

Superior expression of self-complementary AAV and comparable functionality of mini- and full-length MeCP2 support the design of TSHA-102 gene therapy for Rett syndrome

Emdadul Haque, Andrew D. Wallace, Ryan R. Chaparian, Rayvon Moore, Ian Jones, Annika N. Alicardi, Carlos D. Robles, Frederick Porter

Taysha Gene Therapies, 3000 Pegasus Park Dr., Dallas, Texas 75274, United States

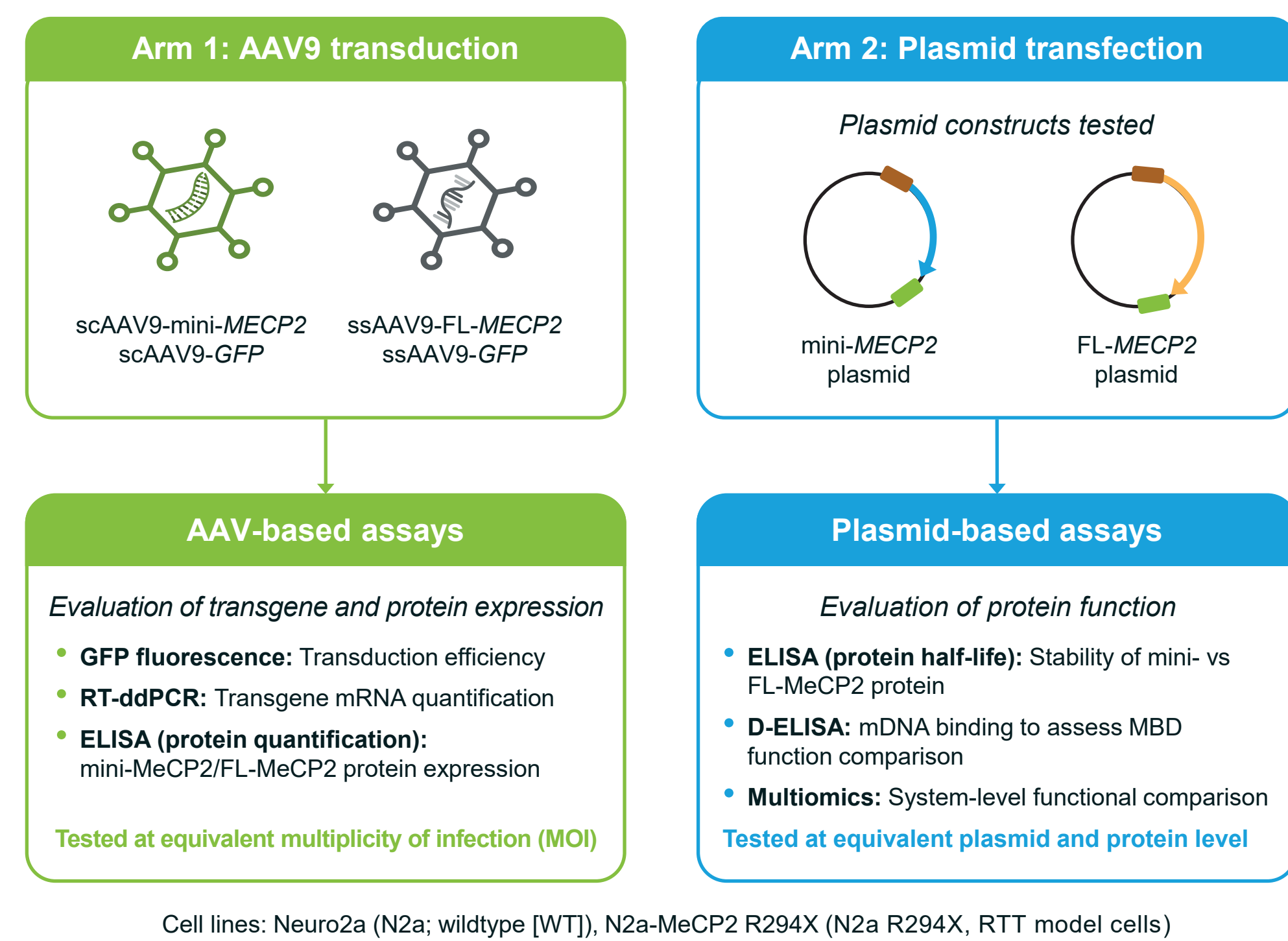
Aims

To investigate expression of transgenes/proteins with self-complementary (sc) adeno-associated virus serotype 9 (AAV9) vs single-stranded (ss) AAV9 vectors, and to compare functionality of miniaturized and full-length methyl-CpG-binding protein 2 (MeCP2) proteins.

Background

- Rett syndrome (RTT) is a rare, neurodevelopmental disorder caused by pathologic loss-of-function mutations in the X-linked *MECP2* gene, encoding the MeCP2 protein¹⁻³
- For gene regulation, MeCP2 binds to both methylated (m) and unmethylated DNA and functions with partner proteins, including DNA methyltransferases (DNMTs), to modulate chromatin structure, DNA methylation and transcriptional activity⁴⁻⁷
- A truncated MeCP2 protein retaining the essential methyl-CpG-binding domain (MBD) and the transcriptional repressor NCoR/SMRT interaction domain (NID) restored a normal phenotype in *Mecp2*^{-/-} mice and showed comparable functionality to full-length MeCP2⁸
 - These findings established RTT is reversible in this model and the mechanistic basis for miniaturized *MECP2* gene therapy to restore expression in MeCP2-deficient cells
