A flow cytometry-based screen identifies MBNL1 modulators that rescue splicing defects in myotonic dystrophy type I

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Abstract

Myotonic dystrophy Type 1 (DM1) is a rare genetic disease caused by the expansion of CTG trinucleotide repeats ((CTG)\textsubscript{exp}) in the 3' untranslated region of the DMPK gene. The repeat transcripts sequester the RNA binding protein Muscleblind-like protein 1 (MBNL1) and hamper its normal function in pre-mRNA splicing. Overexpressing exogenous MBNL1 in the DM1 mouse model has been shown to rescue the splicing defects and reverse myotonia. Although a viable therapeutic strategy, pharmacological modulators of MBNL1 expression have not been identified. Here, we engineered a ZsGreen tag into the endogenous MBNL1 locus in HeLa cells and established a flow cytometry-based screening system to identify compounds that increase MBNL1 expression. The initial screen of small molecule compound libraries identified more than thirty hits that increased MBNL1 expression greater than double the baseline levels. Further characterization of two hits revealed that the small molecule HDAC inhibitors, ISOX and vorinostat, increased MBNL1 expression in DM1 patient-derived fibroblasts and partially rescued the splicing defect caused by (CUG)\textsubscript{exp} repeats in these cells. These findings demonstrate the feasibility of this flow-based cytometry screen to identify both small molecule compounds and druggable targets for MBNL1 upregulation.

Introduction

Myotonic dystrophy (DM) is a genetic disorder that affects at least 1 in every 8,000 people worldwide and is the most common muscular dystrophy in adults (1,2). Patients suffer from multi-systemic symptoms including myotonia, muscle wasting, cardiac arrhythmia, dysphagia, cataracts, insulin resistance, sleep dysregulation, cognitive decline and premature death (3). Currently, there is no approved treatment. Genetically, there are two sub-types of DM.
Type 1 (DM1) is caused by the CTG-trinucleotide repeat expansion ((CTG)_{exp}) in the 3’ untranslated region (UTR) of Dystrophia Myotonica Protein Kinase (DMPK) gene (4–7). Type 2 (DM2) is associated with a (CCTG)_{exp} in intron 1 of Zinc Finger Nuclease 9 (ZNF9) (8). Both types are autosomally dominantly inherited with overlapping symptoms but different prevalence. DM1 is more common among patients with more severe symptoms and earlier onset (9,10). In vivo studies indicate that the toxic RNA gain-of-function is the main cause of DM1 rather than the DMPK loss of function (11,12). In affected cells, (CUG)_{exp} transcripts sequester RNA-binding protein Muscleblind-like proteins (MBNL) into nuclear aggregates, up-regulate CUGBP and Elav-like family members (CELF), and further disrupts alternative splicing (13–16). These splicing perturbations have a physiological connection to DM symptoms and highlight their potential use as biological markers for both disease characterization and drug treatment. In particular, Sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (SERCA1) protein is responsible for regulating calcium concentration in cells, and the splicing disruption of its transcript could contribute to muscle weakness (17–19). Furthermore, the exon 11 skipped insulin receptor (INSR) isoform has lower tyrosine kinase signaling capability which is implicated in the insulin resistance phenotype in DM1 (20). Although other mechanisms such as Repeat Associated Non-ATG Translation (RAN translation), microRNA deregulation and transcription misregulation are also involved in DM1 (21–23), the spliceopathy induced by MBNL1 sequestration and CELF up-regulation is believed to be the primary mechanism of (CUG)_{exp} RNA toxicity and the common cause of the multi-systemic symptoms.

