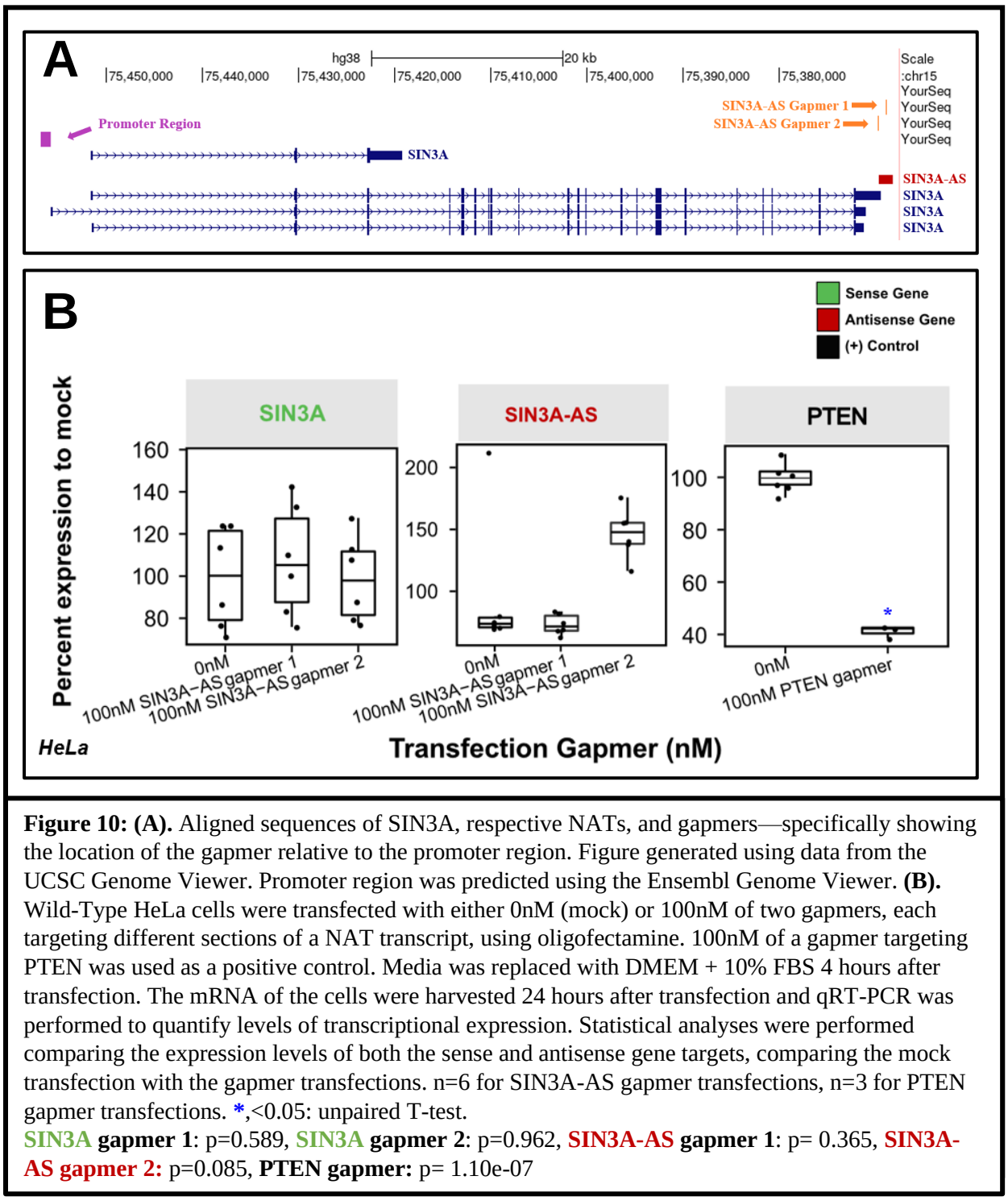

Figure 10: (A). Aligned sequences of SIN3A, respective NATs, and gapmers—specifically showing the location of the gapmer relative to the promoter region. Figure generated using data from the UCSC Genome Viewer. Promoter region was predicted using the Ensembl Genome Viewer. **(B).** Wild-Type HeLa cells were transfected with either 0nM (mock) or 100nM of two gapmers, each targeting different sections of a NAT transcript, using oligofectamine. 100nM of a gapmer targeting PTEN was used as a positive control. Media was replaced with DMEM + 10% FBS 4 hours after transfection. The mRNA of the cells were harvested 24 hours after transfection and qRT-PCR was performed to quantify levels of transcriptional expression. Statistical analyses were performed comparing the expression levels of both the sense and antisense gene targets, comparing the mock transfection with the gapmer transfections. n=6 for SIN3A-AS gapmer transfections, n=3 for PTEN gapmer transfections. *,<0.05: unpaired T-test.
SIN3A gapmer 1: p=0.589, **SIN3A gapmer 2**: p=0.962, **SIN3A-AS gapmer 1**: p= 0.365, **SIN3A-AS gapmer 2:** p=0.085, **PTEN gapmer:** p= 1.10e-07

that were treated with a mock transfection containing no gapmers **(Fig. 9B-11B)**. Given the transfection of the gapmer against the KMT2E NAT (AC005070.3) was a pilot experiment, we did not have the PTEN positive control at this point **(Fig. 9B)**. All subsequent ASO transfections

