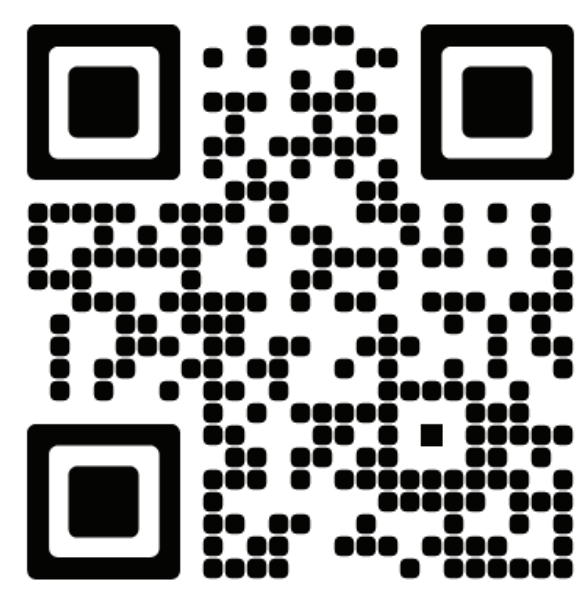




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INTRODUCTION

- USH2A* variants are a leading cause of non-syndromic autosomal recessive retinitis pigmentosa and of Usher syndrome type 2A, in which retinitis pigmentosa occurs with congenital hearing loss.¹
- Exon 13 is one of the most frequently mutated regions of the *USH2A* gene.²
- The most common mutations in exon 13 introduce a premature stop codon in messenger RNA (mRNA), which in turn triggers nonsense-mediated decay of the transcript, resulting in no usherin protein production (Figure 1).
 - Upon exon 13 skipping, a truncated protein is generated, which has been reported to retain functionality.³
- Results of preclinical studies have shown that antisense oligonucleotide-mediated *USH2A* exon 13 skipping can produce a shorter but functional usherin protein.³
- A neutrally charged phosphorodiamidate morpholino oligomer (PMO) approach was developed to induce exon 13 skipping and address safety considerations associated with negatively charged ASOs.