- TSHA-102 is a recombinant scAAV9 gene therapy that encodes a functional, miniaturized *MECP2* transgene that retains the essential MBD and NID^{9,10} currently under investigation in the REVEAL pivotal trials¹¹
 - In contrast to ssAAV9 vectors that require conversion of the single-stranded DNA to double-stranded DNA prior to expression, TSHA-102 contains complementary strands of the *MECP2* transgene, delivering a transcription-ready transgene to MeCP2-deficient cells for enhanced stability and efficiency^{9,12}
 - A miRNA-Responsive Auto-Regulatory Element (miRARE) further refines expression by silencing transgene expression in MeCP2-healthy cells while permitting expression in MeCP2-deficient cells^{9,10}
- scAAV9 vectors have been shown to drive robust transgene expression across disease models and genetic payloads¹²⁻¹⁴ yet a direct comparison with ssAAV9 for *MECP2* delivery in RTT models is lacking. Furthermore, although miniaturized (mini-)MeCP2 is sufficient to rescue the disease phenotype in MeCP2-deficient mice^{9,10} a comprehensive comparison of the biochemical and molecular properties of mini-MeCP2 and full-length (FL-MeCP2) versions of MeCP2 have not been fully characterized
- The current study addresses both of these gaps through a multi-level panel of functional assays. Complementary assays compared scAAV9 vs ssAAV9 and mini-MeCP2 vs FL-MeCP2 at three levels: vector delivery, transgene expression, and biochemical/molecular function (Figure 1)

Figure 1: Overview of experimental design for scAAV9 vs ssAAV9 and mini- vs FL-MeCP2 comparisons



