SUPPORTING INFORMATION

Combination Treatment of Erythromycin and Furamidine Provides Additive and Synergistic Rescue of Mis-Splicing in Myotonic Dystrophy Type 1 Models

Jana R. Jenquin,[1] Hongfen Yang,[2] Robert W. Huigens III,[2] Masayuki Nakamori[3] and J. Andrew Berglund[1,4]*

[1] Department of Biochemistry & Molecular Biology, Center for NeuroGenetics, College of Medicine, University of Florida, Gainesville, Florida, 32610, USA. Email: aburglund@ufl.edu

[2] Department of Medicinal Chemistry, Center for Natural Products Drug Discovery and Development, College of Pharmacy, University of Florida, Gainesville, FL, 32610, USA

[3] Department of Neurology, Osaka University Graduate School of Medicine, Osaka, 565-0871, Japan

[4] Department of Biological Sciences, RNA Institute, College of Arts and Sciences, University at Albany-SUNY, Albany, New York, 12222, USA

* Correspondence should be addressed to J. Andrew Berglund. Email: aberglund@ufl.edu

Supporting methods:

General information about chemical synthesis of pafuramidine: All reagents for chemical synthesis were purchased from commercial sources and used without further purification. Reagents were purchased at ≥ 95% purity and commercially available controls were used in our biological investigations without further purification Analytical thin layer chromatography (TLC) was performed using 250 µm Silica Gel 60 F254 pre-coated plates (EMD Chemicals Inc.). Flash column chromatography was performed using 230-400 Mesh 60Å Silica Gel from Sorbent Technologies. All melting points were obtained, uncorrected, using a Mel-Temp capillary melting point apparatus from Laboratory Services, Inc.

NMR experiments were recorded using broadband probes on a Bruker Avance III (500 MHz for ^{1}H NMR; 125 MHz for ^{13}C NMR) and Bruker Avance II (600 MHz for ^{1}H NMR). All spectra are presented using MestReNova 11.0 (Mnova) software and are displayed without the use of the signal suppression function. Spectra were obtained in the following solvents (reference peaks also included for ^{1}H and ^{13}C NMRs): $CDCl_3$ (^{1}H NMR: 7.26 ppm; ^{13}C NMR: 77.23 ppm) and *d*$_6$-DMSO (^{1}H NMR: 2.50 ppm; ^{13}C NMR: 39.52 ppm). All NMR experiments were performed at room temperature. Chemical shift values (δ) are reported in parts per million (ppm) for all ^{1}H NMR and ^{13}C NMR spectra. ^{1}H NMR multiplicities are reported as: s = singlet, br. s = broad singlet, d = doublet, m = multiplet. High-Resolution Mass Spectrometry (HRMS) were obtained from the Chemistry Department at the University of Florida

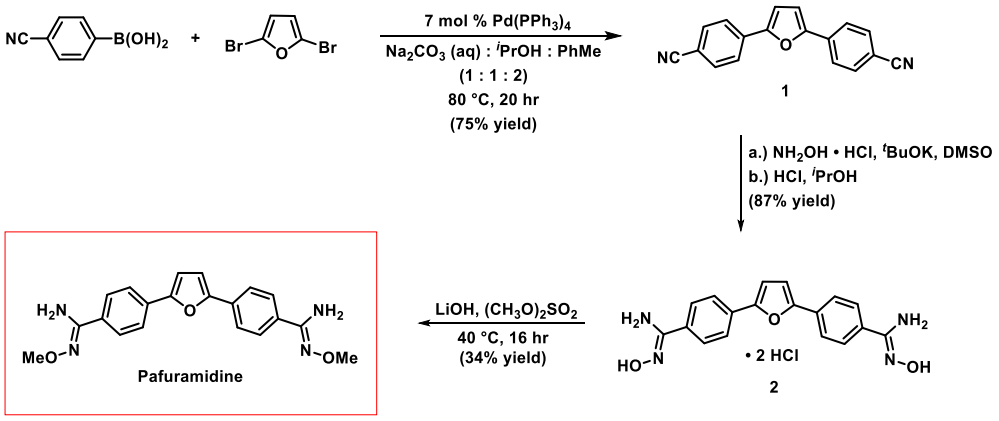

Figure 1: Synthesis scheme for pafuramidine.

*Synthesis of Compound **1**:* 4-Cyanophenylboronic acid (1.11 g, 7.50 mmol), 2,5-dibromofuran (847 mg, 3.75 mmol) and tetrakis(triphenylphosphine)palladium(0) (300 mg, 0.26 mmol) were

added to a round bottom flask. The flask was then flushed with argon for 3 minutes before adding 15 mL toluene, 7.5 mL isopropanol and 7.5 mL of an aqueous 2 M sodium carbonate solution. The reaction mixture was then heated to 80 °C and allowed to stir for 20 hours to allow the reaction to occur. Upon completion, the reaction mixture was allowed to cool to room temperature resulting in a precipitate that was filtered to give **1** as yellow green solid (758 mg, 75% yield). **Note:** Compound **1** is a known compound (CAS No.: 55368-37-1) and was synthesized according to a modified literature procedure (*63*). The ^{1}H NMR tabulation data and melting point (>250 °C) that we obtained for **1** matched those previously reported (*64*).

Synthesis of Compound 2: Hydroxylamine hydrochloride (2.57 g, 37 mmol) was dissolved in anhydrous dimethyl sulfoxide under an argon atmosphere. Then potassium *tert*-butoxide (3.56 g, 37mmol) was added slowly to this mixture at 0 °C, which was partially frozen. The resulting mixture was allowed to stir at 0 °C for 20 minutes before the addition of compound **1**. The reaction was then stirred at room temperature for 21.5 hours. Upon completion, the reaction mixture was slowly poured into a beaker containing ice water forming a white precipitate. This mixture was allowed to stir for 15 minutes, then the white precipitate was filtered and washed with water to afford compound **2** as a free base. Finally, the free base of **2** was added to a solution of 5 M hydrochloric acid in isopropanol (20 mL) and allowed to stir. After a few minutes, this solution was then diluted with ether and the precipitate that formed was collected by filtration to give bis-hydrochloride salt of **2** (671 mg, 87% yield). **Note:** Compound **2** is a known compound (CAS No.: 186953-55-9) and was synthesized according to a modified procedure (*65*). No spectra for **2** were found for comparison in our search, so we have provided our data in this report. MP: > 250 °C, lit. > 350 °C (*66*).