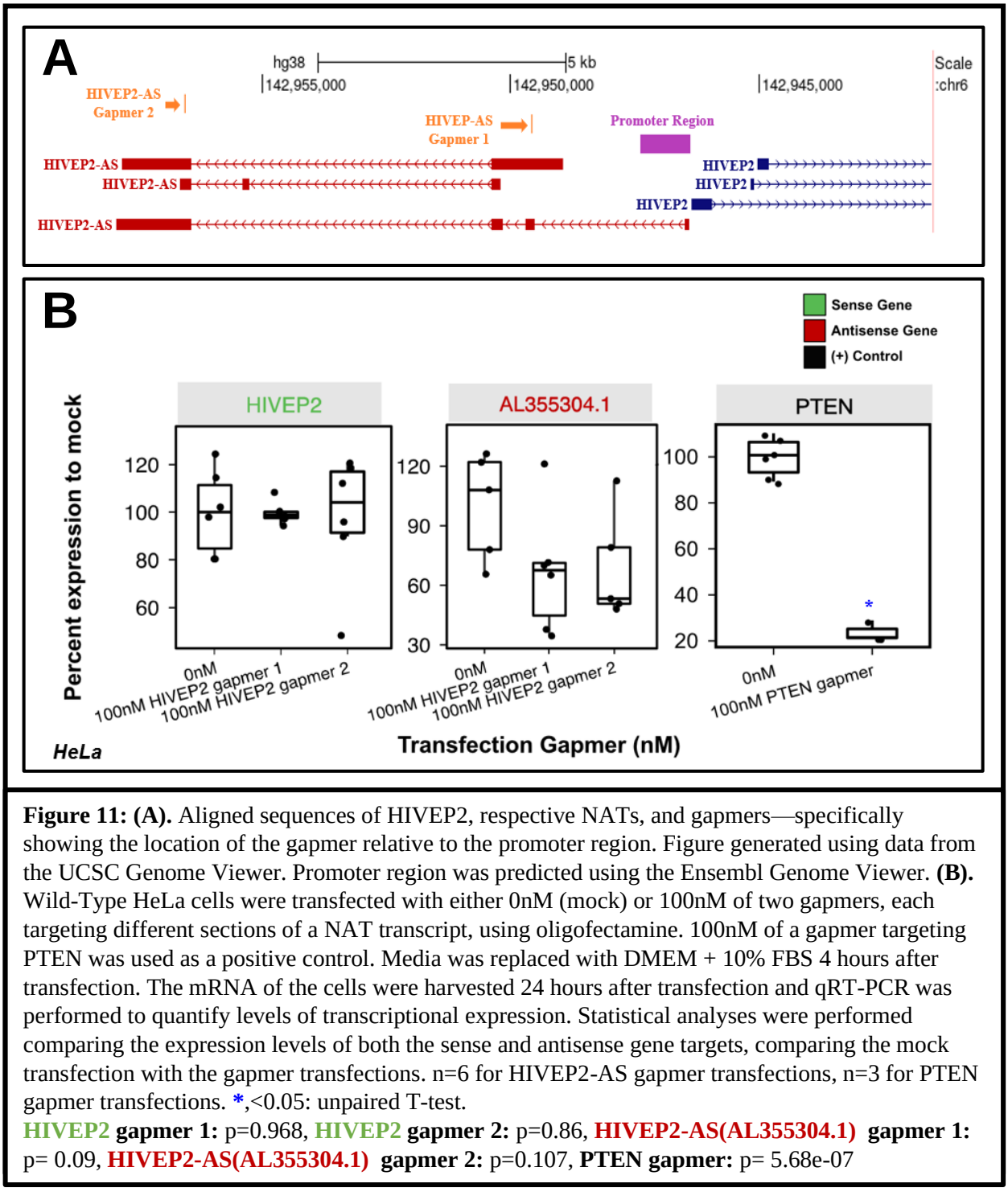

Figure 11: (A). Aligned sequences of HIVEP2, respective NATs, and gapmers—specifically showing the location of the gapmer relative to the promoter region. Figure generated using data from the UCSC Genome Viewer. Promoter region was predicted using the Ensembl Genome Viewer. **(B).** Wild-Type HeLa cells were transfected with either 0nM (mock) or 100nM of two gapmers, each targeting different sections of a NAT transcript, using oligofectamine. 100nM of a gapmer targeting PTEN was used as a positive control. Media was replaced with DMEM + 10% FBS 4 hours after transfection. The mRNA of the cells were harvested 24 hours after transfection and qRT-PCR was performed to quantify levels of transcriptional expression. Statistical analyses were performed comparing the expression levels of both the sense and antisense gene targets, comparing the mock transfection with the gapmer transfections. n=6 for HIVEP2-AS gapmer transfections, n=3 for PTEN gapmer transfections. *,<0.05: unpaired T-test.
HIVEP2 gapmer 1: p=0.968, **HIVEP2 gapmer 2:** p=0.86, **HIVEP2-AS(AL355304.1) gapmer 1:** p= 0.09, **HIVEP2-AS(AL355304.1) gapmer 2:** p=0.107, **PTEN gapmer:** p= 5.68e-07

included a gapmer that targeted PTEN to act as a positive control, with robust, statistically significant decreases in PTEN expression in the SIN3A and HIVEP2 experiments **(Fig. 9B-11B)**.

Gapmers targeting the IRF2BPL NAT increased IRF2BPL and reduced IRF2BPL-AS mRNA expression

When targeting the NAT of IRF2BPL (IRF2BPL-AS), both gapmer 1 and gapmer 2 successfully increased the sense IRF2BPL mRNA expression relative to the mock transfection by 100% and 50% with statistical significance, respectively **(Fig. 12B)**. However, gapmer 2 reduced the mRNA expression of IRF2BPL-AS by 40% with statistical significance, while gapmer 1 had a trend showing a 15% decrease in mRNA expression of IRF2BPL-AS without statistical significance **(Fig. 12B)**.

