# Upregulating disease-relevant genes via regulatory RNA-targeting antisense oligonucleotides

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## Abstract

Diseases caused by haploinsufficiencies or hypomorphic mutations could be potentially addressed by modest (<2-fold) increases in expression of the healthy alleles of the affected disease-associated genes. Transcription of all active protein-coding genes is associated with transcription of non-coding RNAs, a subset of which have well-characterized cis-regulatory roles and are termed regulatory RNAs (regRNAs). While many regRNAs have been functionally validated, it remains unknown whether they can be successfully modulated to up-regulate expression of disease-associated genes and alleviate hypomorphic disease phenotypes.

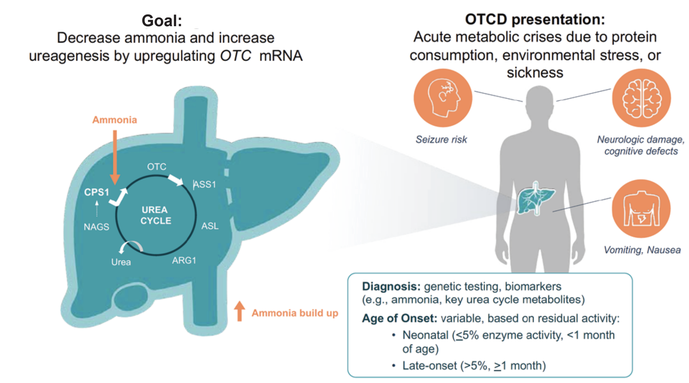
As part of our RAP Platform™, we developed a high-throughput regRNA capture-seq method and annotated sequences of tens of thousands of regRNA species transcribed from hundreds of different enhancers in primary human hepatocytes. We then applied the regRNA capture-seq to characterize a genetically validated enhancer for the human ornithine transcarbamylase gene (*OTC*), the most frequently mutated gene in patients with urea cycle disorders. Screens of antisense oligonucleotides (ASOs) targeting regRNAs transcribed from the *OTC* enhancer identified several ASOs that induced dose-dependent upregulation of *OTC* gene expression in primary human hepatocytes. Using a humanized liver mouse model we further demonstrated the efficacy of selected ASOs to induce *OTC* upregulation, a significant reduction in plasma ammonia levels, as well as a concomitant increase in urea production.

To investigate the mechanisms by which select ASOs induced *OTC* upregulation, we evaluated their ability to modify the steady-state levels and secondary structure of the targeted regRNAs, as well as changes in transcription factor binding to the targeted *OTC* enhancer and chromatin modifications using primary human hepatocytes. We identified ASO-induced local and distal effects on the regRNA's secondary structure *in vitro* that corresponded to elevation of the steady-state and nascent regRNA levels, increase in levels of H3K27 acetylation, and reduction in binding of negative transcriptional regulators to the targeted *OTC* enhancer. Our results support a mechanism whereby ASO binding to the targeted regRNA induces structural changes in regRNA that result in depletion of negative regulators from the targeted enhancer and increase in enhancer activity leading to increased mRNA transcription.

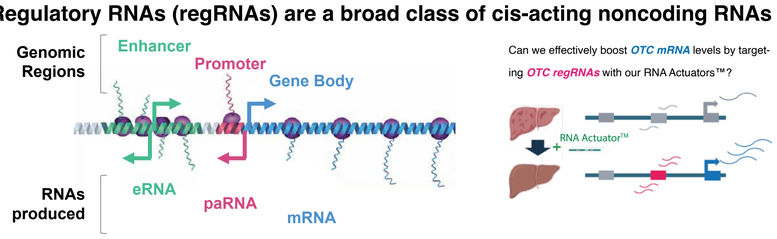
**Our results support a mechanism to harness the regulatory roles of non-coding RNAs to upregulate therapeutically-relevant genes using regRNA-targeting ASOs.**

## Background

**Ornithine Transcarbamylase Deficiency (OTCD) is an X-linked urea cycle disorder resulting in an inability to adequately process ammonia to urea**