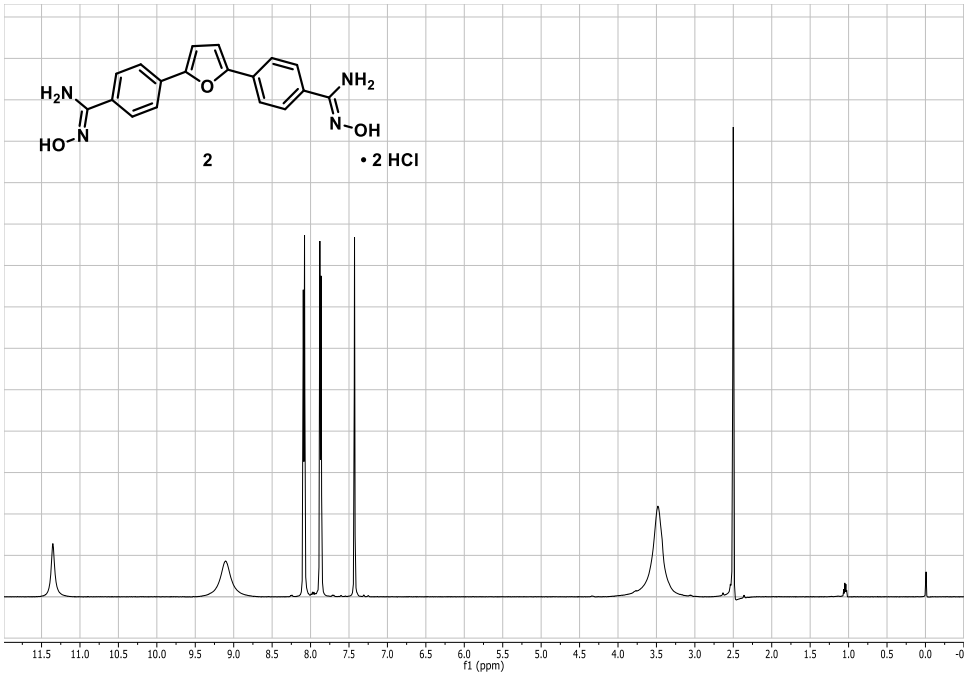

Figure 2: ^{1}H NMR spectrum of compound 2. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.35 (br. s, 2H), 9.10 (br. s, 4H), 8.09 (d, J = 8.1 Hz, 4H), 7.87 (d, J = 8.1 Hz, 4H), 7.43 (s, 2H).

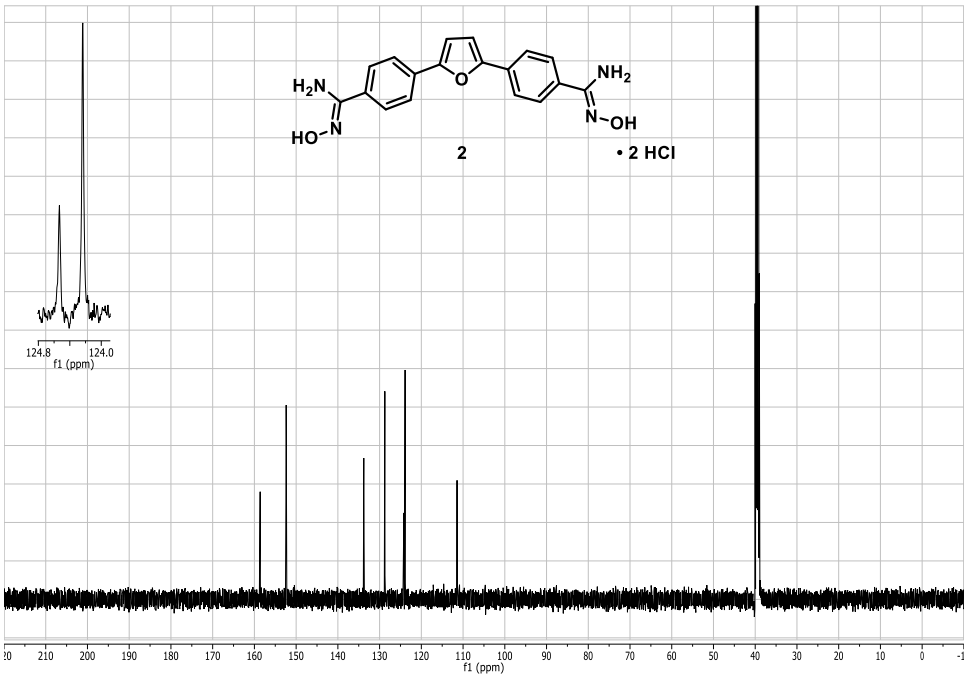

Figure 3: ^{13}C NMR spectrum of compound 2. ^{13}C NMR (125 MHz, DMSO-d_6): δ 158.6, 152.4, 133.8, 128.7, 124.2, 123.9, 111.4.

Synthesis of Pafuramidine: Compound **2** (233 mg, 0.57 mmol) was dissolved in dimethylformamide (14 mL). Then, a solution of lithium hydroxide (144 mg, 3.42 mmol) in water (2.1 mL) was added to the stirring solution of **2** in dimethylformamide and allowed to stir for 30 minutes before dimethylsulfate (135 µL, 1.43 mmol) was added dropwise at room temperature. The reaction was then heated to 40 °C and stirred for 20 hours. Upon completion of this reaction, the mixture was allowed to cool to room temperature. The reaction mixture was then poured slowly into a beaker containing ice water resulting in the formation of a precipitate. The resulting mixture was allowed to stir for 15 minutes, afterwards the white precipitate was collected by filtration and purified using flash column chromatography using 99.5:0.5 dichloromethane:methanol as the eluent to afford Pafuramidine as a white solid (102 mg, 49% yield). **Note:** Pafuramidine is a known compound (CAS No.: 186953-56-0) and was synthesized according to a modified literature procedure (*65*). No spectra of pafuramidine were found for comparison in our search, so we have provided our data in this report. **HRMS (ESI):** calc. for $C_{20}H_{21}N_4O_3$ $[M+H]^+$: 365.1608, found: 365.1621. **MP:** 188 - 190 °C, lit. 192.5 - 193 °C (*66*).

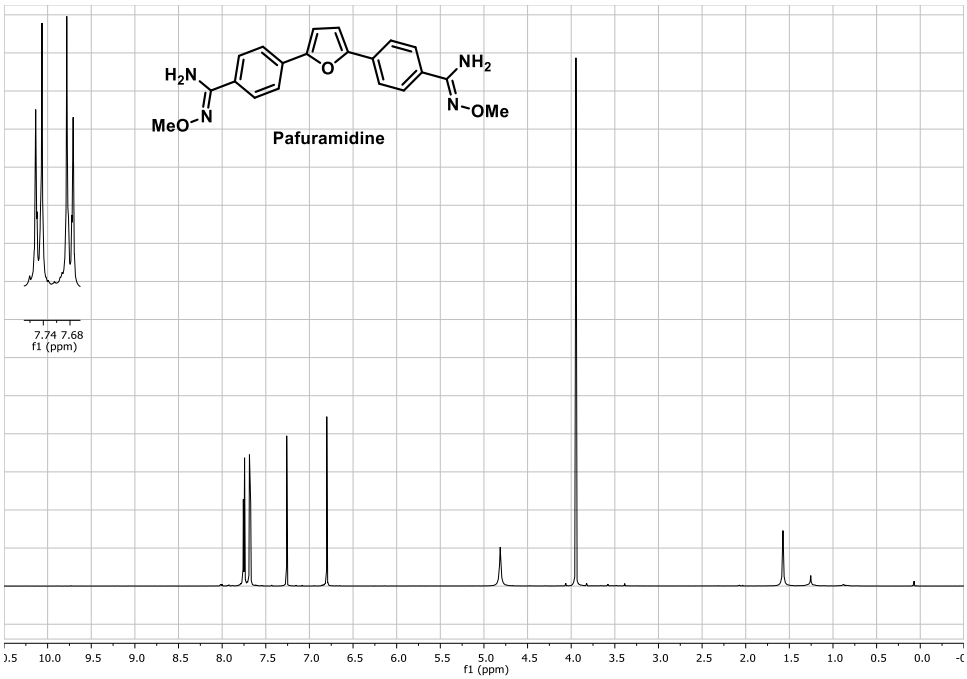

Figure 4: ^{1}H NMR spectrum of pafuramidine. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.77 – 7.73 (m, 4H), 7.70 – 7.66 (m, 4H), 6.80 (s, 2H), 4.81 (br. s, 4H), 3.95 (s, 6H).