To confirm these results, a repeat experiment was performed using the same two gapmers targeting IRF2BPL-AS. Although similar trends were observed in which gapmers 1 and 2 increased the expression of the sense IRF2BPL mRNA with statistical significance, the effect size was noticeably lower—with an increase of 40% from gapmer 1 treatment and 20% from gapmer 2 treatment **(Fig. 12C)**. Comparably, the IRF2BPL-AS exhibited a similar pattern with a 30% statistically significant reduction after gapmer 2 treatment and a trend showing a 15% decrease in mRNA expression of IRF2BPL-AS without statistical significance **(Fig. 12C)**. The positive control PTEN gapmer effectively decreased the expression of PTEN in both experiments with statistical significance **(Fig. 12B, C)**.

Gapmers targeting NATs of CTBP1 and ARID1B elucidated Sense-Antisense ASD gene relationships

When targeting the NAT of CTBP1 (CTBP1-DT), gapmer 1 had no effect while gapmer 2 produced a 30% statistically significant reduction in the mRNA of the CTBP1 sense transcript

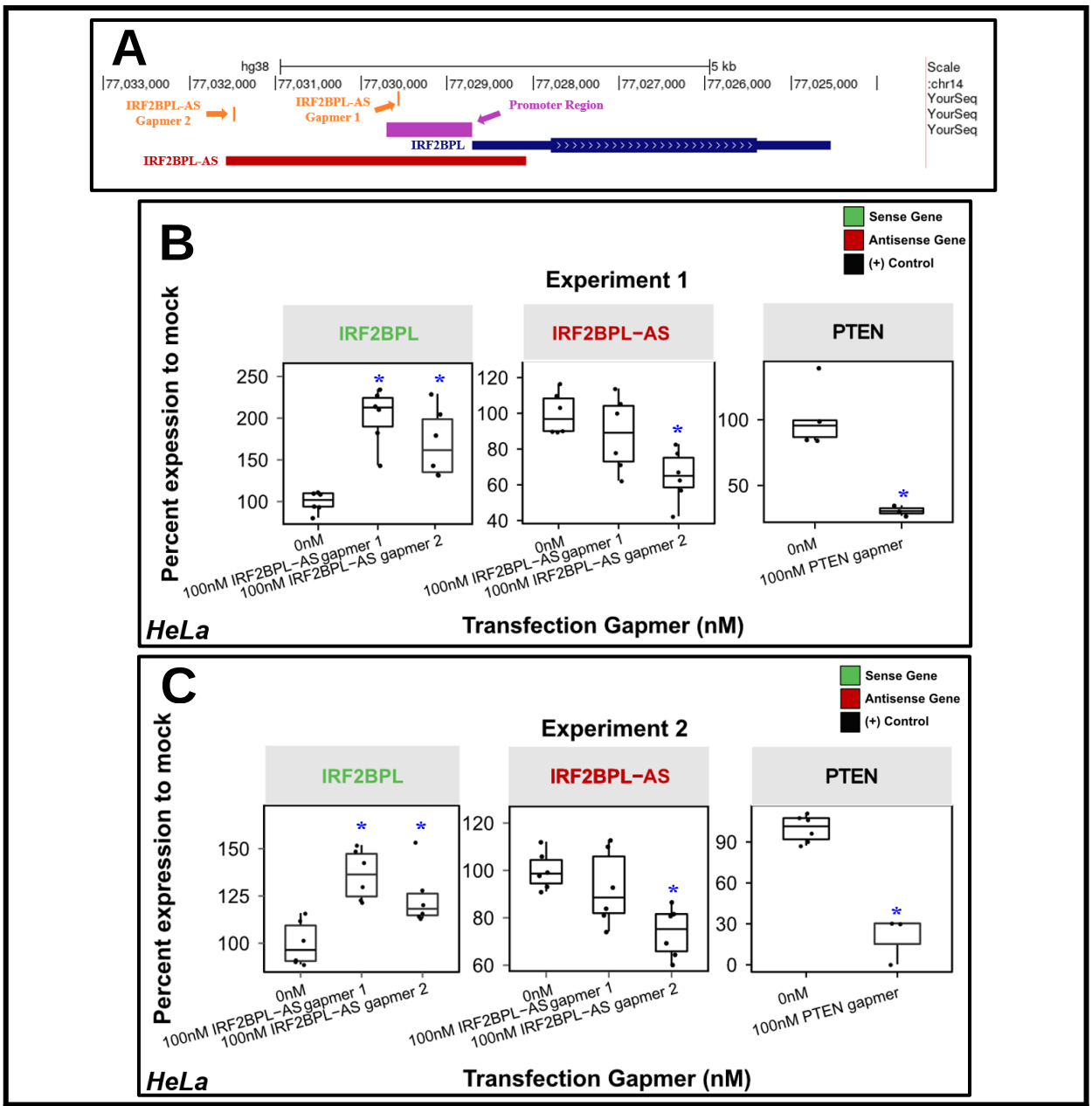

Figure 12: (A). Aligned sequences of IRF2BPL, respective NATs, and gapmers—specifically showing the location of the gapmer relative to the promoter region. Figure generated using data from the UCSC Genome Viewer. Promoter region was predicted using the Ensembl Genome Viewer. **(B, C).** Wild-Type HeLa cells were transfected with either 0nM (mock) or 100nM of two gapmers, each targeting different sections of the IRF2BPL-AS transcript, using oligofectamine. Media was replaced with DMEM + 10% FBS 4 hours after transfection. The mRNA of the cells were harvested 24 hours after transfection and qRT-PCR was performed to quantify levels of transcriptional expression. Statistical analyses were performed comparing the expression levels of both the sense and antisense gene targets, comparing the mock transfection with the gapmer transfections. n=6 for IRF2BPL-AS gapmer transfections, n=3 for PTEN gapmer transfections. *,<0.05: unpaired T-test.

