Therapeutic Approach to Angelman Syndrome Using CRISPR Cas13d/CasRx Programmable Targeting

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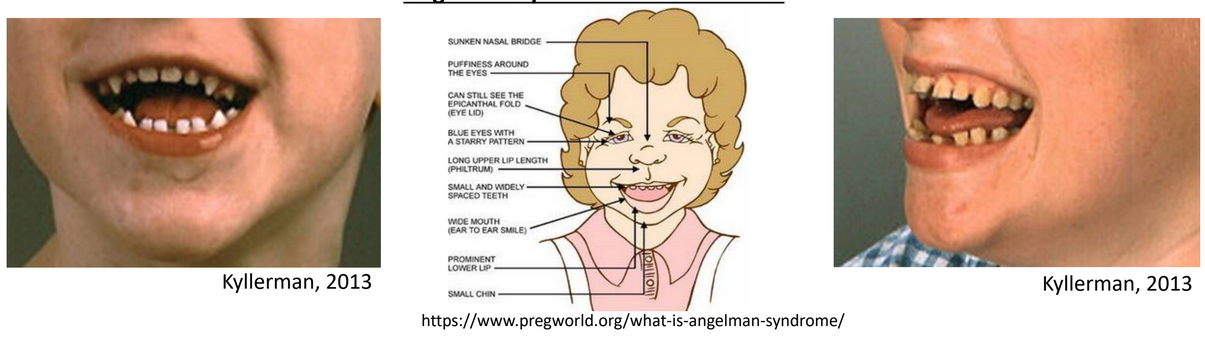
INTRODUCTION

- Angelman Syndrome (AS) is a rare neurogenetic disorder (~1:15,000) characterized by severe intellectual disability, lack of speech, autistic tendencies, seizures, ataxia, muscle hypotonia and overall happy dispositions.
- Patients frequently display craniofacial malformations such as prognathism, bruxism and widely spaced teeth.
- AS is a monogenic disorder caused by loss of function of the UBE3A gene (a ubiquitin ligase) on the maternally inherited allele (chromosome 15). The paternal allele is intact but epigenetically imprinted by a long non-coding antisense RNA, SNHG14 (UBE3A-ATS).
- Unsilencing the intact but silenced paternal UBE3A presents a therapeutic opportunity.
- **We hypothesize** that applying a novel CRISPR based genome engineering approach, Cas13d/CasRx with designed guide RNAs will precisely target and degrade the long non-coding RNA (UBE3A-ATS), effectively unsilencing UBE3A.

Angelman Syndrome Characteristics



Kyllerman, 2013

https://www.pregworld.org/what-is-angelman-syndrome/

Kyllerman, 2013

Figure 1. Facial characteristics of Angelman Syndrome individuals. Patients frequently display craniofacial malformations such as prognathism, bruxism and widely spaced teeth.

OBJECTIVES

To unsilence the UBE3A gene by designing and constructing CRISPR Cas13d/CasRx components and multiple guide RNAs to precisely target and degrade the antisense non-coding RNA UBE3A-ATS responsible for epigenetically imprinting the UBE3A gene.