Methods

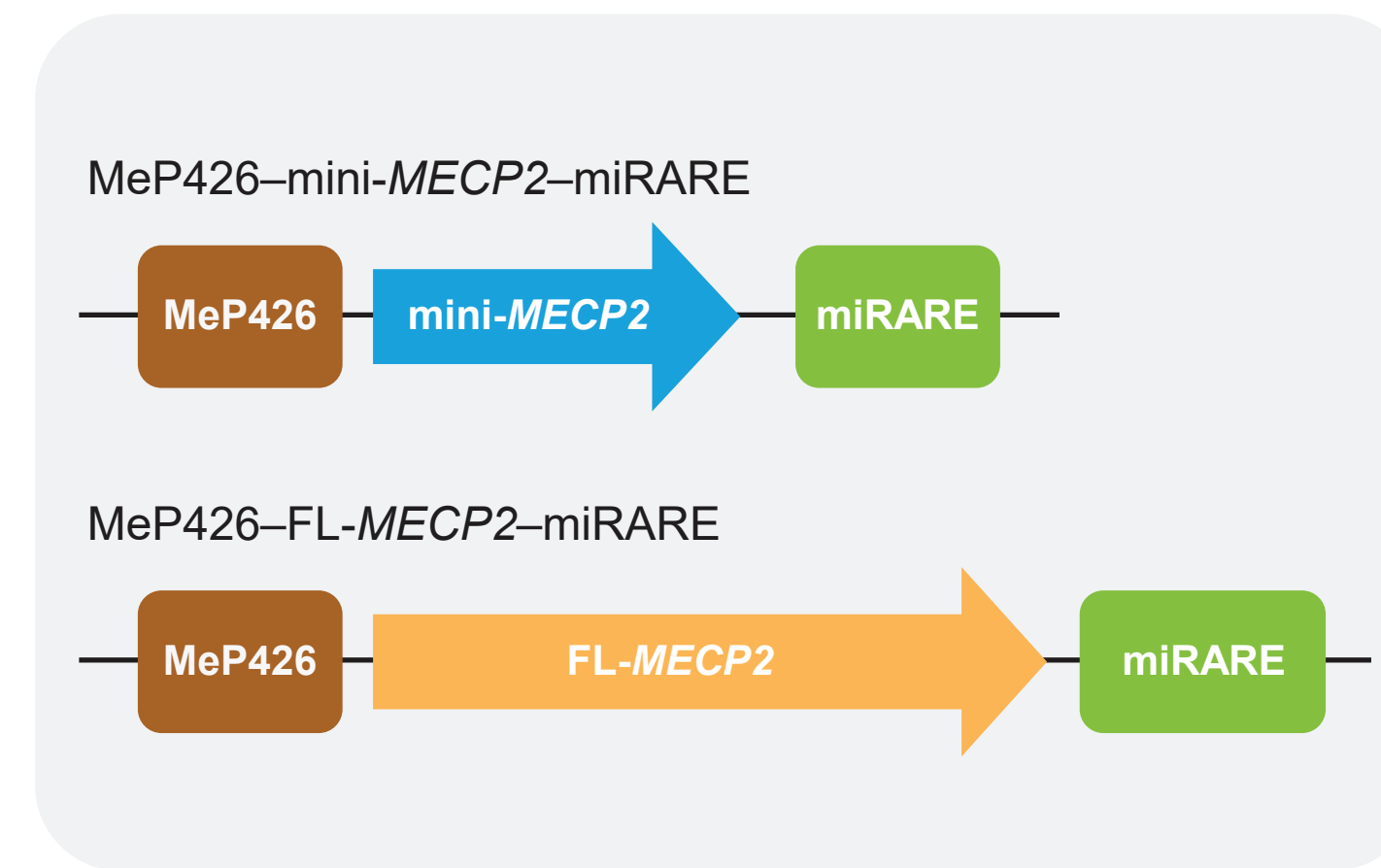
scAAV and ssAAV vectors

- TSHA-102 (scAAV9-mini-*MECP2*) was evaluated against an analogous ssAAV9 construct in which the mini-*MECP2* and mutant inverted terminal repeat (ITR) were replaced with FL-*MECP2* and WT ITR, while maintaining identical backbone and regulatory elements
- N2a cells expressing either WT *MECP2* or the clinically relevant R294X allele (N2a R294X, RTT model cells) were transduced with either TSHA-102 or the ssAAV9-FL-*MECP2* construct
- Transgene and protein expression were quantified using reverse transcription droplet digital polymerase chain reaction (RT-ddPCR) and enzyme-linked immunosorbent assay (ELISA), respectively. The measured concentration of MeCP2 protein was within 25% of the coefficient of variation
- N2a and N2a R294X cells were also transduced with ssAAV9-green fluorescent protein (GFP) or scAAV9-GFP vector. Transgene and protein expression were measured by RT-ddPCR and GFP fluorescence from live cells, respectively