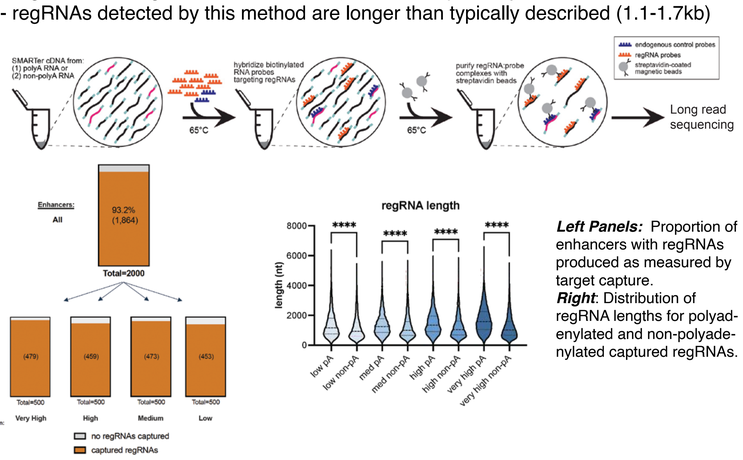

**Regulatory RNAs (regRNAs) are a broad class of cis-acting noncoding RNAs**




## Cataloging regRNAs at thousands of enhancers

**Identifying regRNAs transcribed from 2,000 enhancers in human hepatocytes**

- Target-capture sequencing method developed to identify regRNA species
- regRNAs cataloged for >93% of the 2,000 transcriptionally active enhancers
- regRNAs detected by this method are longer than typically described (1.1-1.7kb)

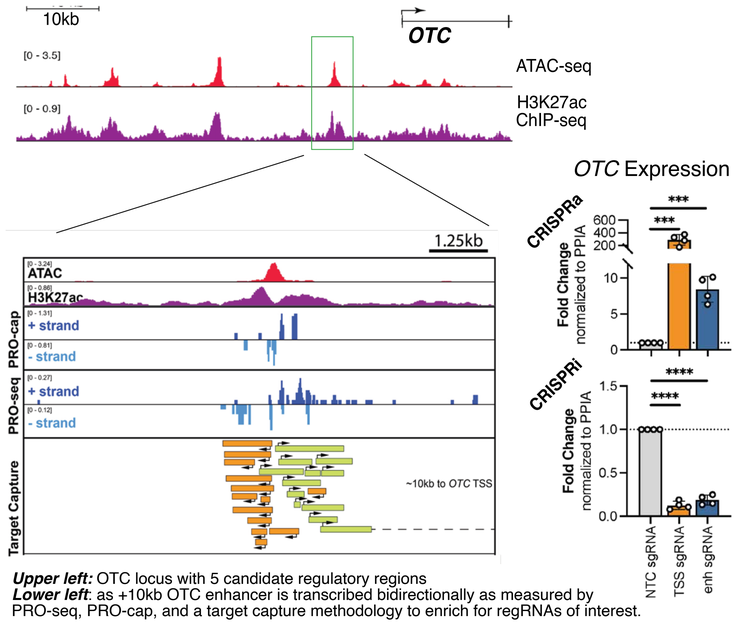



***Left Panels:*** Proportion of enhancers with regRNAs produced as measured by target capture.
***Right***: Distribution of regRNA lengths for polyadenylated and non-polyadenylated captured regRNAs.

## Mapping *OTC* locus in primary human hepatocytes

**Upstream OTC enhancer is a functionally-validated regulatory region**

- OTC enhancer exhibits hallmark open chromatin and histone modification
- regRNA is produced bidirectionally from enhancer
- CRISPRa/i targeting the functional enhancer modulates *OTC* expression



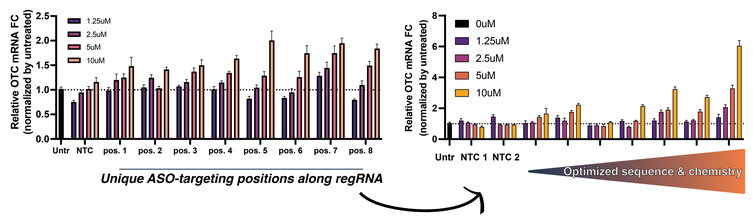

***Upper left:*** OTC locus with 5 candidate regulatory regions
***Lower left***: as +10kb OTC enhancer is transcribed bidirectionally as measured by PRO-seq, PRO-cap, and a target capture methodology to enrich for regRNAs of interest.