MATERIALS AND METHODS

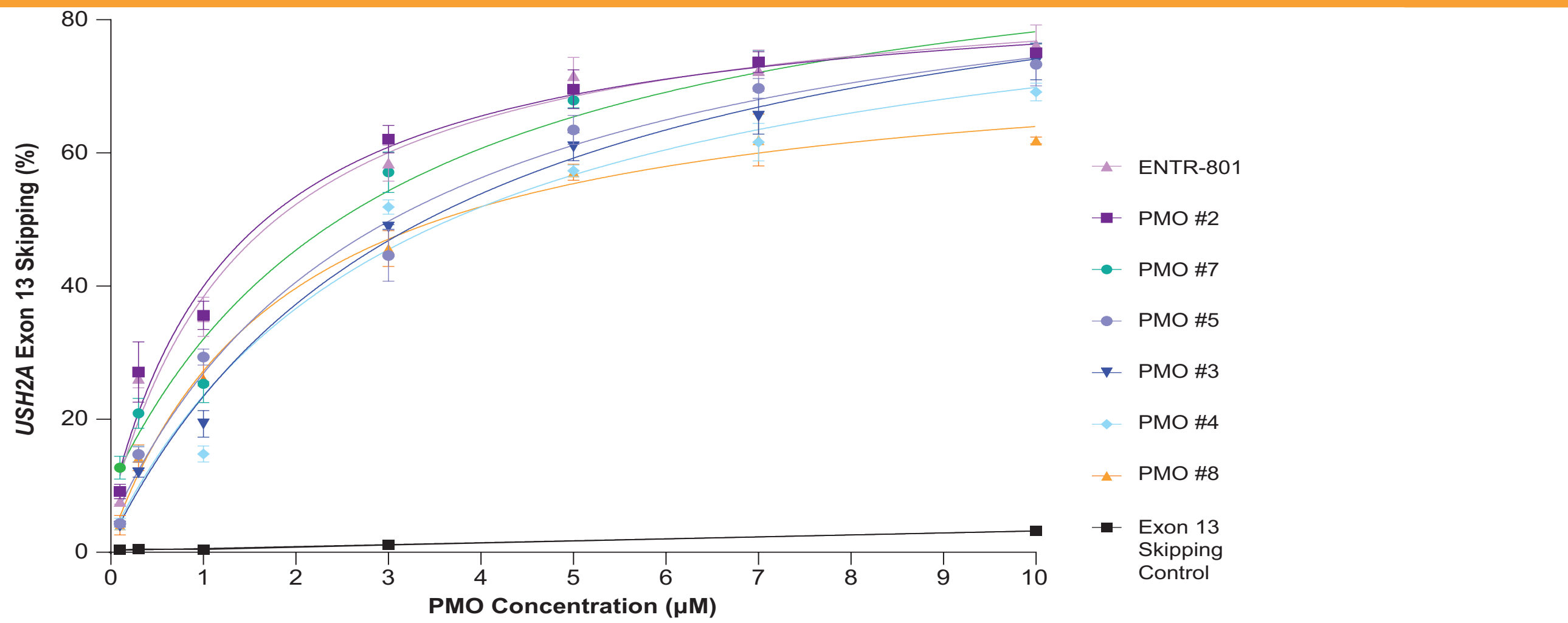
- Neutrally charged PMOs, including ENTR-801, were synthesized and evaluated for *USH2A* exon 13 skipping potency and efficacy.
- USH2A* exon 13 skipping was assessed by digital droplet reverse transcription-polymerase chain reaction in a human retinoblastoma cell line (WERI-Rb-1), human induced pluripotent stem cell-derived retinal organoids (iPSC-ROs), human retinal explants, and partially humanized mice expressing wild-type human *USH2A* (ph*USH2A* mice).
- Usherin protein production in ph*USH2A* mice following a single intravitreal (IVT) injection of ENTR-801 was evaluated via Western immunoblot and quantified via densitometric analysis in ImageJ.
- PMO biodistribution in the retina of ph*USH2A* mice after a single IVT injection of ENTR-801 was evaluated via immunofluorescence with a custom-made anti-PMO antibody.
- Human explants were obtained from VisionGift (Portland, OR) or Vision Share (Chicago, IL). Wild-type iPSCs were licensed from StemCell Technologies (Cambridge, MA) and engineered by EditCo Bio (Redwood City, CA) using CRISPR-Cas9 to introduce the mutation.
- All animal procedures were conducted in accordance with guidelines for the care and use of laboratory animals.

RESULTS

Evaluation of PMOs for Exon 13 Skipping Potency in a Human Retinoblastoma Cell Line

- When comparing EC-50 (ie, half-maximal effective concentration), ENTR-801 was among the top performing PMOs tested for *USH2A* exon 13 skipping in WERI-Rb1 cells (Figure 2).

Figure 2. Exon 13 Skipping Potency of ENTR-801 and Other PMO Candidates in Human Retinoblastoma Cells