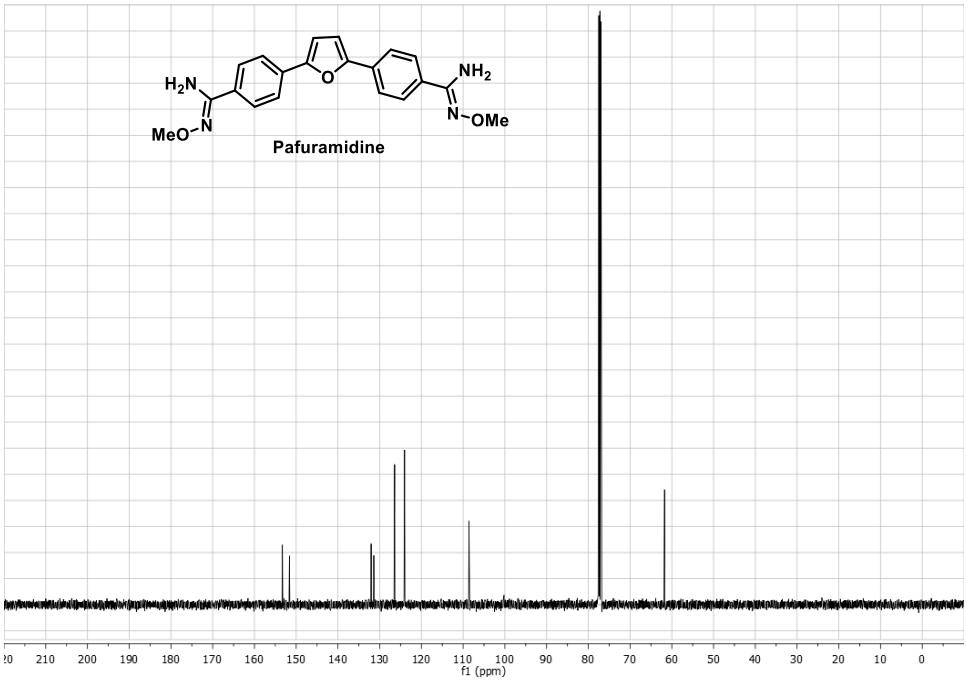

Figure 5: ^{13}C NMR spectrum of pafuramidine. ^{13}C NMR (125 MHz, $CDCl_3$): δ 153.3, 151.6, 132.0, 131.4, 126.4, 124.0, 108.6, 61.7.

Supporting tables:

TABLE S1. Differential gene expression analysis of treated DM1 patient-derived myotubes.

Sample	Genes up (% of total)*	Genes down (% of total)*	Genes Rescued (%)¶	Genes Mis-rescued (%)§	Genes Mis-rescued (%)#	Off-targets[œ]
Non-DM1 vs DM1	4474 (14.4)	4413 (14.2)	--	--	--	--
DM1 vs DM1 + EM	2505 (8.1)	1798 (5.8)	910 (21)	1421 (33)	374 (9)	1598
DM1 vs DM1 + FM	2323 (7.5)	2382 (7.7)	853 (18)	1809 (38)	329 (7)	1714
DM1 vs DM1 + EM + FM	4437 (14.3)	4393 (14.1)	1275 (14)	2690 (30)	904 (10)	3962

* Out of 31054 genes with nonzero total read count with an adjusted p-value < 0.1.
¶ Of genes mis-regulated between non-DM1 and DM1 cells, number of genes showing rescue between 1-110 %.
§ Of genes mis-regulated between non-DM1 and DM1 cells, number of genes showing mis-rescue < -1 %.
# Of genes mis-regulated between non-DM1 and DM1 cells, number of genes showing over-rescue > 110 %.
œ Number of genes differentially expressed that are not mis-regulated between non-DM1 and DM1 cells.

Supporting figures:

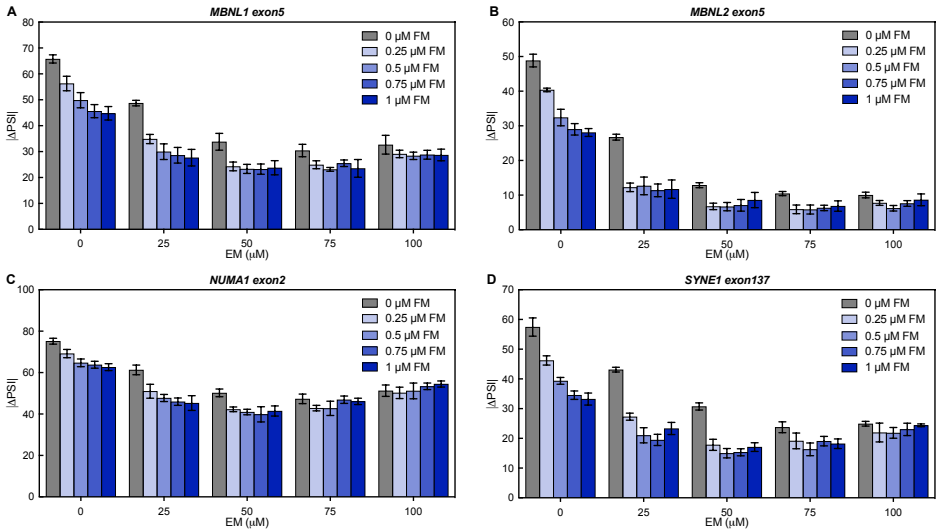

Figure S1. Combination of furamidine and erythromycin displays an additive rescue of mis-splicing in DM1 patient-derived myotubes. Absolute value of the Percent Spliced-In difference between non-DM control and DM1 myotubes (|ΔPSI|) with and without treatment determined via RT-PCR. Cells were treated with either furamidine (FM) alone or erythromycin (EM) or a combination of both. **(A)** *MBNL1 exon5*, **(B)** *MBNL2 exon5*, **(C)** *NUMA1 exon2* and **(D)** *SYNE1 exon137* events all displayed additive mis-splicing rescue after 4 days of treatment with the combination ($p<0.05$ or better for all treatments).

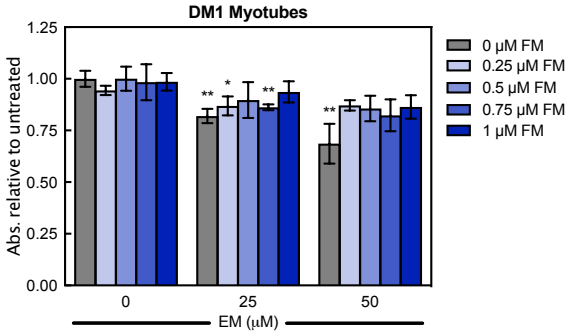

Figure S2. Combination treatment displays little to no toxicity in DM1 patient-derived myotubes. Cell viability determined via absorbance assay testing the reducing power of DM1 myotubes with erythromycin (EM) and/or furamidine (FM) treatment relative untreated DM1 myotubes (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$).