(B). IRF2BPL gapmer 1: p=3.55e-04, **IRF2BPL gapmer 2**: p=0.007, **IRF2BPL-AS gapmer 1:** p=0.275, **IRF2BPL-AS gapmer 2:** p=0.001, **PTEN gapmer:** p=2.64e-04

(C). IRF2BPL gapmer 1: p=5.35e-04, **IRF2BPL gapmer 2: p**=0.013, **IRF2BPL-AS gapmer 1:** p=0.342, **IRF2BPL-AS gapmer 2: p**=9.13e-4, **PTEN gapmer:** p=0.007

and 30% reduction in the mRNA of the NATs, respectively. As a result, it appears that CTBP1-DT plays a role in concordantly regulating the CTBP1 sense transcript **(Fig. 13B)**.

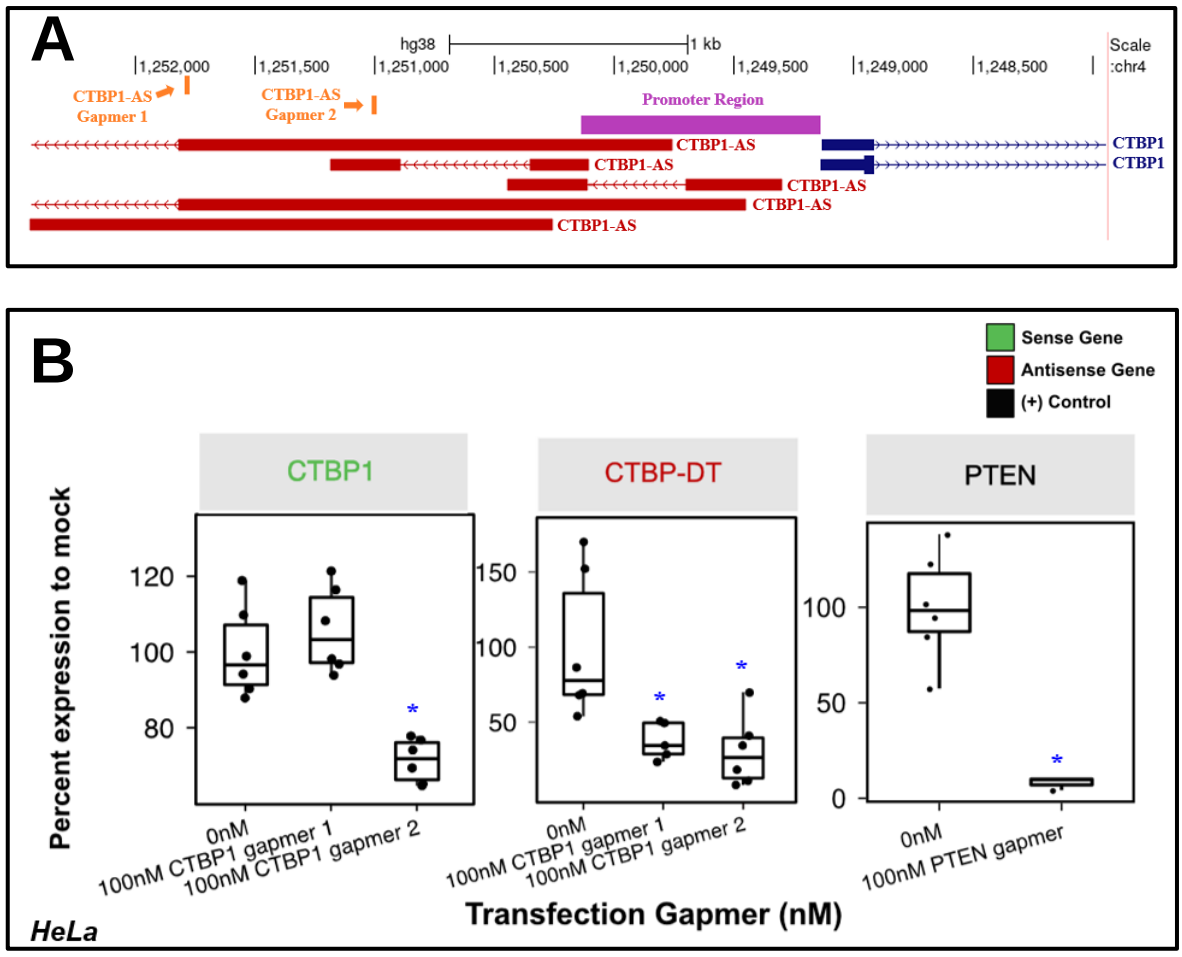

Figure 13: (A). Aligned sequences of CTBP1, respective NATs, and gapmer—specifically showing the location of the gapmer relative to the promoter region. Figure generated using data from the UCSC Genome Viewer. Promoter region was predicted using the Ensembl Genome Viewer. **(B).** Wild-Type HeLa cells were transfected with either 0nM (mock) or 100nM of two gapmers, each targeting different sections of a NAT, using oligofectamine. Media was replaced with DMEM + 10% FBS 4 hours after transfection. The mRNA of the cells were harvested 24 hours after transfection and qRT-PCR was performed to quantify levels of transcriptional expression. Statistical analyses were performed comparing the expression levels of both the sense and antisense gene targets, comparing the mock transfection with the gapmer transfections. n=6 for CTBP1 gapmer transfections, n=3 for PTEN gapmer transfections. *,<0.05: unpaired T-test.
(B). **CTBP1** gapmer 1: p=0.408, **CTBP1** gapmer 2: p=0.001, **CTBP-DT** gapmer 1: p=0.024, **CTBP-DT** gapmer 2: p=0.016, **PTEN** gapmer: p=4.37e-04