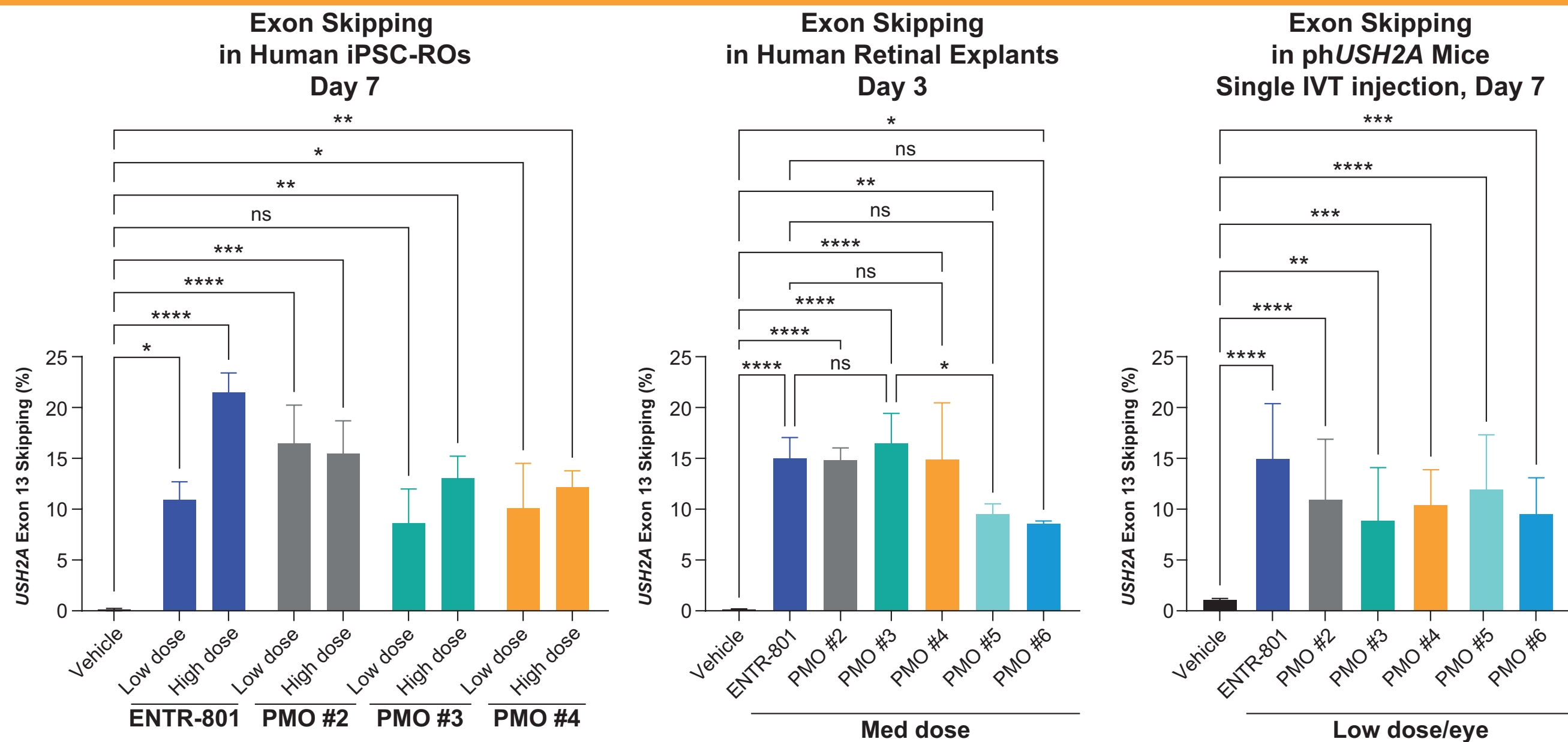


WERI-Rb-1 human retinoblastoma cells were transfected (Endoport, 6 μg/μL) with PMOs for 48 hours; exon skipping was evaluated by RT-ddPCR. Data are mean ± standard error of the mean. PMO, phosphorodiamidate morpholino oligomer; RT-ddPCR, reverse transcriptase-digital droplet polymerase chain reaction.

Evaluation of PMOs in Human iPSC-ROs, Human Retinal Explants, and phUSH2A Mice

- Exon skipping efficacy of the top PMOs was further compared in multiple human model systems (Figure 3).
- ENTR-801 showed consistently high efficacy across model systems compared with other PMO candidates.