MBNL proteins are a family of multifunctional RNA binding proteins that regulate precursor alternative splicing and polyadenylation, RNA stability, localization and microRNA biogenesis (24–30). In mammals, there are three highly conserved paralogues MBNL1, MBNL2 and MBNL3 (31). They share the structure similarity and recognize the common consensus sequences in both pre-mRNAs and (C/U)G_{exp} transcripts (30–34). MBNL3 is primarily expressed in reproductive organs. MBNL1 and MBNL2 are ubiquitously expressed, while MBNL1 is highly abundant and plays a major role in most tissues except brain where MBNL2 is predominantly expressed (35–37). The single- and compound-loss of MBNL(s) in mouse models recapitulate many of the DM multi-systemic phenotypes including splicing disruptions (38–41). A splicing microarray analysis comparing splicing changes in MBNL1 knockout (Mbnl1^{Δ3/Δ3}) mice to a DM1 mouse model (Hsa^{1A} poly(CUC) mice) revealed that ~80% of splicing pathology is MBNL1 dependent (42). Most of these splicing disruptions can be rescued by AAV-mediated MBNL1 overexpression in the Hsa^{1A} mouse model along with the reversion of myotonia (43). Moreover, a transgenic MBNL1 mouse model using a ubiquitous promoter for constitutively high expression demonstrated that both early and long-term increase of MBNL1 levels are well-tolerated suggesting that this is a practical therapeutic strategy for DM1 treatment (44). Based on this evidence, we established a flow-based cytometry screen using a ZsGreen-MBNL1 reporter cell line monitoring the endogenous MBNL1 expression and its functional activity. Our initial findings demonstrate the feasibility of using this cell-based screening system to identify small molecule modulators and druggable targets for MBNL1 upregulation.

**Results**

**Generation of ZsGreen-MBNL1 reporter cells to measure endogenous MBNL1 expression**

Previous work demonstrated that modifying a MBNL1 transgene with an N-terminal GFP did not affect its splicing capability in murine adult skeletal tissue (43). Based on this evidence, we took advantage of the CRISPR/Cas9 gene-editing system to insert a ZsGreen fluorescent tag into the N-terminus of the MBNL1 coding sequence in HeLa cells. We selected HeLa cells to build the reporter system for the following three reasons: 1) as an alternative splicing regulator, the molecular mechanism of MBNL1 function is universal and has been studied in cancer cell lines (26); 2) HeLa cells express MBNL1 at a moderate level which sets a lower signal starting point and allows a signal increase to be measured; 3) HeLa cells are easy to engineer and compatible with most cell-based screening platforms at medium to high throughput.

To increase specificity of the insertion, the D10A double nickase strategy was used to generate two staggered cuts on DNA strands using two guide RNAs targeting sequences upstream and downstream of human MBNL1 exon 2 start codon and the ZsGreen-MBNL1 construct containing the donor sequences was co-transfected (Fig. 1A) (45). After integration, the cells expressing ZsGreen-MBNL1 fusion protein showed medium level green fluorescent signal accumulated in the nuclei (Supplementary Material, Fig. S1A). Flow cytometry quantification revealed a modest but distinguishable fluorescent signal from the non-fluorescent parental HeLa cells that were enriched following fluorescence-activated cell sorting (FACS) (Supplementary Material, Fig. S1B). Next, single cell clones were isolated via FACS and expanded to establish stable cell lines.

To confirm the site-specific integration, we isolated genomic DNA from both parental HeLa cells and colonized ZsGreen-MBNL1 cells followed by PCR amplification using primers spanning the insertion sites in MBNL1 gene and performed gel electrophoresis analysis. Both HeLa and ZsGreen-MBNL1 cells carried the unmodified MBNL1 allele indicated by the 1.5 kb fragment amplified by the primer set F2038 and F2041, while the ZsGreen-MBNL1 cells had an additional 2.2 kb fragment (Fig. 1B). Two fragments (0.9 kb and 1.1 kb) were detected in ZsGreen-MBNL1 cells but not in HeLa cells using ZsGreen specific primers (Fig. 1B). The sequences at the insertion junction were confirmed by Sanger sequencing. To test if this integration was unique to the MBNL1 gene, we used Droplet Digital PCR (ddPCR) to quantitate the copy numbers of MBNL1 and ZsGreen in the genome of ZsGreen-MBNL1 cells. Both parental HeLa and ZsGreen-MBNL1 cells had two copies of MBNL1 while only ZsGreen-MBNL1 cells carried ZsGreen with its copy number close to one (Fig. 1C). Collectively, these data demonstrated that our CRISPR/Cas9 system uniquely integrated ZsGreen into one MBNL1 allele in HeLa cells. Several of the ZsGreen-MBNL1 expressing clones showed similar fluorescence and integration PCR results, so we decided to perform further characterization and screening on a single clone, #27.