Similarly, targeting the NAT of ARID1B (AL355297_3) with gapmer 1 resulted in a 25% statistically significant reduction in the mRNA of the ARID1B sense transcript while treatment with gapmer 2 revealed a trend in which there was a 12% reduction in the mRNA of the ARID1B

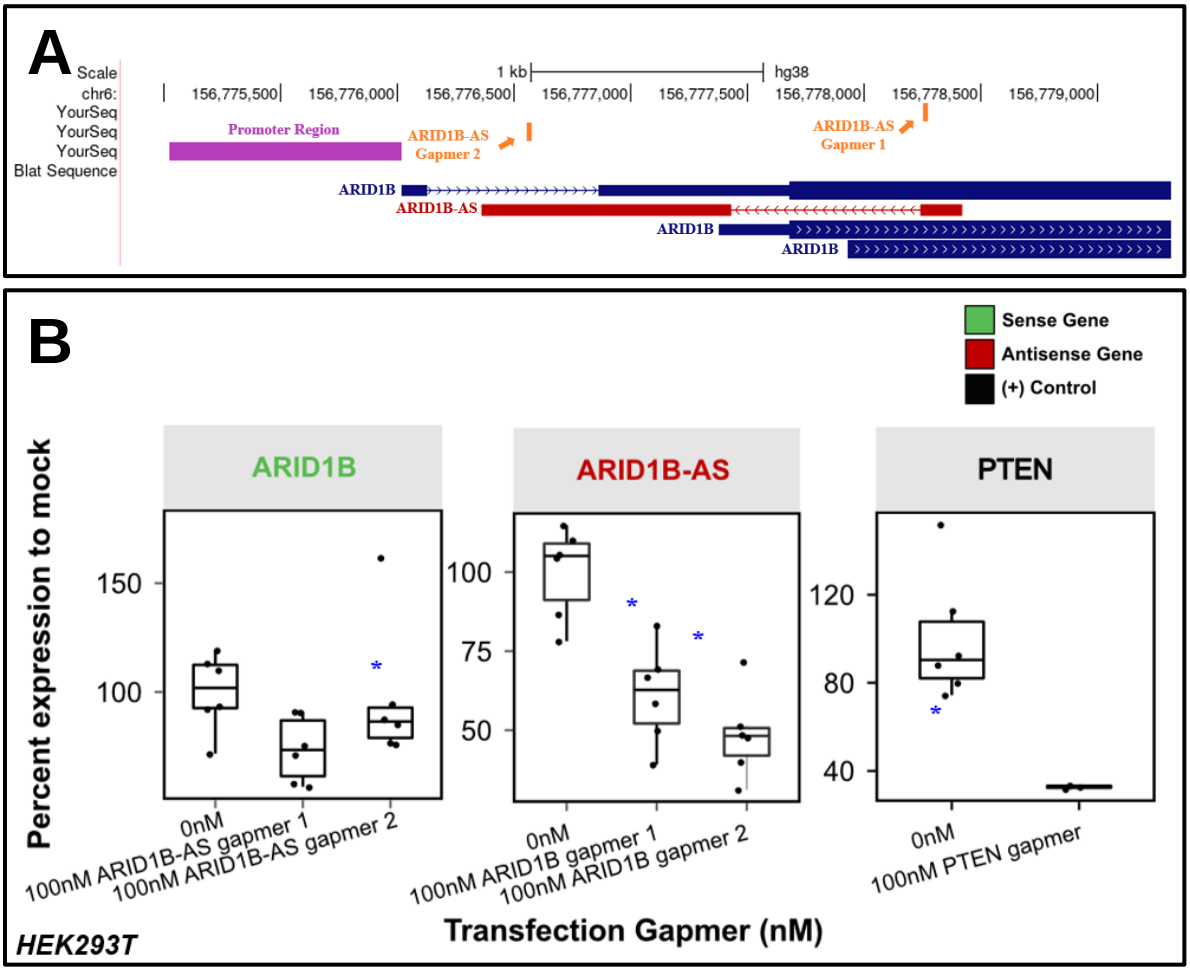

Figure 14: (A). Aligned sequences of ARID1B, respective NATs, and gapmer—specifically showing the location of the gapmer relative to the promoter region. Figure generated using data from the UCSC Genome Viewer. Promoter region was predicted using the Ensembl Genome Viewer. **(B).** Wild-Type HEK293T cells were transfected with either 0nM (mock) or 100nM of two gapmers, each targeting different sections of a NAT, using oligofectamine. Media was replaced with DMEM + 10% FBS 4 hours after transfection. The mRNA of the cells were harvested 24 hours after transfection and qRT-PCR was performed to quantify levels of transcriptional expression. Statistical analyses were performed comparing the expression levels of both the sense and antisense gene targets, comparing the mock transfection with the gapmer transfections. n=6 for ARID1B gapmer transfections, n=3 for PTEN gapmer transfections. *,<0.05: unpaired T-test.