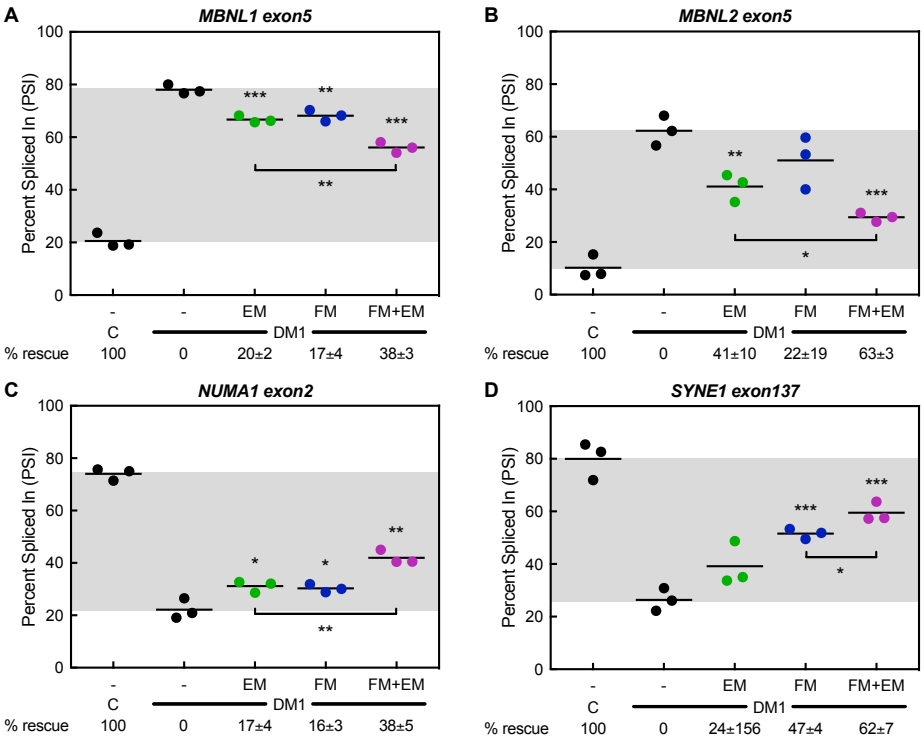

Figure S3. RNA-seq validation of additive mis-splicing rescue in DM1 patient-derived myotubes. Splicing analysis of exon-skipping (ES) events determined via RNA-seq of non-DM control and DM1 myotubes treated with either 25 μM erythromycin (EM, green) or 0.5 μM furamidine (FM, blue) alone or a combination of both (EM+FM, magenta). The Percent Spliced-In (PSI) values are shown for **(A)** *MBNL1 exon5*, **(B)** *MBNL2 exon5*, **(C)** *NUMA1 exon2* and **(D)** *SYNE1 exon137* events. Mean % rescue ± standard deviation values are displayed below each graph. All events display additive mis-splicing rescue after 4 days of treatment with the combination (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$).

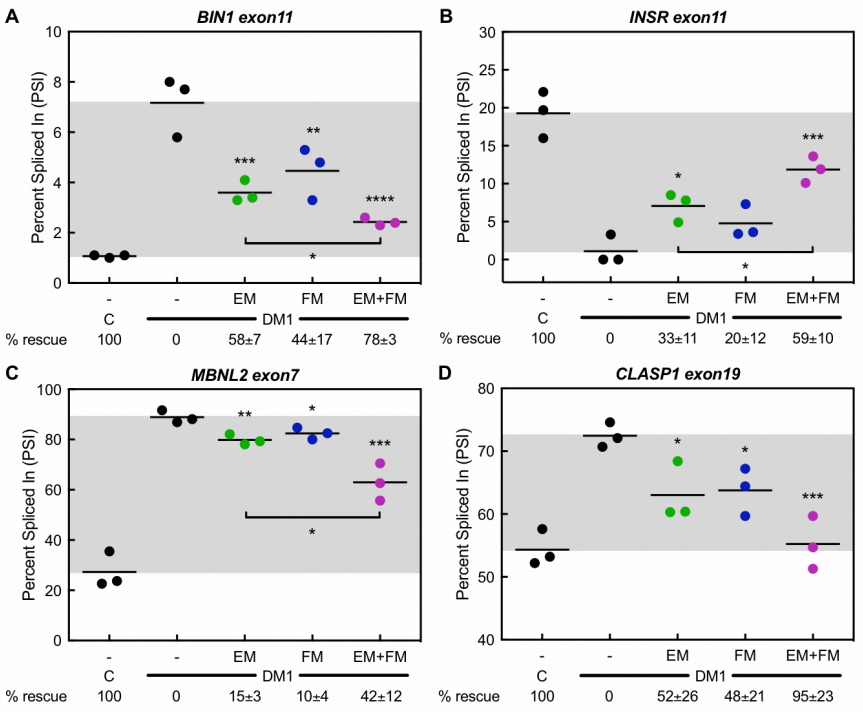

Figure S4. Combination treatment displays an additive rescue of mis-splicing of MBNL-dependent events in DM1 patient-derived myotubes. Splicing analysis of exon-skipping (ES) events determined via RNA-seq of non-DM control and DM1 myotubes treated with either 25 μM erythromycin (EM, green) or 0.5 μM furamidine (FM, blue) alone or a combination of both (EM+FM, magenta). The Percent Spliced-In (PSI) values are shown for **(A)** *BIN1 exon11*, **(B)** *INSR exon11*, **(C)** *MBNL2 exon7* and **(D)** *CLASP1 exon19* events. Mean % rescue ± standard deviation values are displayed below each graph. All events display additive mis-splicing rescue after 4 days of treatment with the combination (* = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001).

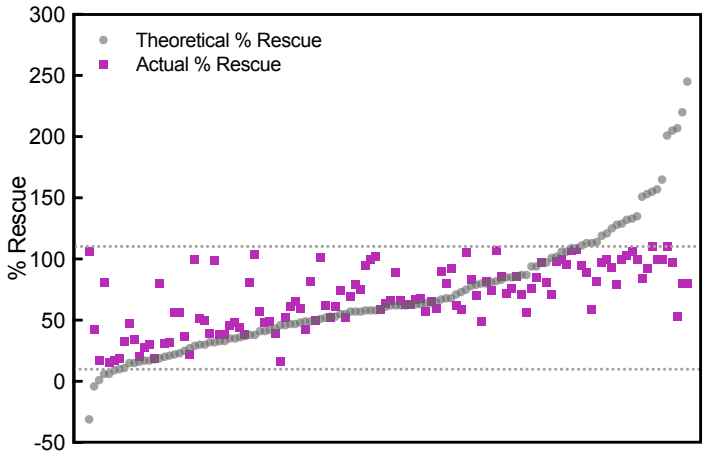

Figure S5. Mis-splicing events over- or mis-rescued by either drug alone are rescued by the combination in DM1 patient-derived myotubes. A scatter plot displaying the theoretical percent rescue based upon a purely additive effect by adding the percent rescues of erythromycin and furamidine alone (grey circles) and the actual percent rescue of the combination treatment (magenta squares) for the 121 ES events rescued by the combination treatment ordered by increasing theoretical percent rescue. Grey dotted lines mark 10 – 110 % rescue.