Functional analyses

- N2a R294X cells were transiently transfected with equimolar plasmids using FuGENE 6 (Promega) to express a similar amount of MeCP2 protein (Figure 2)

Figure 2: Plasmid constructs
TSHA-102 vector map was used for mini- and FL-MeCP2



- Half-life:** Transfected cells were treated with 15 μM cycloheximide, and cellular extracts were prepared at various time intervals (cycloheximide chase experiment¹⁵). MeCP2 levels were quantified by ELISA
- Binding to mDNA:** Cell lysates were prepared from transfected cells and DNA-binding (D-)ELISA performed^{16,17} using microplates coated with custom biotin-labeled 45 base pair (bp) oligonucleotides containing three methylated CpG sites
- DNMT inhibition:** DNMT3A/3L complexes (Active Motif) were incubated with different molar amounts of either mini-MeCP2 or FL-MeCP2 recombinant proteins. The Epiquick DNMT Activity/Inhibition Assay Ultra kit (Epigentek) was used to quantify DNMT activity

Global biomolecule rescue analysis

- Multomics analysis was performed on cell lysates of transfected cells using liquid chromatography/mass spectrometry (LC/MS) to define biomolecule profiles
- Statistical associations between biochemicals and transfection groups were tested using linear regression models. Biomolecules were considered significant hits if the adjusted p-value was ≤0.05
- The fold change (FC) in protein abundance was compared from baseline to post-transfection; biomolecule regulation was considered improved if their FC shifted closer to 1 (indicating no dysregulation) by at least 20%
- To compare mini-MeCP2 and FL-MeCP2, the improvement ratio was calculated by dividing the ΔFC of mini-MeCP2 by the ΔFC of MeCP2, with a ratio of 1 indicating the same molecular activity

Results

Figure 3: Transduction with scAAV9 vs ssAAV9 vectors demonstrated greater transgene expression and ~30-fold greater protein expression across MeCP2 and GFP in RTT model cells

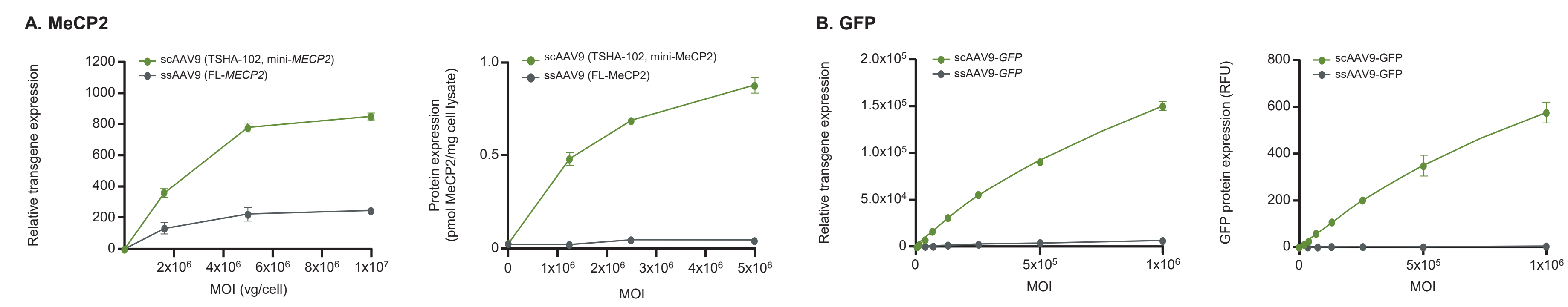


Figure 4: Following plasmid transfection, mini-MeCP2 protein expression was comparable to FL-MeCP2 and upregulated 3.5- to 4-fold in RTT model vs WT cells via miRARE-driven regulation

