Targeting miRNAs as a novel therapy for STXBP1 epileptic encephalopathy

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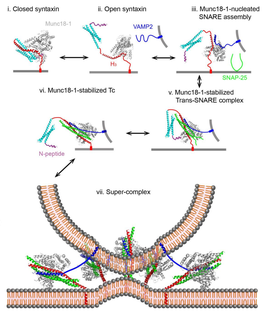
Abstract

STXBP1 epileptic encephalopathy is caused by mutations in the STXBP1 gene. In neurons, STXBP1 regulates the release of neurotransmitters from synaptic vesicles. Reducing the amount of functional STXBP1 protein impairs neurotransmitter release, which in turn leads to uncontrolled neuronal activation, epilepsy, intellectual disability, and motor impairments. In cases of haploinsufficiency of STXBP1, the upregulation of functional STXBP1 protein could be therapeutic for STXBP1 epileptic encephalopathy. As the majority of human mRNAs are at least partly repressed by microRNAs (miRs), blocking the interaction between miRNA and STXBP1 mRNA could upregulate expression of STXBP1 and presumably provide a neuroprotective therapeutic effect. Here based on bioinformatic analysis we hypothesized that STXBP1 is under endogenous repression by miR-218 and miR-424. Using antogomiRs (siRNA against miRNA), we found that inhibiting either miR-218 or miR-424 is sufficient to upregulate STXBP1 mRNA and protein in a human neuroblastoma cell line (SHSY-5Y cells). We generated lentivirus encoding shRNA targeting these miRNAs and a Luciferase-STXBP1-3'UTR Reporter Gene construct to test if inhibition of miR-218 or miR-424 in SHSY-5Y cells increases luciferase expression. Further, we designed and used 2OMe-phosphorothioate backbone antisense oligonucleotides (ASOs) to bind and block miRNAs target sites in STXBP1 3'UTR directly, and as such prevent miRNA mediated repression. These studies identify miR-218 and miR-424 targeting as a promising therapeutic development for STXBP1 epileptic encephalopathy caused by haploinsufficiency.

BACKGROUND

What is STXBP1?

- Syntaxin Binding Protein 1 (aka Munc18-1)
- Located on chromosome 9, coding region 1.7kb
- Protein size – 594 amino acids, ~68kDa
- Forms a complex with the SNARE protein syntaxin-1 to play an **essential role in synaptic vesicle release.**
- Initiates SNARE assembly and stabilizes the half-zippered complex.
- STXBP1 deficient neurons/mice have a complete loss of neurotransmitter release.



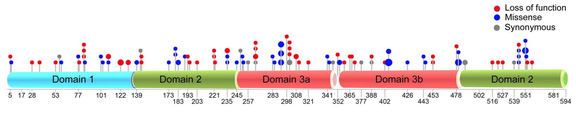
SNARE proteins (SNAP Receptor) complex of 60 members which is responsible for fusion of vesicles with target membrane bound compartments in neurons

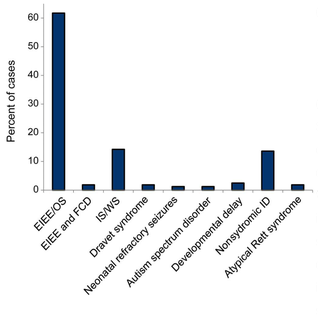
STXBP1 Encephalopathy

- Complex neurodevelopmental disorder first described in 2008
- 1 in 90,000 kids (likely an underestimate)
- Clinical Features:
  - Severe to profound **intellectual disability** (almost all are non-verbal)
  - **Epilepsy** (seizures controlled in ~30% of kids)
  - Varying levels of autistic features and **motor dysfunction** (40% learn to walk assisted, hypotonia, ataxia, tremor commonly seen)
  - Brain MRI normal in ~50% of kids (cerebral atrophy, hypomyelination, frequent age related findings)

Mapping of mutations within the STXBP1 gene

Uddin et al., Neurology Genetics, 2017



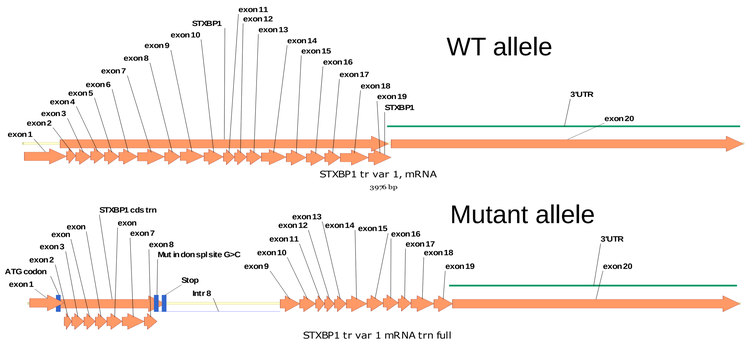


(A) Mutations mapped within the 3 protein domains (cyan—domain 1, green—domain 2, and red—domain 3a/b) of the STXBP1 gene.