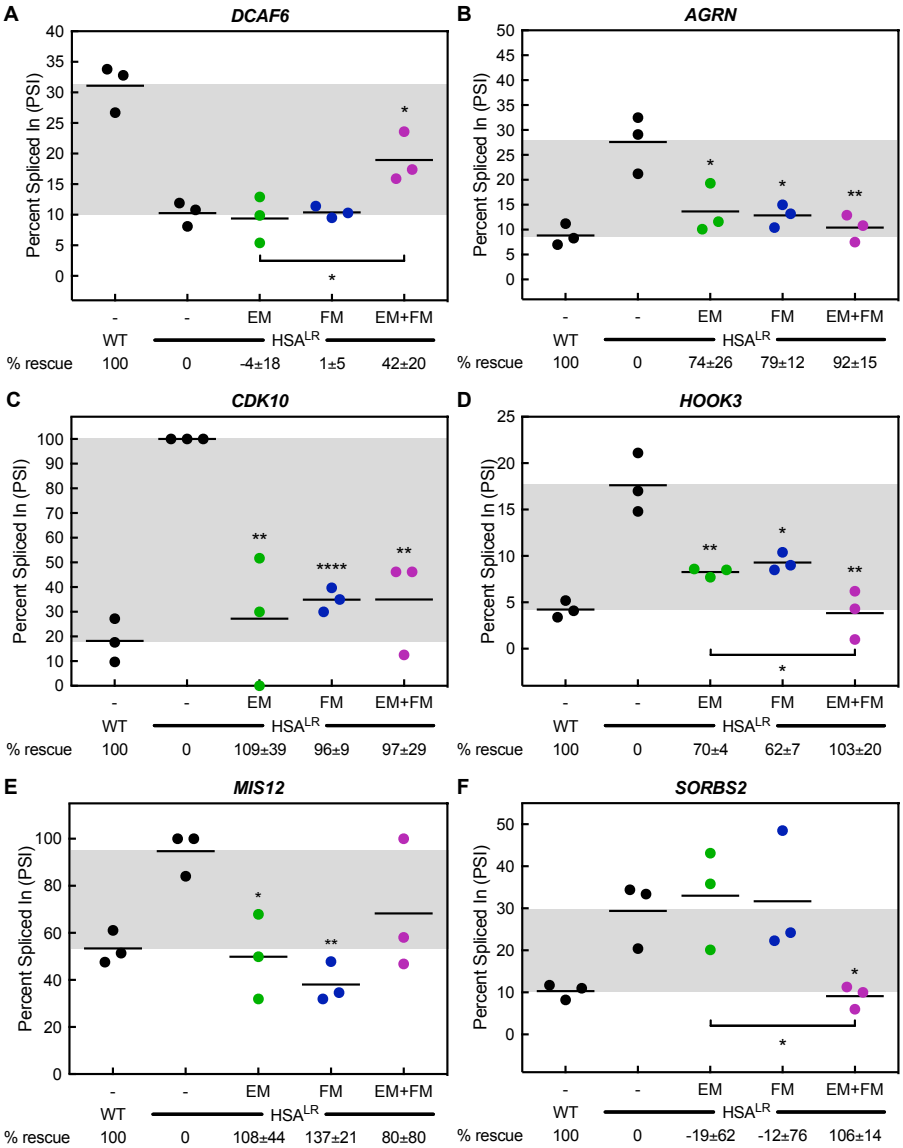

Figure S6. Several mis-splicing events suggest synergistic rescue by the combination in DM1 patient-derived myotubes. Splicing analysis of exon-skipping (ES) events determined via RNA-seq of non-DM control and DM1 myotubes treated with either 25 μM erythromycin (EM, green) or 0.5 μM furamidine (FM, blue) alone or a combination of both (EM+FM, magenta). The Percent Spliced-In (PSI) values are shown for **(A)** *DCAF6*, **(B)** *AGRN*, **(C)** *CDK10*, **(D)** *HOOK3*, **(E)** *MIS12* and **(F)** *SORBS2* events. Mean % rescue ± standard deviation values are displayed below each graph (* = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001).

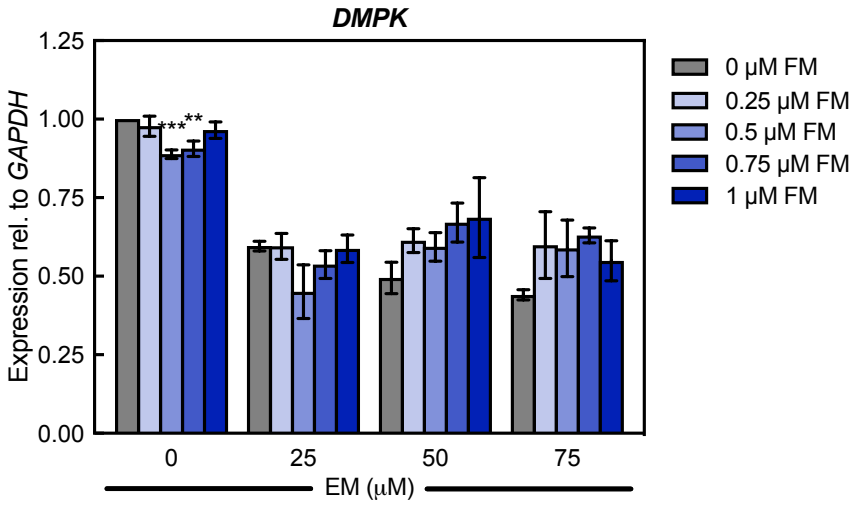

Figure S7. Combination treatment reduces *DMPK* transcript levels in DM1 patient-derived myoblasts. RT-qPCR data showing *DMPK* expression levels in DM1 patient-derived myotubes treated with either furamidine (FM) or erythromycin (EM) or a combination of both. FM treatment caused decreased *DMPK* transcripts at 0.5 and 0.75 μM. EM treatment significant decreased *DMPK* transcript levels alone and in combination with FM ($p<0.05$ or better for all treatments with erythromycin present, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$).

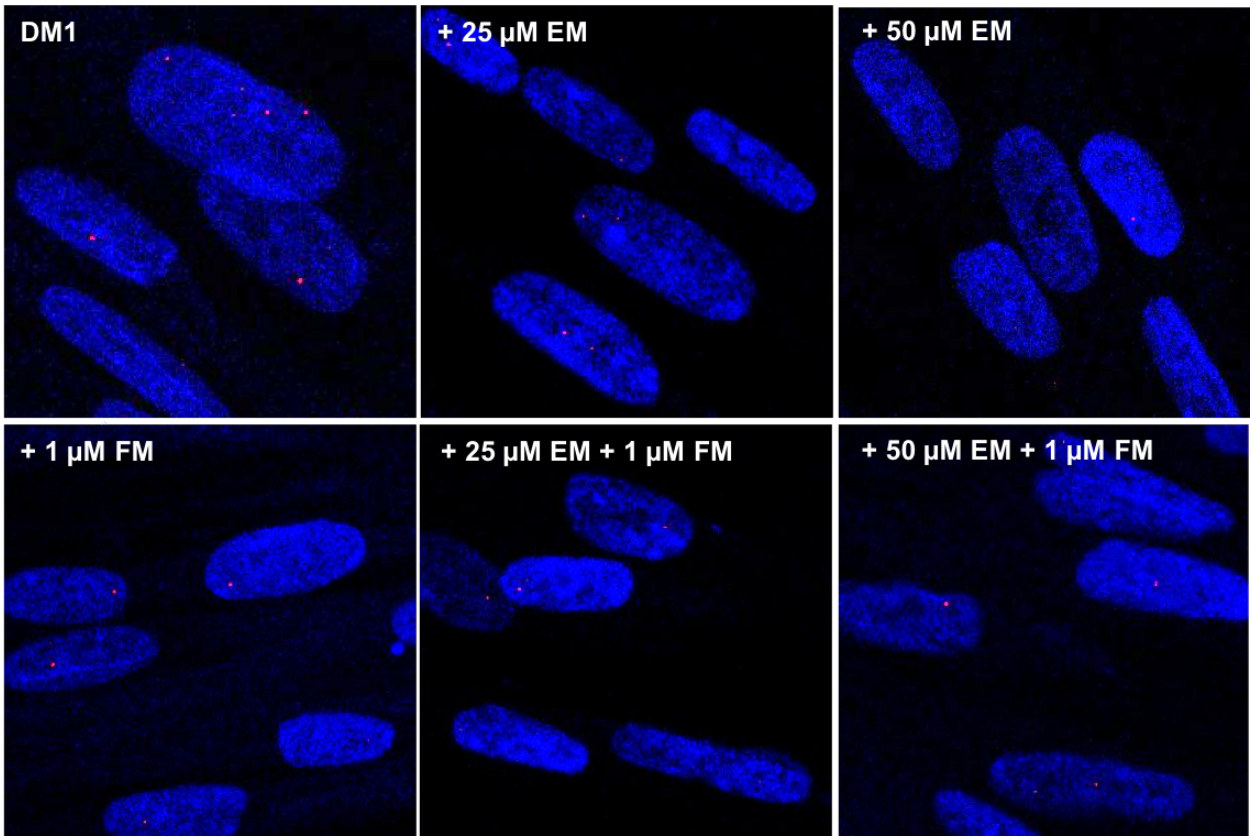

Figure S8. Combination treatment reduces ribonuclear foci abundance in DM1 patient-derived myoblasts. Representative fluorescent in situ hybridization (FISH) images using a Cy3-$(CAG)_8$ probe for CUG-repeat RNA foci (red) hybridization and DAPI to stain the nucleus (blue) in DM1 patient-derived myotubes. DM1 myoblasts were differentiated for 7 days and then treated with the indicated concentration of erythromycin (EM) and/or furamidine (FM) for 4 days.