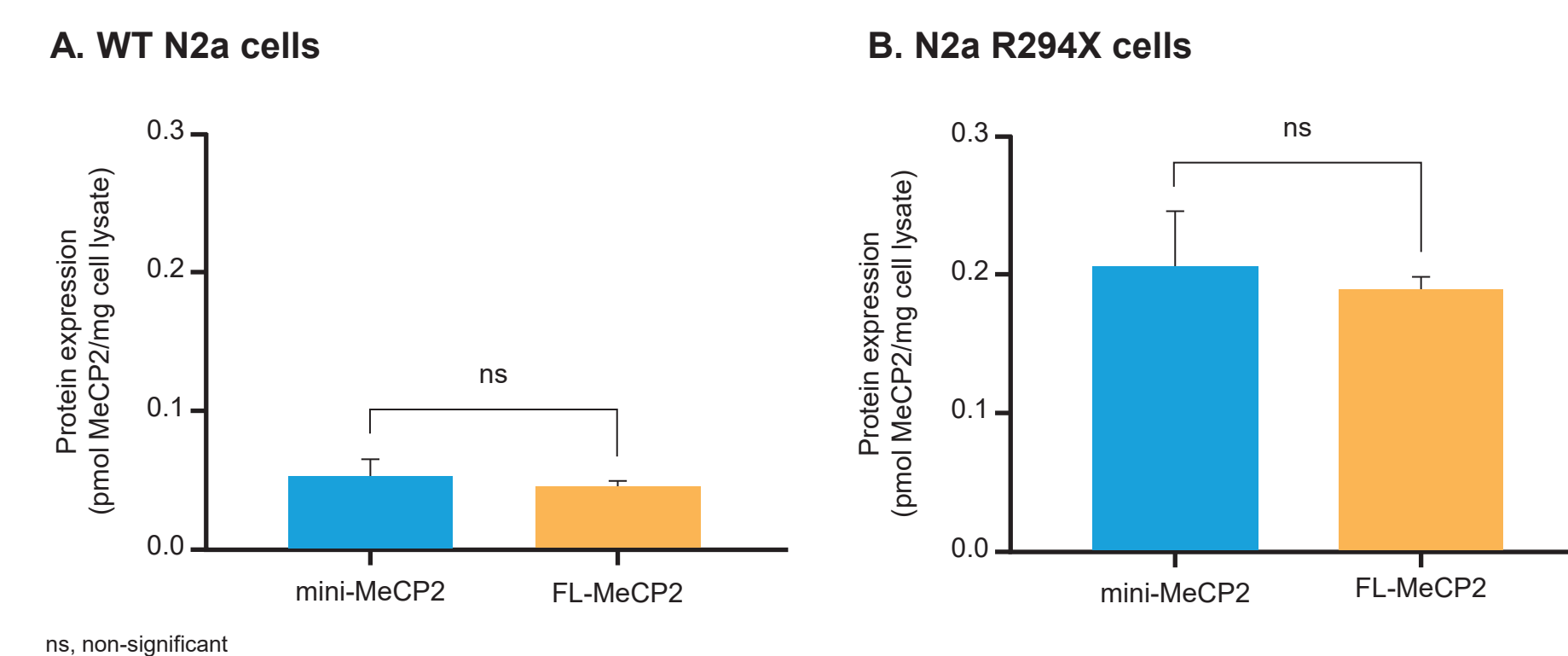
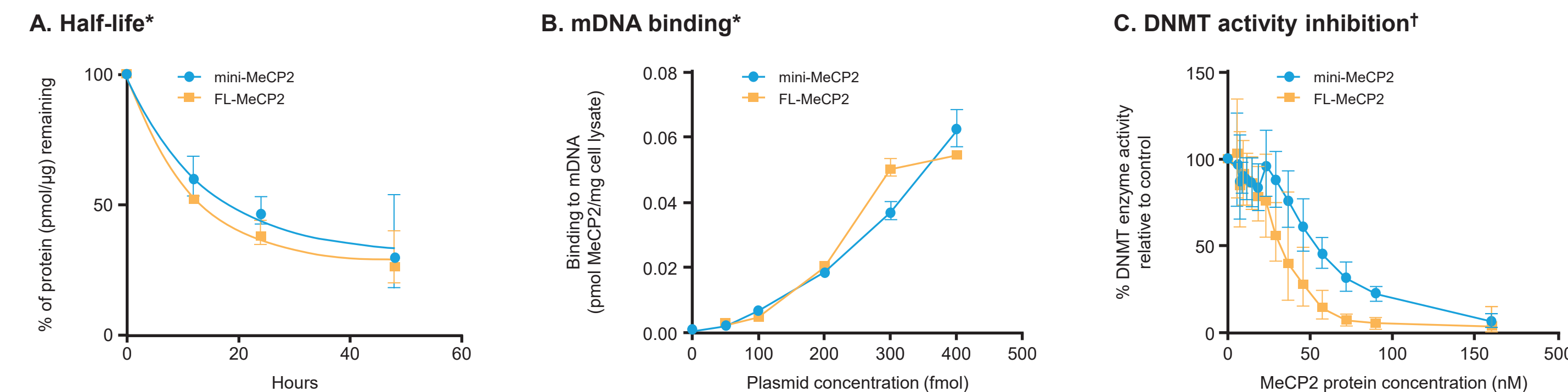