(B) Clinical spectrum associated with de novo STXBP1 mutations reported for early infantile epileptic encephalopathy (EIEE), focal cortical dysplasia (FCD), Ohtahara syndrome (OS), West syndrome (WS), Dravet syndrome, infantile spasms (ISs), neonatal refractory seizures, autism spectrum disorder, developmental delay, nonsyndromic intellectual disabilities (IDs), and atypical Rett syndrome.

TARGET MUTATION: UNDERLYING GENETICS

663+1G>T substitution in donor splice site of exon 8

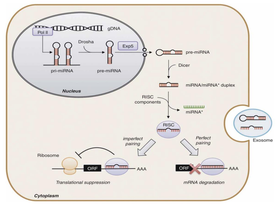


- Artificial STOP codon introduced into intron 8, predicted to be degraded due to non-sense mediated decay
- Mutation in same splice site (663+1G) found in three other patients.
- Evidence of non-sense mediated decay in cells from patient with similar mutation (663+5G, Saitsu *Epilepsia* 2010)
- Likely pathomechanism is **haploinsufficiency**

STXBP1 PROTEIN UPREGULATION: STRATEGY

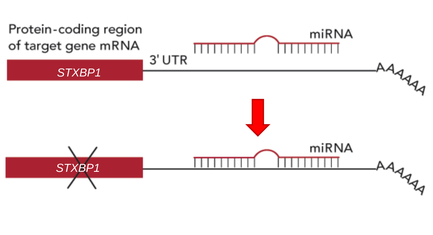
Transcriptional upregulation by repressing the repressors – miR-based therapy

- Small (22 nucleotide) non-coding RNAs that function in RNA silencing
- miRs base-pair with complementary mRNA molecules, and then silence these mRNAs by cleavage, destabilization, or less efficient translation
- ~60% of genes in humans are targeted by miRs

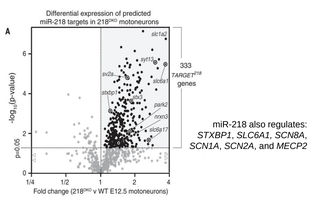


•Three basic strategies – anti-sense oligonucleotide mimics, antogomiRs, or site blockers (SBOs) (depending on if you want less or more of your target(s))
•Several miRNA-based therapies are in clinical testing (cancer, liver disease, cardiovascular disease, diabetes)
•Challenges are **identification** of the best miR candidates or targets for each disease, and the **delivery** of compounds with high stability, tissue-specific targeting, and minimal off-target effects
•Pros are that this is titratable (not a one-time on off switch), and so far, a very promising toxicity-profile, which provides a quick route from bench to bedside

IS STXBP1 REGULATED BY ANY MICRO-RNAS?



miR-218 KO mice
Amin et al., *Science* 2015

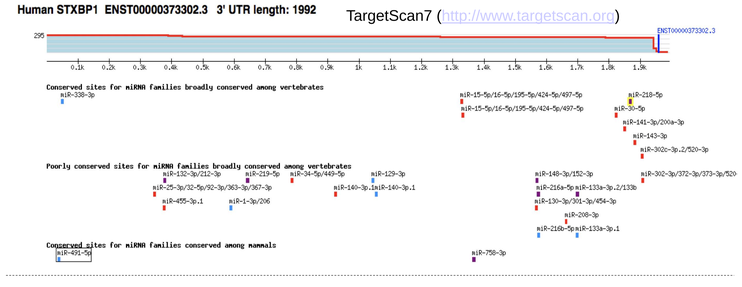


miR-218 also regulates: *STXBP1, SLC6A1, SCN8A, SCN1A, SCN2A*, and *MECP2*

In silico approaches predict miR-218 and miR-424 may regulate STXBP1

Human STXBP1 ENST00000373302.3 3' UTR length: 1992

TargetScan7 (http://www.targetscan.org)