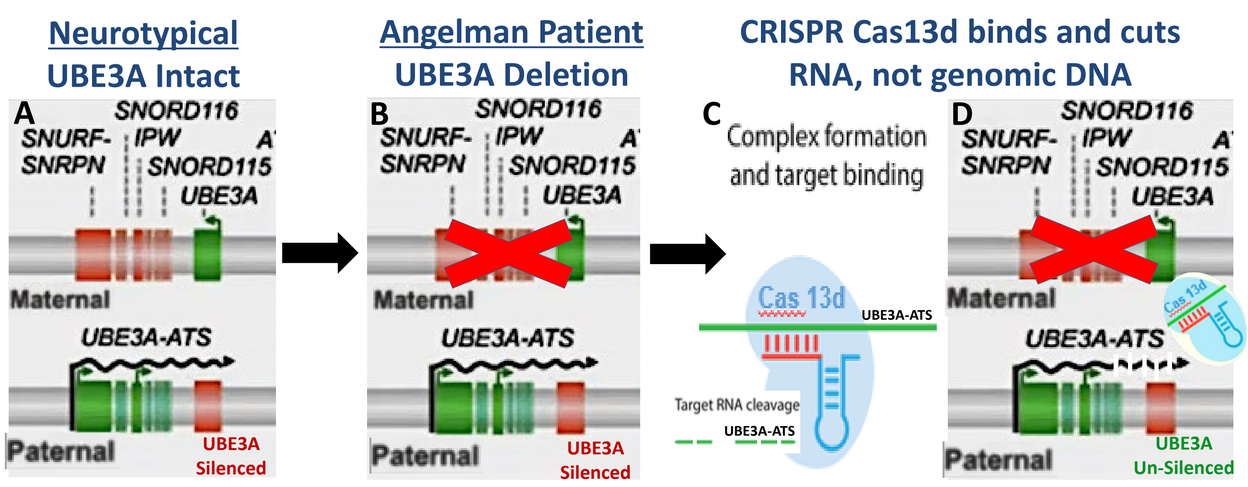


Figure 2. Model of Angelman Syndrome chromosomal deletion in the 15q11-q13 region. (A) A neurotypical individual with one expressed copy of UBE3A from the maternal allele. The paternal copy is epigenetically imprinted. **(B)** Angelman neurons contain a maternal deletion of the UBE3A gene in addition to a silenced paternal UBE3A as a result of the long non-coding UBE3A-ATS antisense RNA, leaving no functional copy of gene. **(C)** Depiction of Cas13d/CasRx annealing to UBE3A-ATS causing precise RNA cleavage. **(D)** Our Cas13d/CasRx approach to precisely target and degrade UBE3A-ATS, unsilencing the UBE3A gene.

METHODS

Genome Target Region
- Distal regions of the long non-coding antisense RNA SNHG14 (UBE3A-ATS) were located and targeted.

Map the Target Regions
- Target regions were mapped onto chromosome 15 using Ensembl genome browser (Figure 3A).

Design CRISPR Cas13d guide RNAs
- Guide RNAs were designed targeting distal regions of long non-coding RNA SNHG14 (UBE3A-ATS) (Figure 3B,C).
- gRNAs were scored & ranked for specificity and off target activity.

Clone Plasmids
- Molecularly cloned 29 Cas13d/CasRx guide RNAs into pLenti-RfxCas13d vector (Addgene #138147) with Gibson cloning (Figure 5).

Transfect gRNAs for Initial Screening
- Transfected ~29 gRNAs using Cas13d enzyme that only targets and degrades RNA
- Will screen gRNAs with two cell culture lines, HEK293-T and an induced pluripotent stem cell line expressing UBE3A-ATS.

Second Screening of gRNAs
- Will take best performing gRNAs to target UBE3A-ATS in Angelman Syndrome patient specific stem cell derived neurons.

RESULTS

Visualizing the distal targeting region of human long non-coding RNA SNHG14 (UBE3A-ATS) on chromosome 15

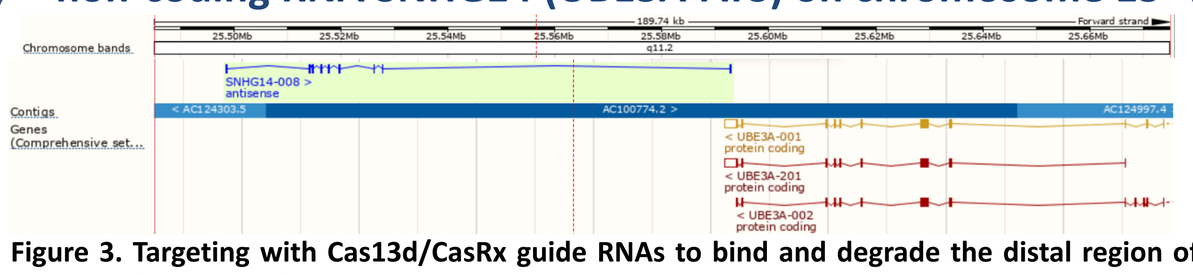


Figure 3. Targeting with Cas13d/CasRx guide RNAs to bind and degrade the distal region of SNHG14 (UBE3A-ATS) RNA transcripts. Visual representation of the distal region of the SNHG14 (UBE3A-ATS) overlapping the UBE3A gene.