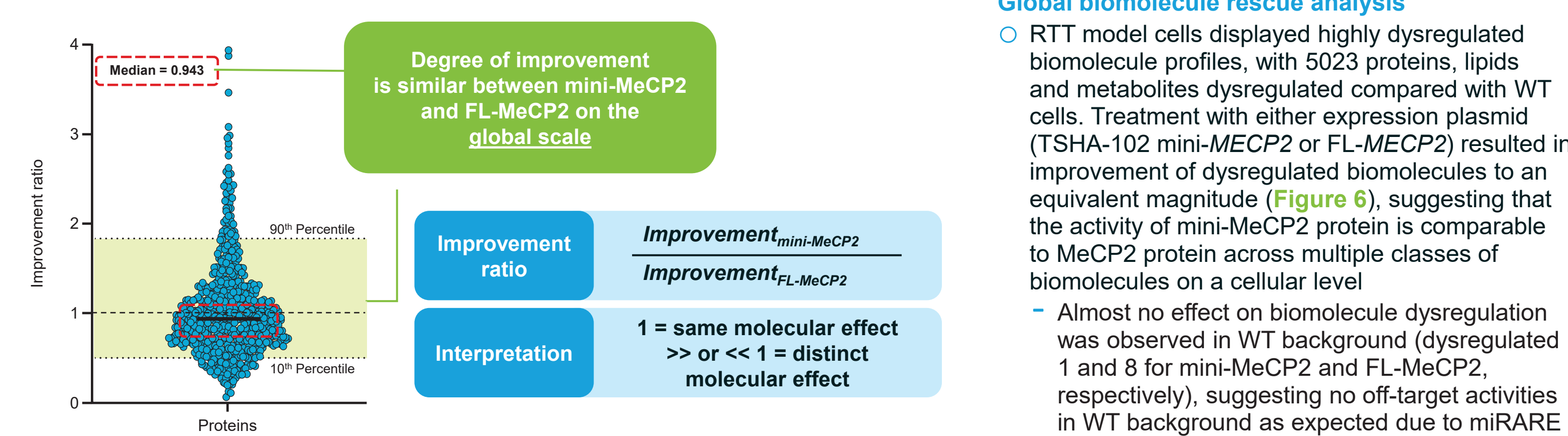