(B). **ARID1B** gapmer 1: p=0.02, **ARID1B** gapmer 2: p=0.846, **ARID1B-AS** gapmer 1: p=0.001, **ARID1B-AS** gapmer 2: p=7.92e-5, **PTEN** gapmer: p=0.002

sense transcript **(Fig. 14B)**. Gapmers 1 and 2 resulted in a statistically significant reduction in the mRNA expression of AL355297_3 by 12% and 25%, respectively **(Fig. 14B)**. This implies an activating relationship between the AL355297_3 ARID1B NAT and the ARID1B sense transcript. In both CTBP1 and ARID1B experiments, the positive control gapmer targeting PTEN decreased the expression of PTEN with statistical significance **(Fig. 14B)**.

Validation of Cas13d had reduced effect size relative to established literature

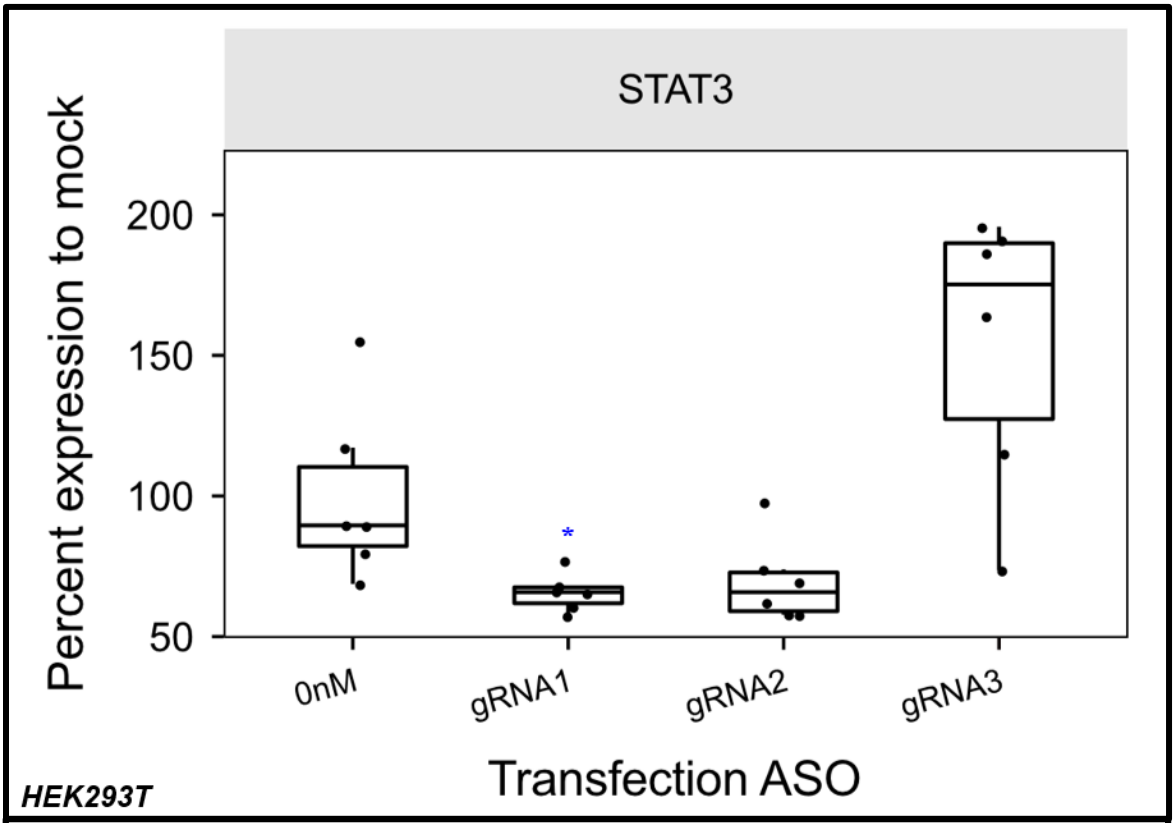

Figure 15: Wild-Type HEK293T cells were co-transfected with 2.5 ng of Cas13, and either no gRNA plasmid or 2.5 ng of various gRNAs, each targeting different sections of a STAT3 transcript, using lipofectamine. Media was replaced with DMEM + 10% FBS 4 hours after transfection. The mRNA of the cells were harvested 48 hours after transfection and qRT-PCR was performed to quantify levels of transcriptional expression. Statistical analyses were performed comparing the expression of STAT3 between STAT3-targeting gRNAs and the mock transfection. n=6 for every gRNA condition. *****,<0.05: unpaired T-test.

gRNA1: p=0.044 , **gRNA2:** p=0.071 , **gRNA3:** p= 0.051

Attempts to validate the Cas13d system using the STAT3 positive control from previous studies [181] were unsuccessful as only gRNA1 was able to reduce the expression of STAT3 by 30% with statistical significance while gRNAs 2 and 3 had no significant effect on STAT3 expression, relative to the mock transfection **(Fig. 15)**. In the previous study, Konermann demonstrated that gRNAs 1-3 were able to reduce the expression of STAT3 by nearly 100% relative to the nontargeting gRNA. Therefore, we proceeded to run an experiment transfecting different ratios of the plasmids and lipofectamine to assess whether this would influence the efficacy of our Cas13d system. The following ratios were tested: 3.75µL lipofectamine/2.5µg plasmid, 7.5µL lipofectamine/2.5µg plasmid, and 7.5µL lipofectamine/5µg plasmid. The only statistically significant effect was a 50% increase in STAT3 mRNA in gRNA2 within the 7.5µL