Figure 3. Target Engagement of PMOs Across Human-Relevant Model Systems

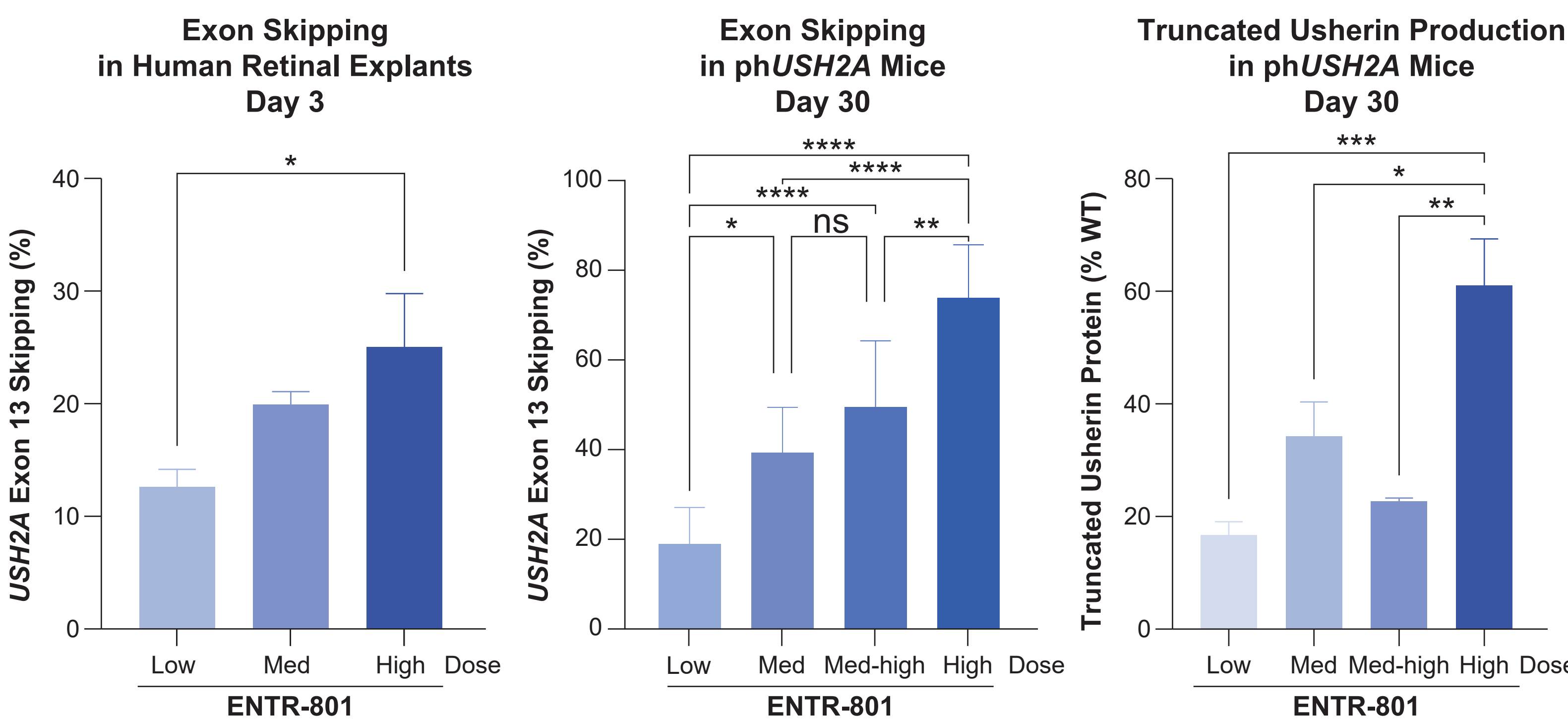


Exon skipping was evaluated with RT-ddPCR on (left) day 7 after treatment of wild-type human iPSC-ROs (n=3) with low- and high-dose PMO, with no statistically significant difference between PMOs at the same doses; (middle) on day 3 after treatment of human retinal explants (n=3) with med-dose PMO, (right) and on day 7 after a single bilateral IVT injection at a low dose/eye in ph*USH2A* mice (n=10). Data are mean ± standard error of the mean. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. iPSC-RO, induced pluripotent stem cell-derived retinal organoid; IVT, intravitreal; Med, medium; ns, not significant; ph*USH2A*, partially humanized mice expressing wild-type human *USH2A*; PMO, phosphorodiamidate morpholino oligomer; RT-ddPCR, reverse transcriptase-digital droplet polymerase chain reaction; WT, wild-type.