TarBase v.8

Gene name	miRNA name	Experiments throughput	Publications	Cell lines	Tissues	Pred. Score
STXBP1	hsa-miR-218-5p	low: 0 high: 5	2	2	2	0.994
STXBP1	hsa-miR-424-5p	low: 0 high: 1	1	1	1	0.782

RESULTS: ANTAGOMIRS

Inhibition of miR-218 or miR-424 with antogomiRs increase STXBP1 mRNA and protein

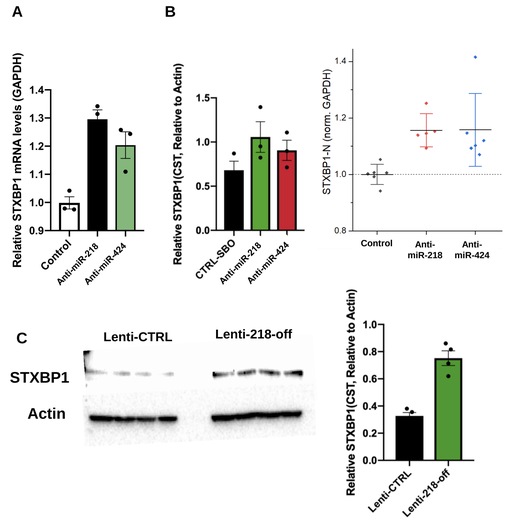


Fig. 1 Relative levels of STXBP mRNA (A) and protein (B) expression in SHSY5Y cells transfected by synthetic miRNA Inhibitors, 48 hours (Sigma). (Protein data from 2 independent experiments). Transduction of SHSY-5Y cells for 48hrs by Sigma's Mission Lenti anti miRNA-218 leads to a more robust upregulation of STXBP1 (C).

Luciferase reporter assay for expression of STXBP1 3'UTR

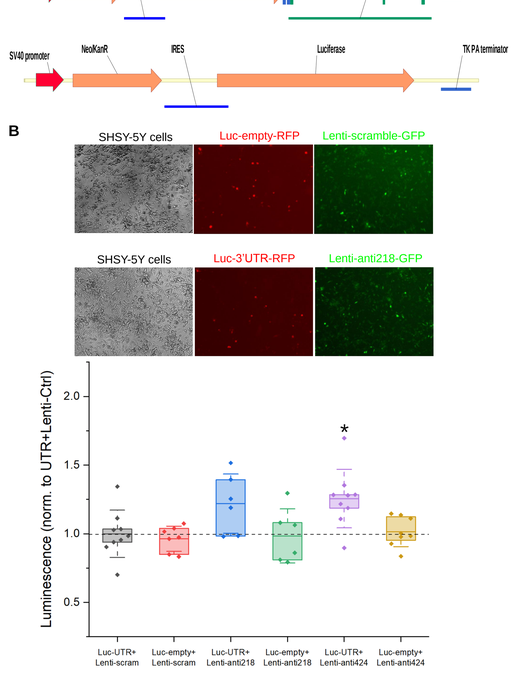


Fig. 2 (A) Linear plasmid map of Luciferase-STXBP1 3'UTR + RFP construct. (B) SHSY-5Y cells were cultured in 6-well plates one day before transfection. On the next day, when cells reached 30% to 40% confluence, they were transduced with the anti miR218- and anti miR-424-5p lentiviruses. After 24 hours cell were transfected by Luciferase-3'UTRSTXBP1 and Luciferase control constructs . (C) Luciferase activity was increased in the cells transduced with anti miR-218- 5p and anti miR-424-5p lentiviruses compared to Lenti miRNA scrambled control and Luciferase control construct. These results indicate that the 3'UTR site of mRNA from the STXBP1 gene consists of conserved binding sites for miR-218-5p and anti miR-424-5p. With higher transfection efficiency, this assay should be useful for high-throughput screening of SBOs.