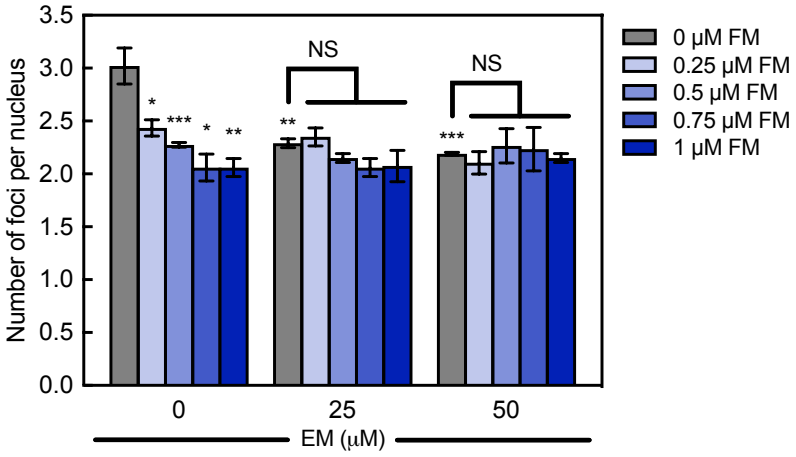

Figure S9. Combination treatment does not display an additive effect on ribonuclear foci formation in DM1 patient-derived myotubes. Quantification of the average number of ribonuclear foci per nucleus in DM1 myotubes determined via *in situ* hybridization of CUG-repeat RNA. A reduction in the number of ribonuclear foci per nucleus was observed for all furamidine (FM), erythromycin (EM) or combination concentrations tested (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$).

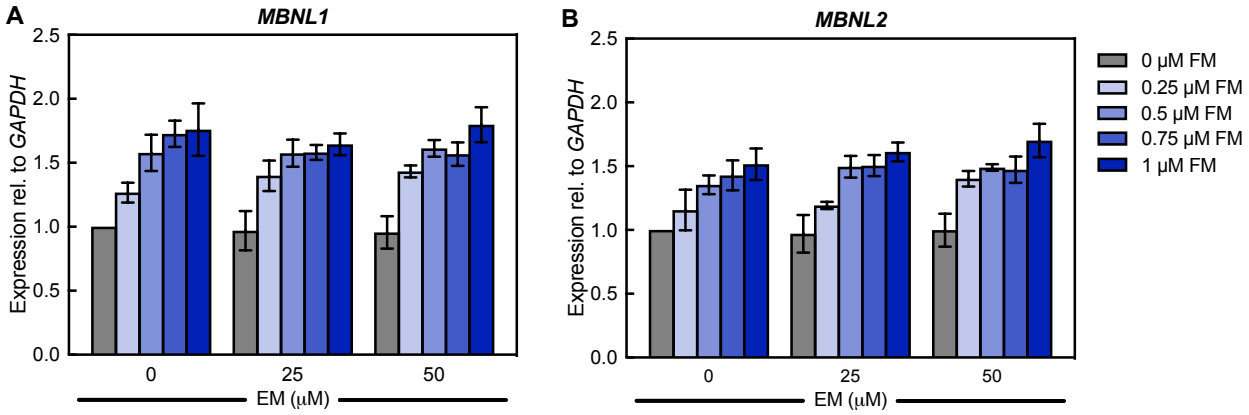

Figure S10. MBNL transcripts increase with combination treatment in DM1 patient-derived myotubes. RT-qPCR data showing **A)** *MBNL1* and **B)** *MBNL2* expression levels in DM1 patient-derived myotubes treated with either furamidine (FM) or erythromycin (EM) or a combination of both. FM treatment caused increased levels of both *MBNL1* and *MBNL2* transcripts regardless of EM addition in the combination ($p<0.05$ or better for all treatments with furamidine present, NS for treatments with erythromycin only).

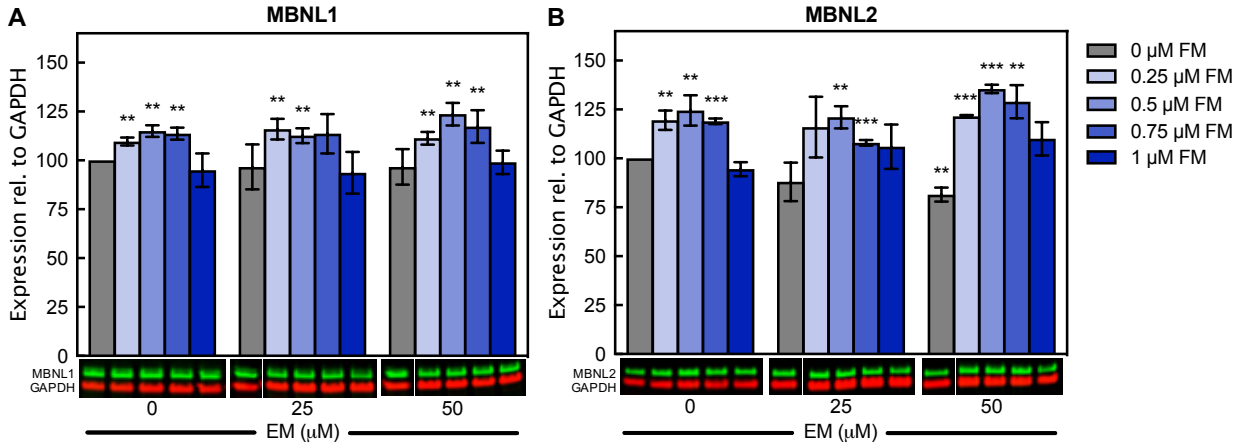

Figure S11. MBNL protein levels increased with combination treatment in DM1 myotubes. A) MBNL1 and **B)** MBNL2 protein levels in DM1 patient-derived myotubes treated with combination treatment. Furamidine treatment alone increased levels of MBNL1 and MBNL2 protein levels at 0.25, 0.5, and 0.75 μM furamidine. Erythromycin treatment alone did not affect MBNL1 protein levels, but it did decrease MBNL2 protein levels at 50 μM. All combination treatments showed the same trends in MBNL1 and 2 protein levels as the corresponding furamidine treatment (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$).