Figure 5: Protein functional analysis confirms mini-MeCP2 is functionally equivalent to FL-MeCP2 demonstrating a comparable half-life, mDNA binding, and DNMT3 inhibition



- mini-MeCP2 and FL-MeCP2 proteins showed similar half-lives in the RTT model cells (12 h vs 9 h, respectively; Figure 5A), indicating comparable steady-state abundance and protein synthesis rates
- mini-MeCP2 and FL-MeCP2 proteins both demonstrated binding to mDNA with a similar magnitude (Figure 5B)
- Both proteins inhibited DNMT3 activity in a dose-responsive manner, with similar levels of inhibition observed (Figure 5C), suggesting that both proteins directly interacted with the DNMT enzyme

Figure 6: RTT model cells demonstrate mini-MeCP2 normalizes the RTT multiomic signature resulting in improvement of dysregulated biomolecules to an equivalent magnitude to FL-MeCP2



Global biomolecule rescue analysis

- RTT model cells displayed highly dysregulated biomolecule profiles, with 5023 proteins, lipids and metabolites dysregulated compared with WT cells. Treatment with either expression plasmid (TSHA-102 mini-*MECP2* or FL-*MECP2*) resulted in improvement of dysregulated biomolecules to an equivalent magnitude (Figure 6), suggesting that the activity of mini-MeCP2 protein is comparable to MeCP2 protein across multiple classes of biomolecules on a cellular level
- Almost no effect on biomolecule dysregulation was observed in WT background (dysregulated 1 and 8 for mini-MeCP2 and FL-MeCP2, respectively), suggesting no off-target activities in WT background as expected due to miRARE

scAAV vs ssAAV expression

- When looking at MeCP2 expression in RTT model cells, the scAAV9 vector (TSHA-102) produced up to 30-fold more MeCP2 protein than the ssAAV9 vector (ssAAV9-FL-*MECP2*), which expressed an insignificant amount of MeCP2 protein (Figure 3A, right panel)
- Using a GFP model transgene in RTT model cells, scAAV9 expressed ~30-fold more GFP transgene and GFP protein compared with ssAAV9, for which expression was negligible (Figure 3B)

Conclusions

- scAAV9 enables markedly higher (30x) MeCP2 protein expression than an analogous ssAAV9 for MeCP2 delivery, consistent with the literature from other sc- and ss-model vector comparisons¹²⁻¹⁴
- mini-MeCP2 is functionally comparable to FL-MeCP2 across biochemical functions, with both proteins exhibiting similar behavior in RTT model cells:
 - Both mini-MeCP2 and FL-MeCP2 are stably expressed in neuronal cells. miRARE controls protein expression in a genotype-dependent manner, independent of whether the expressed protein is mini- or FL-MeCP2
 - The half-life of the mini-MeCP2 protein is similar to the FL-MeCP2 protein, suggesting comparable protein stability and supporting the likelihood of similar biological functions
 - Both mini-MeCP2 and FL-MeCP2 similarly bind to mDNA, which is a fundamental process for establishing and maintaining normal gene expression patterns, essential for brain health¹⁸
 - mini-MeCP2 inhibits DNMT enzymatic activity, further showing that mini-MeCP2 is functionally analogous to FL-MeCP2
 - mini- and FL-MeCP2 proteins produce similar improvements across dysregulated multiomics signatures, indicating preservation of core MeCP2 function, resulting in broad cellular correction with mini-MeCP2 equivalent to that of FL-MeCP2

Key takeaway

The molecular functions of mini-MeCP2 and FL-MeCP2 are analogous, with both proteins exhibiting similar, stable expression in neuronal cells. scAAV9 enables superior MeCP2 expression compared with ssAAV9, supporting effective delivery of TSHA-102 to the central nervous system using a minimally invasive lumbar intrathecal administration. Together, the findings provide strong mechanistic support for the scAAV9 + mini-MECP2 + miRARE design of TSHA-102 and translational support for the ongoing REVEAL trial, indicating TSHA-102 is generally well tolerated with multidomain developmental milestone gain/regain across core RTT domains¹¹