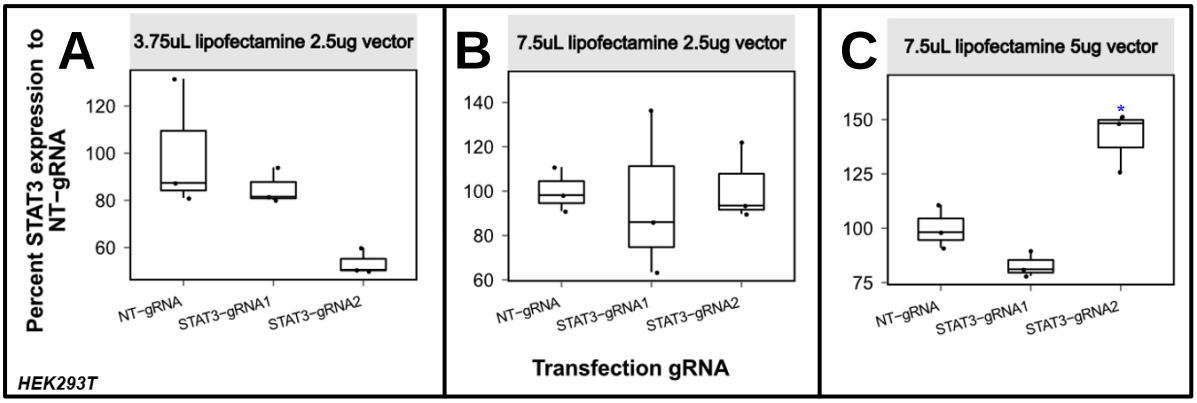

Figure 16: Wild-Type HEK293T cells were co-transfected with varying ratios of Cas13/gRNA plasmids and lipofectamine. Media was replaced with DMEM + 10% FBS 4 hours after transfection. The mRNA of the cells were harvested 48 hours after transfection and qRT-PCR was performed to quantify levels of transcriptional expression. Statistical analyses were performed comparing the expression of STAT3 between STAT3-targeting gRNAs and the non-targeting gRNAs. n=3 for every gRNA condition. *,<0.05: unpaired T-test.

(A). STAT3-gRNA1: p=0.455, **STAT3-gRNA2:** p=0.094

(B). STAT3-gRNA1: p=0.851, **STAT3-gRNA2:** p=0.888

(C). STAT3-gRNA1: p=0.08, **STAT3-gRNA2:** p=0.016

lipofectamine/5µg plasmid condition—an effect that is in the opposite direction as expected in a Cas13d system **(Fig. 16)**.

Cas13d was unable to decrease the expression of the IRF2BPL NAT

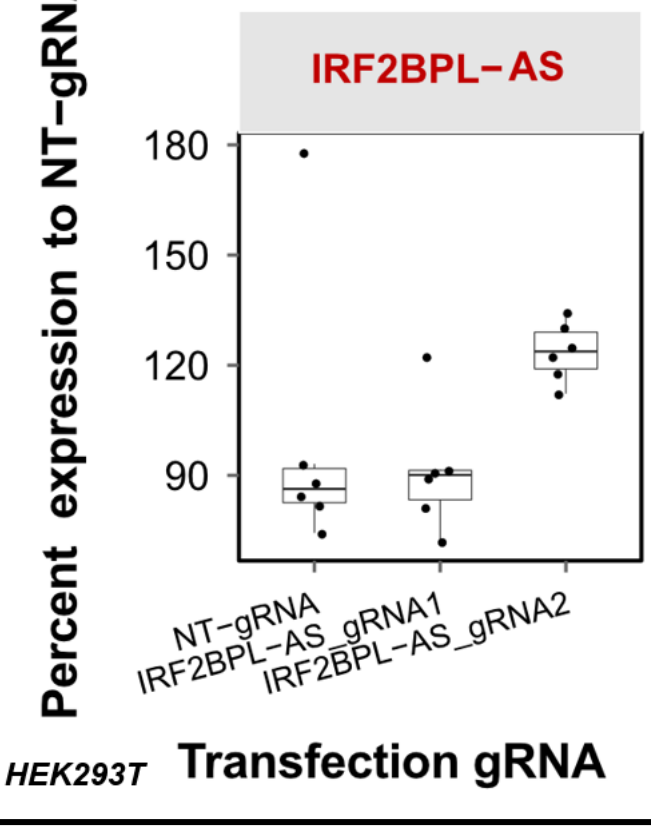

Figure 17: Wild-Type HEK293T cells were co-transfected with 2.5 ng of Cas13, and either no gRNA plasmid or 2.5 ng of various gRNAs, each targeting different sections of a IRF2BPL-AS transcript, using lipofectamine. The mRNA of the cells were harvested 48 hours after transfection and qRT-PCR was performed to quantify levels of transcriptional expression. Statistical analyses were performed comparing the expression of IRF2BPL between IRF2BPL-targeting gRNAs and the non-targeting gRNA. n=6 for every gRNA condition. *,<0.05: unpaired T-test.

IRF2BPL-AS-gRNA1: p=0.629**, IRF2BPL-AS-gRNA2:** p=0.197

Although the STAT3 positive control gRNA was unable to decrease the mRNA of STAT3 with a significant effect size, the mild success with gRNA1 prompted a preliminary experiment assessing whether the system could still work in targeting the IRF2BPL NAT (IRF2BPL-AS). Using gRNAs targeted towards IRF2BPL based on the ASOs, we found that RNA levels of IRF2BPL-AS by these guides had no statistically significant difference compared to the mRNA levels of IRF2BPL-AS treated by a non-targeting guide **(Fig. 17).**

Chapter 3, in part will be prepared in the far future for submission for publication of the material. Hong, Derek; Tran, Stephen; Sebat, Jonathan; Iakoucheva, Lilia. The thesis author was the primary author of this material.