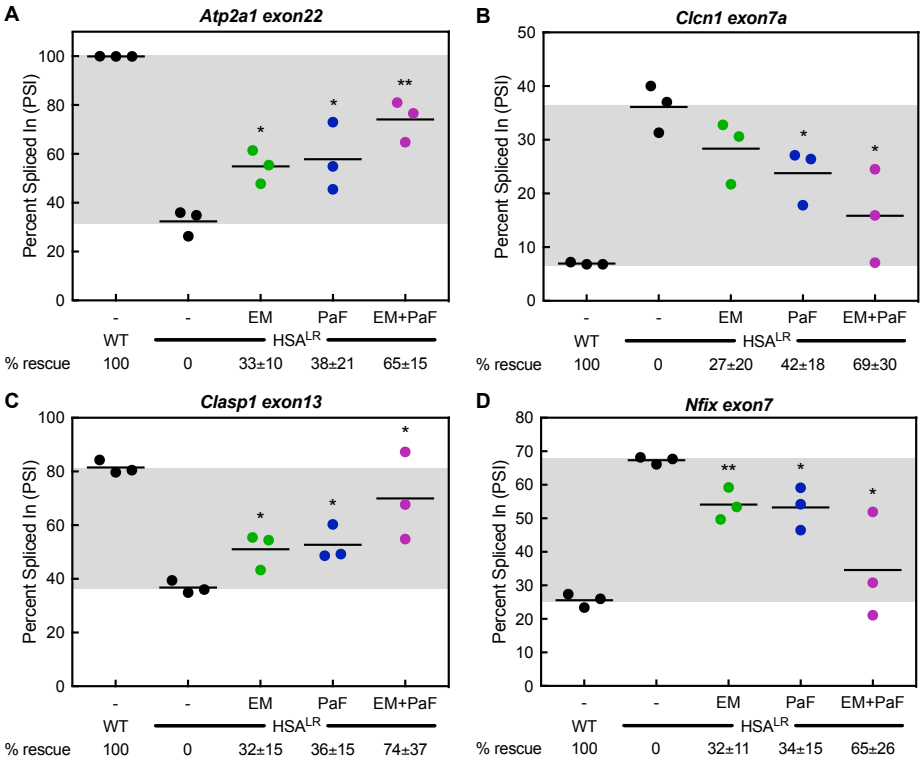

Figure S12. RNA-seq validation of rescue of multiple mis-splicing events with combination treatment in HSA^{LR} DM1 mice. Percent Spliced-In between WT and HSA^{LR} mice (PSI) with and without treatment determined via RNA-seq analysis. Mice were treated with either 600 mg kg^{-1} erythromycin (EM, green) or 10 mg kg^{-1} pafuramidine (PaF, blue) alone or a combination of both (EM+PaF, magenta) per oral administration daily for 14 days. **(A)** *Atp2a1 exon22*, **(B)** *Clcn1 exon7a*, **(C)** *Clasp1 exon13* and **(D)** *Nfix exon7* mis-splicing events all displayed additive rescue with combination treatment (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$).

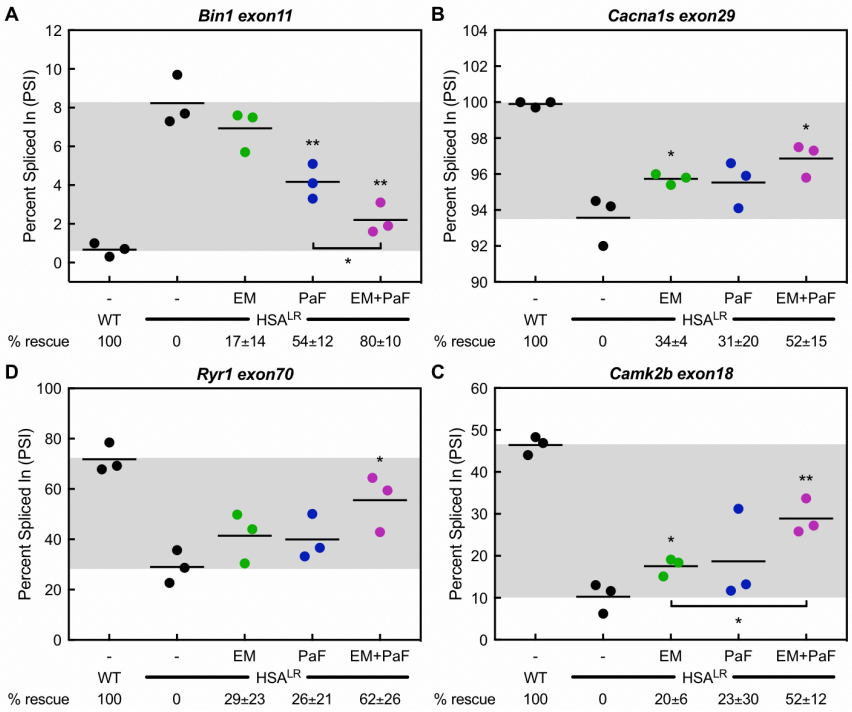

Figure S13. Combination treatment rescues mis-splicing implicated in human disease symptoms in HSA^{LR} DM1 mice. Splicing analysis of exon-skipping (ES) events determined via RNA-seq on HSA^{LR} mice treated with 600 mg kg^{-1} erythromycin (EM, green) or 10 mg kg^{-1} pafuramidine (PaF, blue) or a combination of both (EM+PaF, magenta) per oral administration daily for 14 days. **(A)** *Bin1 exon11*, **(B)** *Cacna1s exon29*, **(C)** *Ryr1 exon70* and **(D)** *Camk2b exon18* mis-splicing events displayed additive rescue with combination treatment (* = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001).

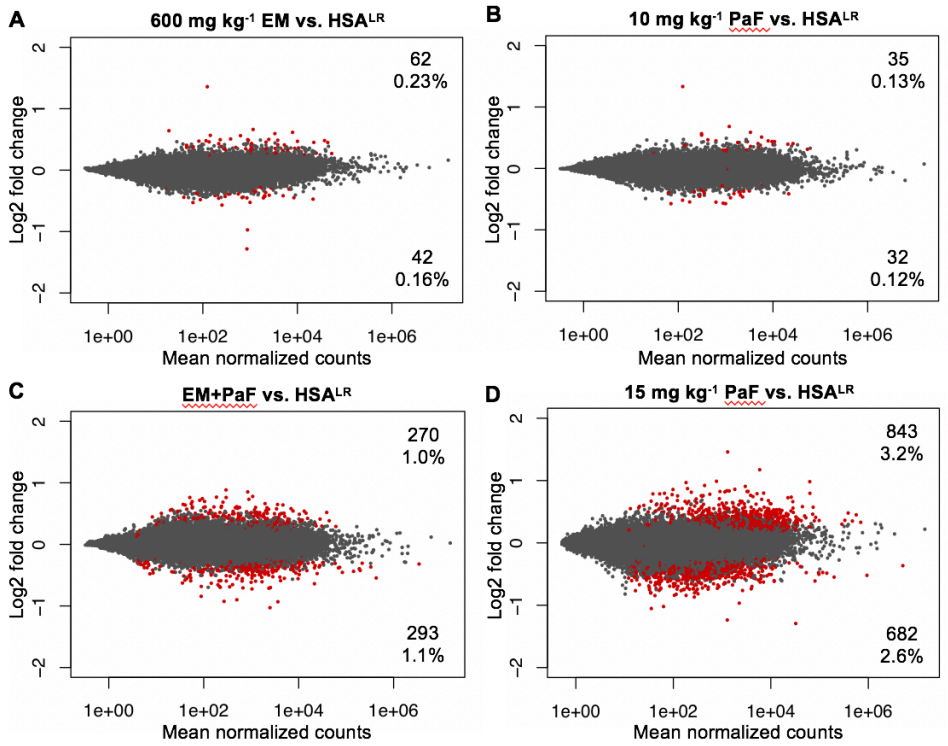

Figure S14. Global differential gene expression for all drug treatments is minimal even without correction for gene expression rescue in HSA^{LR} mice. Global differential gene expression analysis determined via RNA-seq showing MAplots for **(A)** 600 mg kg^{-1} erythromycin, **(B)** 10 mg kg^{-1} pafuramidine (PaF), **(C)** the combination (EM+PaF) and **(D)** 15 mg kg^{-1} pafuramidine (PaF) versus control HSA^{LR} mice NOT CORRECTED for gene expression rescue. Red dots represent gene with significantly altered expression ($p<0.1$). Grey dots represent genes that were not significantly differentially expressed.