Dose Dependency of ENTR-801 Efficacy in Human-Relevant Model Systems

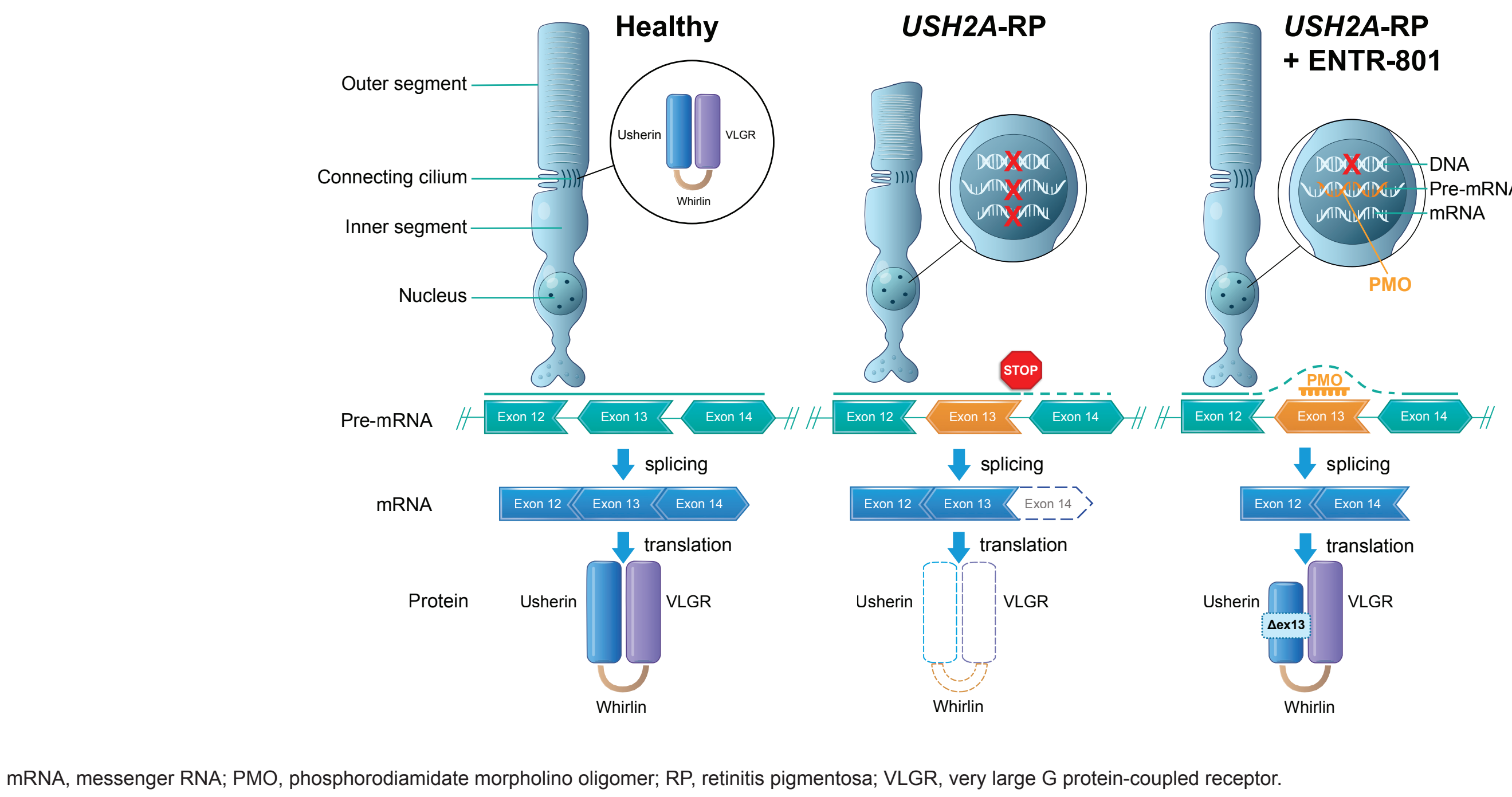
- ENTR-801 demonstrated dose-dependent efficacy across both human retinal explants and humanized mice (Figure 4).

Figure 4. Efficacy of ENTR-801 in Human Retinal Explants and in phUSH2A Mice



Exon skipping was evaluated with RT-ddPCR on (left) day 3 after treatment of human retinal explants (n=3) with low, med or high doses of ENTR-801, (middle) and on day 30 after a single bilateral IVT injection of ENTR-801 at low to high doses per eye in ph*USH2A* mice (n=8). (right) Truncated usherin protein from ph*USH2A* mice (n=2) was assessed at day 30 via WB and quantified via densitometric analysis in ImageJ. One-way ANOVA was used for statistical comparison. Data are mean ± standard error of the mean. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ANOVA, analysis of variance; IVT, intravitreal; Med, medium; ns, not significant; ph*USH2A*, partially humanized mice expressing wild-type human *USH2A*; RT-ddPCR, reverse transcriptase-digital droplet polymerase chain reaction; WB, Western immunoblot; WT, wild-type.

Figure 1. *USH2A*-PMO Construct Mechanism of Action



mRNA, messenger RNA; PMO, phosphorodiamidate morpholino oligomer; RP, retinitis pigmentosa; VLRG, very large G protein-coupled receptor.