***Right:*** Guide RNAs targeting the OTC enhancer in HepG2 cells expressing CRISPRa (VPH) or CRISPRi (KRAB) effectors validate that the enhancer is functional. All indicated p values are < 0.001, student's t-test.

## ID and optimization of *OTC* regRNA-targeting ASOs

**RAP Platform™: Programming medicines targeting regRNAs**


**ASO screening identifies lead dose-responsive ASOs**

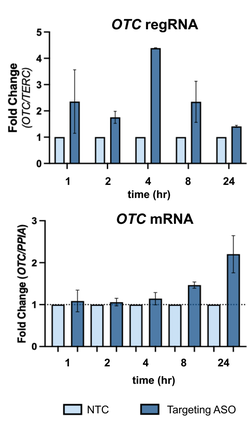



***Left:*** ASO screening of ASOs targeting different positions along the regRNA for their ability to upregulate *OTC* mRNA, resulting in multiple hits with >1.5 Fold-change (FC).
***Right:*** Optimization of lead ASO sequence/chemistry increases *OTC* mRNA upregulation *in vitro*.

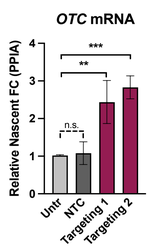
## Impact of ASO treatment on transcription

**Treatment of hepatocytes with regRNA-targeting ASO results in:**

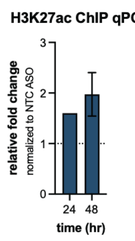
**Upregulation of targeted regRNA preceding *OTC* mRNA changes**




**Increased transcription of *OTC* nascent RNA**



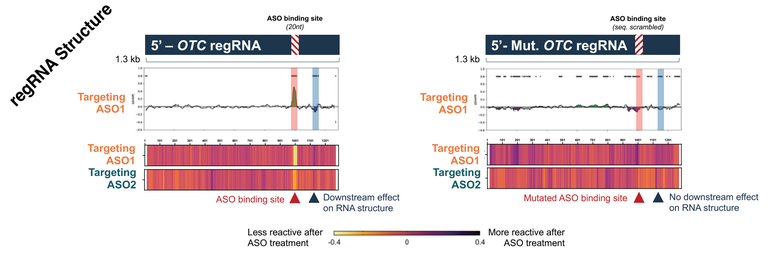

**Increased activate histone mark accumulation at *OTC* enhancer**




***Left:*** Effect of regRNA-targeting ASO on regRNA and mRNA levels in a timecourse. Results are averages of [n=3] biological replicates.
***Center***: Effect of 2 regRNA-targeting ASOs on nascent transcription of OTC mRNA. Results are averages of [n=3] biological replicates. P values are significant as measured by student t-test.
***Right***: Effect of regRNA-targeting ASO on H3K27ac histone mark at the OTC enhancer (ChIP-qPCR).
***All***: OTC regRNA-targeting ASO [5uM] or non-targeting control ASO [5uM] were delivered to primary human hepatocytes via gymnosis.