Guide RNA scoring algorithm identified and top ranked gRNAs

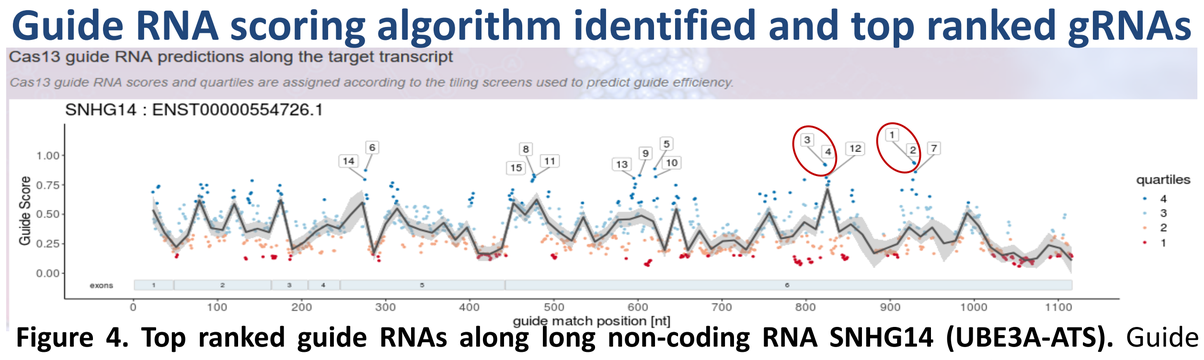


Figure 4. Top ranked guide RNAs along long non-coding RNA SNHG14 (UBE3A-ATS). Guide RNAs were designed targeting distal regions of long non-coding RNA SNHG14 (UBE3A-ATS) This is a visual representation of the transcript with highest ranked gRNAs located in the 4th quadrant. The top 4-6 gRNAs were chosen for molecular cloning into the CRISR Cas13d/CasRx plasmid vector. Gene representation generated from NY Genome Center Cas13d algorithm.

Sequence verification of gRNA transformation

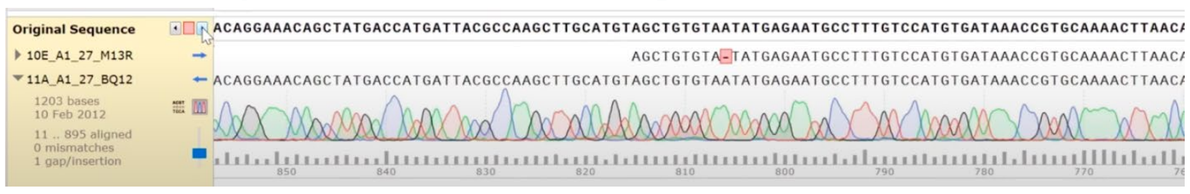


Figure 5. gRNA sequence from transformed plasmid compared to reference gRNA sequence. Guide RNA plasmid clones were verified using Sanger sequencing. Visualization of gRNA sequences in comparison to reference gRNA sequences was performed using SnapGene software. Above is example of gRNA sequence within plasmid backbone compared to reference gRNA sequence. Smooth peaks corresponding to each nucleotide visible, indicating accurate software read of sequence. Comparison reveals exact nucleotide replication, indicating successful transformation of target gRNA.