Chapter 4: Discussion

This review portion of the introduction has investigated three main targets of intervention for NDDs across the central dogma, specifically at the: DNA, mRNA, and protein levels. While there has been much success within in-vitro and animal models at the DNA and protein levels, there has yet to be successful translation of these therapeutics in human clinical trials. At the

Table 2: Table summarizing therapeutics for human disease at different levels of the central dogma.

Therapeutic	Type	Target	Disease	Current Status	Source [doi]
Luxturna	Transgene Delivery	RPE65 Gene	Retinal Dystrophy	**FDA Approved**	10.1016/j.drudis.2019.01.019
Spinraza	Exon-Inclusion ASO	SMN2 mRNA	Spinal Muscular Dystrophy	**FDA Approved**	10.1515/tnsci-2017-0001
Drisapersen	Exon-Skipping ASO	DMD mRNA	Duchenne Muscular Dystrophy	**Failed in Phase 3 Clinical Trials**	10.1016/j.nmd.2017.10.004
Eteplirsen	Exon-Skipping ASO	DMD mRNA	Duchenne Muscular Dystrophy	**FDA Approved**	10.1089/nat.2016.0657
Milasen	Splice-Switching ASO	CLN7 mRNA	Batten's Disease	**FDA Approved**	10.1056/NEJMoa1813279
Arbaclofen	Small Molecule Drug	GABA-B Receptor	Fragile X Syndrome	**Failed in Phase 3 Clinical Trials**	10.1186/s11689-016-9181-6
Mavoglurant	Small Molecule Drug	mGluR5	Fragile X Syndrome	**Failed in Preclinical Trials**	10.1126/scitranslmed.aab4109

mRNA level, though scarce, human clinical success was achieved using the Milasen ASO to rescue pathological phenotypes of Batten's Disease—providing a strong precedent for ASO-based therapies to treat human NDDs [115]. It is important to note that the scarcity of clinical success may not be attributed to the biological inefficacies of the therapies at all three levels, but rather due to the gene identification-targeted efforts of the past two decades in NDD research. With the recent efforts of shifting focus to the therapeutic rescue of genes in NDDs, it may be possible that that the next decade will yield translational success. This success is dependent on further optimization of techniques still in their infancy such as CRISPRa or uORF-based/NMD-inhibiting ASOs. Furthermore, therapeutic mechanisms such as alternative splicing ASOs that have FDA approval for non-NDD pathologies such as Spinraza (SMA) and Eteplirsen (DMD) may provide a strong basis for further studies using these ASOs for genes with splice mutations implicated in NDDs [105, 106, 112]. The therapeutics that are either currently in clinical trials or have successfully been FDA approved in this review serve as a therapeutic wedge in the door of clinical translation, effectively paving the road for future NDD therapeutics **(Table 2)**.

The results elucidating the activating relationships between the ASD-associated CTBP1/ARID1B with their NATs also serves as a bridge between identification-based research and the development of therapeutics. By establishing a relationship, future experiments can begin to investigate the mechanism of which these NATs regulate CTBP1 and ARID1B, allowing for expeditious therapeutic development.

Additionally, in cases of inhibiting relationships such as in IRF2BPL-AS and IRF2BPL, the positive results from the Gapmer transfections have an even greater value. It has been found that LoF mutations in IRF2BPL in humans have resulted in severe neurodevelopmental regression with pathological phenotypes such as seizures, a decrease in coordination, and decreases in muscle

tone [182]. One potential direction based on the gapmer results could be to perform experiments using the same gapmers but within Haploinsufficient IRF2BPL human iPSCs or Haploinsufficient human neural progenitor cells (NPCs) to ensure that the therapeutic is still efficacious in a more accurate disease model. Additionally, based on the design of the gapmers and the subsequent results, it appears that the position of gapmers at promoter regions of sense genes do not influence their ability to induce RNAse H-dependent transcript cleavage **(Fig. 9A-13A)**. Further experiments are needed to optimize our current Cas13d system, and the first step is to obtain a new Cas13d plasmid from laboratories that have had success with their systems, followed by validating the system within our own laboratory by replicating positive controls. However, when the system does indeed work, it could potentially be used to target the NAT of discordantly regulated ASD-associated genes such as IRF2BPL to therapeutically upregulate the expression of the sense gene.

However, one concerning result of the IRF2BPL-AS-targeting NAT is the decreased effect size in the replicated experiment (**Fig. 12C)**. The 60%/30% decrease in IRF2BPL sense transcript rescue efficacy for gapmers 1/2 and 10% decrease in NAT knockdown efficacy for gapmer 2 between the two experiments could possibly be attributed to the fact that NATs are expressed significantly lower than their respective sense genes [67]. A slightly lower transfection efficiency in the second experiment could very well attribute to the discrepancies in NAT knockdown efficiency and this could consequently affect the sense gene expression at an even larger extent as one IRF2BPL-AS transcript could potentially interact with multiple IRF2BPL transcripts.

Finally, another obstacle to clinical translation involves optimization of delivery methods for the therapeutics. When considering the use of viral vectors in NDDs, some important considerations include balancing tissue-specificity, immunogenicity, packaging limits, and

ability to penetrate the BBB. With the advent of technologies such as lipid-based vectors, it may be possible to overcome the obstacles associated with the viral vectors for the development of therapeutics. However, since the lipid-based vectors are still in their infancy, further studies are needed to clearly compare both efficacy and safety between viral and lipid-based vectors.

Chapter 4, in part is currently being prepared for submission for publication of the material. Hong, Derek; Tran, Stephen; Sebat, Jonathan; Iakoucheva, Lilia. The thesis author was the primary author of this material.

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