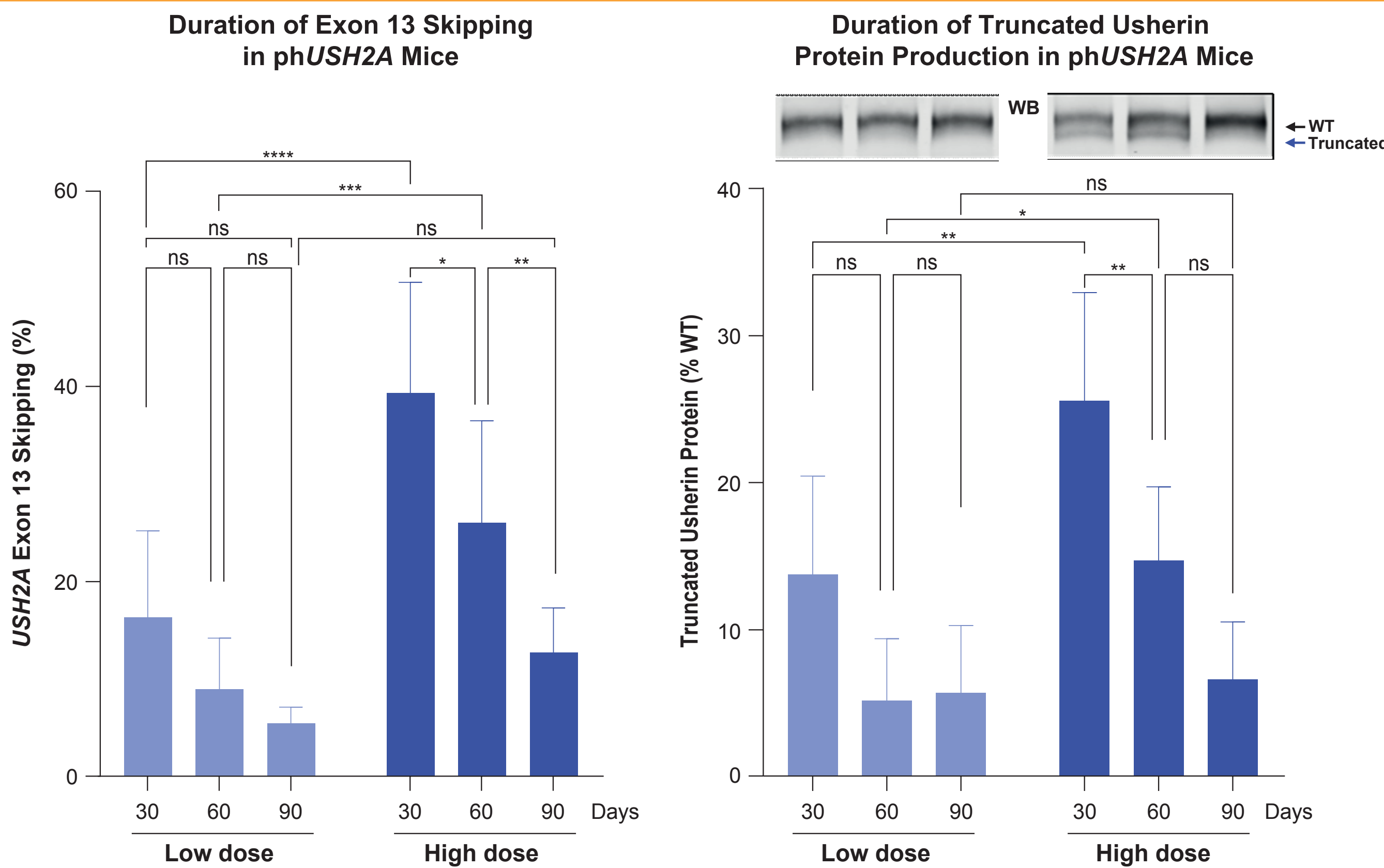
OBJECTIVE

- To evaluate the preclinical efficacy and therapeutic potential of ENTR-801 to induce exon 13 skipping in human *USH2A* mRNA.

Duration of Effect of a Single Dose of ENTR-801 in phUSH2A Mice

- Treatment of partially humanized mice with ENTR-801 resulted in durable exon skipping and truncated usherin protein production (Figure 5).