Robust knockdown of GFP with CRISPR Cas13d/CasRx

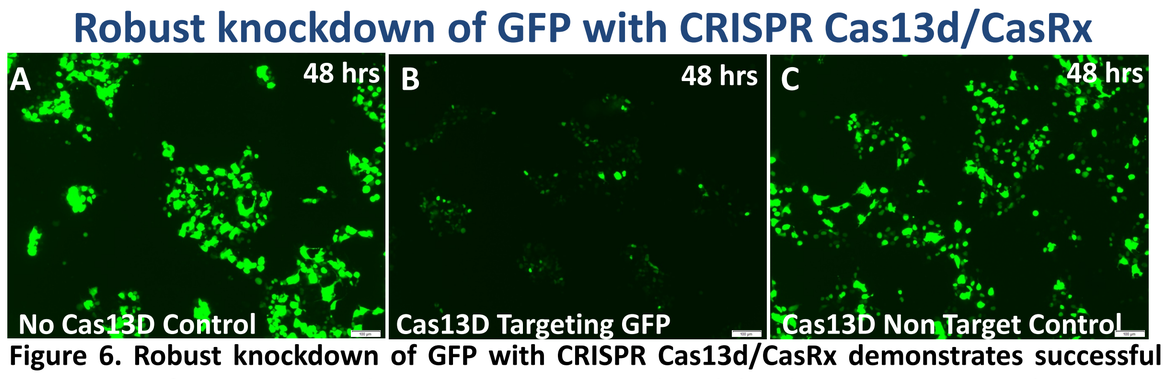


Figure 6. Robust knockdown of GFP with CRISPR Cas13d/CasRx demonstrates successful operation of enzyme. Guide RNAs targeting a destabilized GFP or a control non-GFP targeting region were cloned into the Cas13d/CasRx plasmid vector. Co-transfection of a destabilized GFP plasmid vector were transfected into all HEK293-T cells (20 ng). **(A)** HEK293-T cells transfected with a destabilized GFP (20 ng) only. **(B)** HEK293-T cells co-transfected with a destabilized GFP (20 ng) and a Cas13d/GFP targeting guide RNA (200ng). **(C)** HEK293-T cells co-transfected with a destabilized GFP (20 ng) and a Cas13d/non-targeting guide RNA (200ng). Scale bar = 100 uM

FUTURE DIRECTIONS

- Continue verification of sequences from molecular cloning Cas13d/CasRx plasmids and guide RNAs into the Cas13d/CasRx pLenti-RfxCas13d vector backbone (#138147)
- Screen gRNAs with two human cell culture platforms, HEK293-T and Prader-Willi iPSC line.
- Transfect all cloned 29 gRNAs using Cas13d/CasRx that only targets and degrades RNA.
- Identify and select best performing gRNAs to target UBE3A-ATS in Angelman Syndrome patient specific stem cell derived neurons.
- Compare our CRISPR Cas13d/CasRx targeting approach against the positive control Topotecan, a topoisomerase inhibitor shown to robustly inhibit UBE3A-ATS,
- Quantify UBE3A-ATS and UBE3A expression with gene and protein assays (qPCR, western).

CONCLUSIONS

- We have designed 29 Cas13d/CasRx guide RNAs to precisely target multiple distal regions of UBE3A-ATS. Additionally we have cloned and transfected the gRNAs. RNA extraction for analysis is ongoing.
- We observed robust knockdown of GFP with a precisely designed Cas13d guide RNA.
- Previously reported UBE3A-ATS inhibitors can be toxic, have multiple off target effects or stimulate deleterious DNA repair mechanisms.
- Innovative CRISPR Cas13d/CasRx gene editing targets RNA and not DNA, may be more precise and therefore offers a potentially safer method of gene regulation than traditional CRISPR Cas9 methods or antisense oligo nucleotides (ASOs).
- If paternal UBE3A in Angelman Syndrome is successfully unsilenced, it would provide an innovative treatment for patients suffering from this disorder.

ACKNOWLEDGEMENTS

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