RESULTS: SBOS

RNA-based therapeutic technology: Steric Blocking Oligonucleotides (SBOs)

Employment of 2'-O-methyl-modified phosphorothioate antisense oligonucleotides for blocking of miRNA – mRNA interaction

	Target Sequence(5'-3')	Binding Sites	Tm	G+C	Free Energy	SBO Sequence (DNA,5'-3')	SBO Sequence (RNA,5'-3')
miR491/miR338	CACACATCCTCACCCACAG	23-42	57.9	60	-1.6	CTGTGGGGTGAGGATGTGTG	CUGUGGGGUGAGGAUGUGUG
	ACCCGCACAGAAACTGCTGGA	35-54	58.9	55	-4.1	TCCAGCAGTTTCTGTGCGGGT	UCCAGCAGUUUCUGUGCGGGU
	CAGAACTGCTGGACACACT	40-59	55.1	50	-3.55	AGTGTGTCCAGCAGTTTCTG	AGUGUGUCCAGCAGUUUCUG
miR424	CCATTTGTGCTGCTGCTGTT	1318-1337	56.8	50	-3.14	AACAGCAGCAGCACAAATGG	AACAGCAGCAGCACAAAUGG
	TTGTGCTGCTGCTGTTGTCG	1322-1341	58.8	55	-3.61	CGACAACAGCAGCAGCACAA	CGACAACAGCAGCAGCACAA
	CTGCTGCTGTTGTCGTGAAA	1327-1346	56.1	50	-3.61	TTTCACGACAACAGCAGCAG	UUUCACGACAACAGCAGCAG
miR218/miR143	GAAAGTACTGAAGCACAAAC	1854-1873	49.3	40	-6.64	GTTTGTGCTTCAGTACTTTC	GUUUGUGCUUCAGUACUUUC
	GCACAAACATATATATCATCTC	1866-1885	46.1	35	-6.35	GAGATGATATATATGTTTGTGC	GAGAUGAUAUAUGUUUGUGC
	ATCATCTCTGTACCATTCTG	1878-1897	48.9	40	-3.65	CAGAATGGTACAGAGATGAT	CAGAAUGGUACAGAGAUGAU

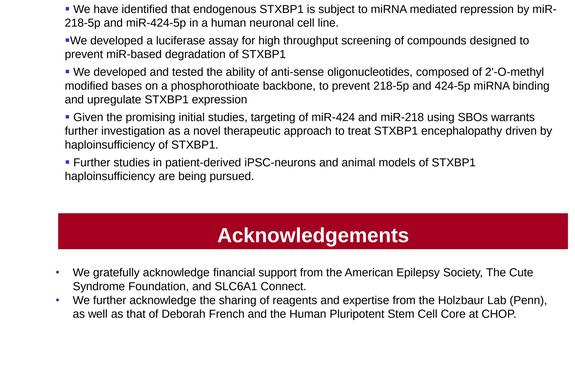


Fig. 3 (A) Initial nine ASO SBOs designed to target various miR binding sites on the 3'UTR of STXBP1. (B) Linear schematic of 3'UTR of STXBP1, miR seed sequences (red), and SBO binding sites.

SBOs targeting the 3'UTR of STXBP1 increase STXBP1 protein expression in SHSY-5Y cells (preliminary)

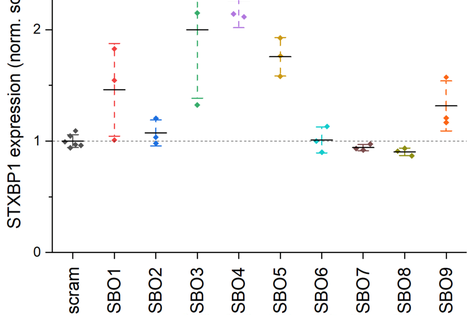


Fig. 4 Effect of SBOs transfection on STXBP1 upregulation in SHSY5Y cells. Cells were transiently transfected 10pmol/well with SBOs/scrambled oligonucleotides using Lipofectamine RNAiMAX Transfection Reagent . Endogenous STXBP1 protein expression 48 hrs after transfection was assayed by western blotting and normalized to GAPDH expression.

Conclusions and Future Directions

- We have identified that endogenous STXBP1 is subject to miRNA mediated repression by miR-218-5p and miR-424-5p in a human neuronal cell line.
- We developed a luciferase assay for high throughput screening of compounds designed to prevent miR-based degradation of STXBP1
- We developed and tested the ability of anti-sense oligonucleotides, composed of 2'-O-methyl modified bases on a phosphorothioate backbone, to prevent 218-5p and 424-5p miRNA binding and upregulate STXBP1 expression
- Given the promising initial studies, targeting of miR-424 and miR-218 using SBOs warrants further investigation as a novel therapeutic approach to treat STXBP1 encephalopathy driven by haploinsufficiency of STXBP1.
- Further studies in patient-derived iPSC-neurons and animal models of STXBP1 haploinsufficiency are being pursued.

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