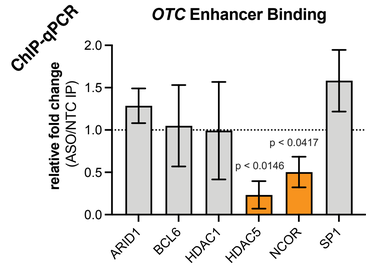
## *OTC* regRNA-targeting ASOs: mechanism of action

**ASO binding results in reproducible changes to regRNA structure**

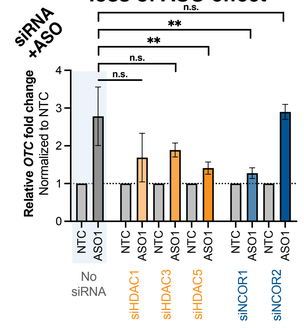



Targeting ASO1 binding alters regRNA secondary structure as measured by SHAPE-MaP (RNA nucleotide reactivity relative to an NTC ASO). Effect was not observed for mutant regRNA lacking the ASO binding site (i.e., ASO2).

**Treatment with ASO reduces repressor binding at enhancer**



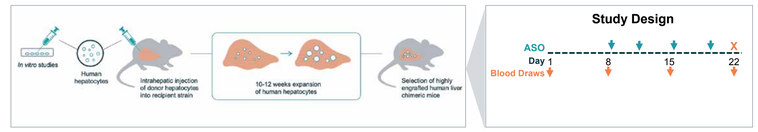

**Repressor knockdown results in loss of ASO effect**



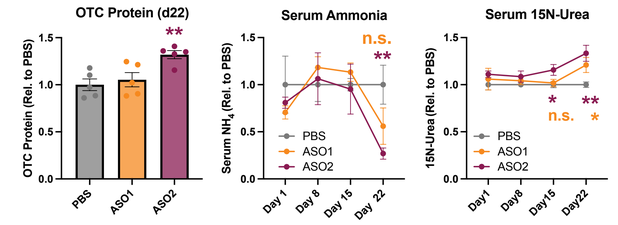

***Left:*** Treatment with targeting ASO1 results in a decrease in binding of HDAC5 and NCOR1 (ChIP-qPCR)
***Right***: Knockdown of HDAC1,3,5 or NCOR1 prior to ASO treatment (48h) ablates ASO1 effect on OTC mRNA

## *In vivo* efficacy of *OTC* regRNA-targeting ASOs

**Humanized liver mouse model allows testing of hOTC-targeting ASOs *in vivo***



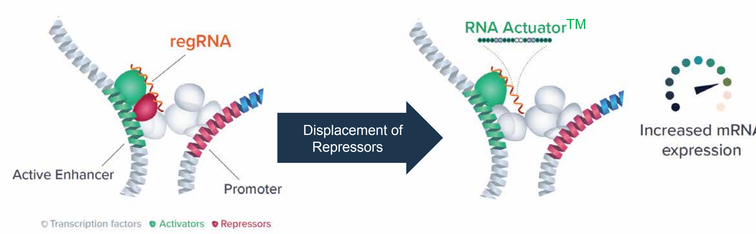

**OTC regRNA-targeting ASO leads to increased OTC protein and ureagenesis**




***Left:*** OTC protein levels were measured at final endpoint, day 22, using capillary-based Western.
***Center:*** Serum ammonia was measured from fresh serum at the indicated day. Data is normalized to the matched timepoint PBS control +/- SEM for n=5 mice per group.
***Right:*** Labeled serum urea following a challenge with 15N-$NH_4$ was measured by LC-MS/MS. Data is normalized to the matched timepoint PBS. P values are one way ANOVA. * p<0.05, **p<0.01

## Model

**regRNA-targeting ASOs displace repressors and increase transcription**




## Takeaways

High throughput regRNA capture-seq method resulted in annotation of tens of thousands of regRNA species expressed from human hepatocyte enhancers

ASOs targeting *OTC* regRNAs upregulate *OTC* mRNA in a dose-dependent manner

*OTC* regRNA upregulation preceded mRNA upregulation after ASO treatment

ASOs modulate repressor binding leading to increased *OTC* transcription

ASOs targeting *OTC* regRNAs increase OTC protein levels and decrease ammonia levels in a humanized liver mouse model

**CAMP4's RAP Platform™ facilitates the development of regRNA targeting ASOs with the potential to upregulate therapeutically-relevant genes**