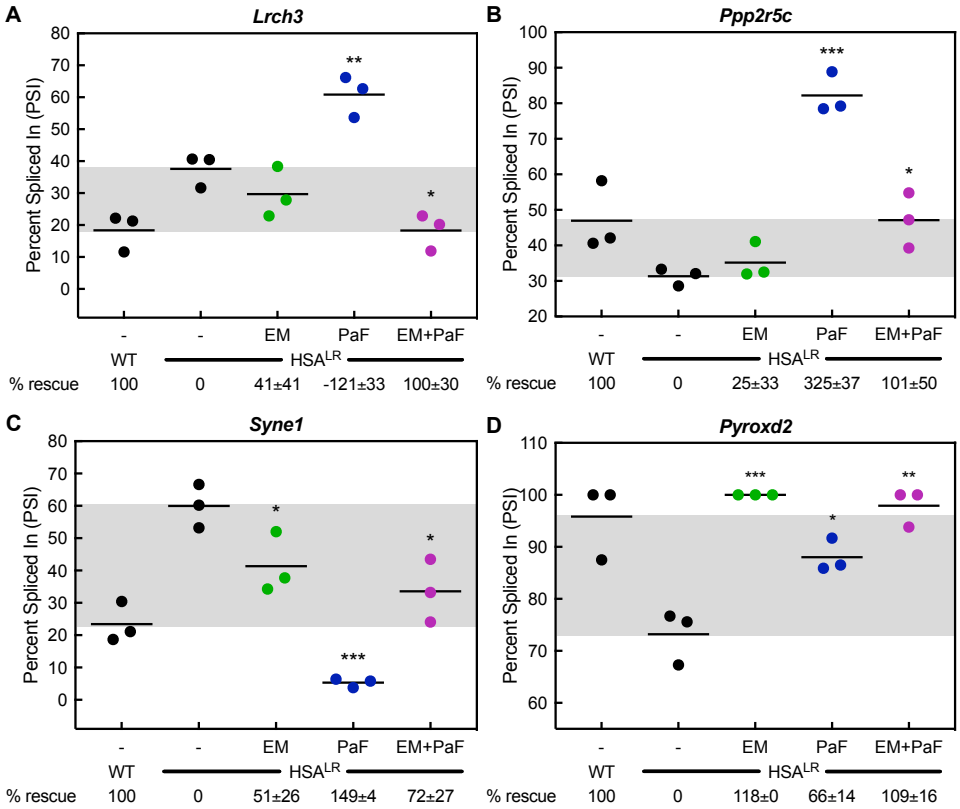

Figure S15. Several mis-splicing events display synergistic rescue with combination treatment in HSA^{LR} DM1 mouse model. Splicing analysis of exon-skipping (ES) events determined via RNA-seq on HSA^{LR} mice treated with 600 mg kg^{-1} erythromycin (EM, green) or 10 mg kg^{-1} pafuramidine (PaF, blue) or a combination of both (EM+PaF, magenta) per oral administration daily for 14 days. **(A)** *Lrch3*, **(B)** *Ppp2r5c*, **(C)** *Syne1* and **(D)** *Pyroxd2* mis-splicing events displayed synergistic rescue with combination treatment. Mean % rescue ± standard deviation values are displayed below each graph (* = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001).

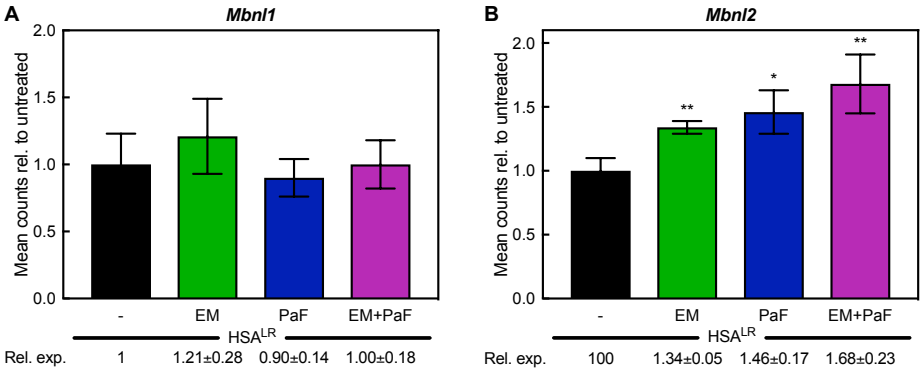

Figure S16. Combination therapy increases *Mbnl2* transcript levels in HSA^{LR} DM1 mice. RNA-seq gene expression analysis showing **A)** *MBNL1* and **B)** *MBNL2* expression levels in HSA^{LR} mice treated with either 600 mg kg^{-1} erythromycin (EM, green) or 10 mg kg^{-1} pafuramidine (PaF, blue) or a combination of both (EM+PaF, magenta) per oral administration daily for 14 days. *MBNL1* transcript levels did not show significant change in expression with any treatment. *MBNL2* transcript levels increased significantly with EM, PaF and combination treatments (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$).

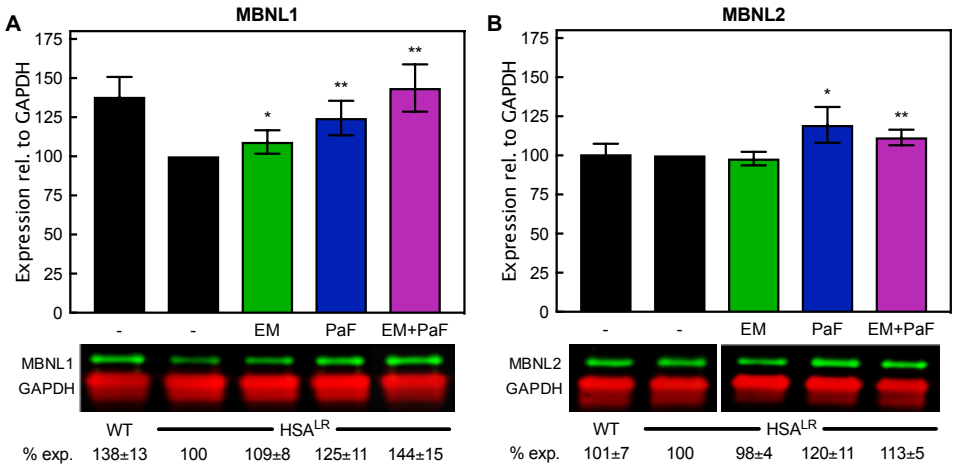

Figure S17. Combination therapy increases Mbnl1 and 2 protein levels in HSA^{LR} DM1 mice. Western blot analysis showing **A)** Mbnl1 and **B)** Mbnl2 protein levels in wild-type (WT) and HSA^{LR} mice treated with either 600 mg kg^{-1} erythromycin (EM, green) or 10 mg kg^{-1} pafuramidine (PaF, blue) or a combination of both (EM+PaF, magenta) per oral administration daily for 14 days. Mbnl1 protein levels showed significant increases with all treatments. Mbnl2 protein levels only showed a significant increase with treatments where PaF was present. (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$).