Figure 5. Duration of Target Engagement and Truncated Usherin Protein Production with ENTR-801 in phUSH2A Mice

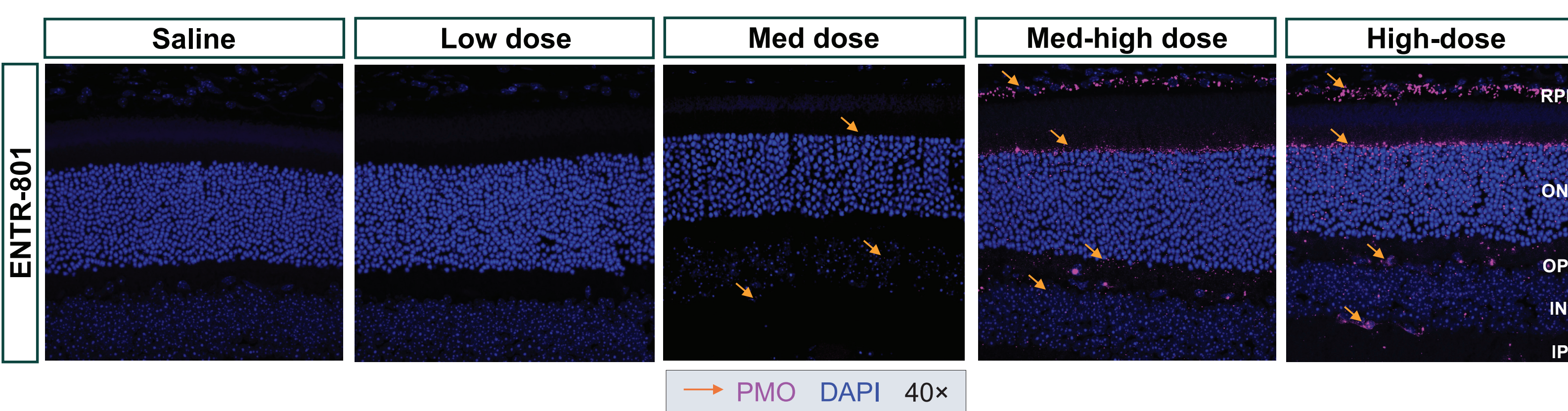


Partially humanized *USH2A* mice (n=6-9) were dosed via a single bilateral IVT injection at multiple concentrations of ENTR-801. At 30, 60, and 90 days post-injection, (left) exon skipping was evaluated by RT-ddPCR (n=9, n=9, and n=10 eyes, respectively), and (right) ImageJ densitometry from representative WBs was semi-quantitative. Total usherin protein was defined as the combined intensity of the WT (n=7) and truncated usherin bands. The percentage of truncated protein was calculated as the intensity of the truncated band divided by the combined intensity of the truncated and WT bands. Data are mean ± standard error of the mean. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. IVT, intravitreal; ns, not significant; ph*USH2A*, partially humanized mice expressing wild-type human *USH2A*; RT-ddPCR, reverse transcriptase-digital droplet polymerase chain reaction; WB, Western immunoblot; WT, wild-type.

Retinal Localization 30 Days After Intravitreal Injection of ENTR-801 in phUSH2A Mice

- Dose-dependent increases in ENTR-801 were detected in mouse retinal target cells 30 days after IVT injection (Figure 6).

Figure 6. Dose-Dependent Biodistribution of ENTR-801 in phUSH2A Mouse Retina



Partially humanized *USH2A* mice were dosed via a single bilateral IVT injection at multiple concentrations. On day 30 post-injection, eyes were fixed overnight with Hartman's fixative, embedded in OCT, and cryosectioned. PMO biodistribution was assessed via immunostaining using custom-made anti-PMO antibody. Immunostained sections were imaged at 40× (LSM-880 Zeiss confocal). DAPI, 4',6-diamidino-2-phenylindole; INL, inner nuclear layer; IPL, inner plexiform layer; IVT, intravitreal; Med, medium; OCT, optimal cutting temperature compound; ONL, outer nuclear layer; OPL, outer plexiform layer; ph*USH2A*, partially humanized mice expressing wild-type human *USH2A*; PMO, phosphorodiamidate morpholino oligomer; RPE, retinal pigment epithelium.

CONCLUSIONS

- ENTR-801 demonstrated substantial exon 13 skipping across human iPSC-ROs, retinal explants, and ph*USH2A* mice, confirming consistent target engagement in vitro, ex vivo, and in vivo.
- At a medium dose per eye of ENTR-801, exon skipping and truncated usherin protein were detectable through 90 days after a single IVT injection.
- ENTR-801 exhibited dose-dependent retinal biodistribution across all layers after a single IVT injection.
- These results support further development of ENTR-801 for *USH2A*-associated retinitis pigmentosa.

ACKNOWLEDGMENTS

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