To confirm our reporter cell line expressed the ZsGreen-MBNL1 fusion protein, we performed immunoblotting analysis using an antibody against MBNL1. Parental HeLa cells expressed the native MBNL1 (40–42 kDa), while the ZsGreen knock-in cells expressed both the wild type and ZsGreen-MBNL1 fusion proteins (65–67 kDa) with the total MBNL1 level equivalent to the parental HeLa cell. All MBNL1 forms were proportionally knocked down by a siRNA against MBNL1 (Fig. 1D). Fluorescent microscopy revealed a homogenous level of green fluorescent signal among ZsGreen-MBNL1 cells that was distinguishable from the minimal cell autofluorescence signal from the parental HeLa cells (Fig. 1E). Furthermore, we observed the enhanced nuclear accumulation of ZsGreen-MBNL1 which is consistent with endogenous MBNL1 functioning as a nuclear splicing factor. Flow cytometry analysis of ZsGreen-MBNL1 cells showed a

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DM1. To determine whether the ZsGreen-MBNL1 fusion protein maintains the same functional properties as native MBNL1, we transfected the stable ZsGreen-MBNL1 line with constructs expressing (CUG)_{400} RNA and then detected the cellular localization of ZsGreen-MBNL1 and native MBNL1 by MBNL1 antibody staining. Both ZsGreen-MBNL1 and native MBNL1 showed diffuse nuclear distribution in mock-transfected cells. Following transfection with (CUG)_{400} RNA constructs, both MBNL1 forms co-localized in nuclear foci (Fig. 2A). Furthermore, the uniform nuclear background signals were significantly lower than the mock-transfected controls suggesting recruitment into foci (Fig. 2A). Detection of the (CUG)_{400} via in situ hybridization with fluorescently labeled probes revealed accumulation in nuclear foci and partial co-localization with ZsGreen-MBNL1 (Fig. 2B). These results demonstrated that our ZsGreen-MBNL1 reporter fusion protein has similar RNA-binding activity to native MBNL1. Alternative splicing of SERCA1 transcripts has been shown to be regulated by MBNL1, and SERCA1 exon 22 skipping increases when MBNL1 is sequestered by (CUG)_{exp} repeats. Peptides in DM1 cells (17,18). To examine the splicing pattern in our ZsGreen-MBNL1 cells, we quantified the spliced SERCA1 mRNA by RT-PCR using primers spanning exon 22. In mock-transfected cells, exon 22 was included in 86% of SERCA1 transcripts (Fig. 2C). However, in cells expressing the (CUG)_{400} the percentage of transcripts containing exon 22 decreased to 63% (Fig. 2C). These data gave us confidence that our ZsGreen-MBNL1 reporter system not only recapitulates the endogenous expression level of MBNL1 but also maintains its essential function as an RNA-binding splicing regulator.

Flow cytometry-based screen identifies small molecule MBNL1 up-regulators

At the time we were developing the screening assay, a positive control tool compound, reported to increase MBNL1 levels, was not available. We hypothesized that epigenetic modulators may likely alter endogenous MBNL1 expression as these compounds control accessibility of the transcriptional machinery to genes and play an important role in gene transcription states (46,47). We initiated screening with a compound set that contains 61 epigenetic modulators covering 44 targets including regulators of histone methyltransferases, demethylases, acetyltransferases and deacetylases, chromodomain proteins, arginine deiminases, sirtuins and DNA methyltransferases and identified nine compounds that showed significant increases in ZsGreen signal (Supplementary Materials, Fig. S2A and Table S1). Seven of these compounds are HDAC inhibitors suggesting histone deacetylation plays an important role in MBNL1 regulation. Each positive compound was confirmed independently, and full dose response curves were generated up to 100 μM with an EC_{50} and maximum fold increase determined (Supplementary Material, Table S1). Using the dose response data, the HDAC inhibitor ISOX showed 2.0 and 1.8 times maximal increase of ZsGreen-MBNL1 signal compared with DMSO treated cells, respectively, with an EC_{50} calculated at 2.9 μM for ISOX and 2.9 μM for vorinostat (Fig. 3A and B). We chose ISOX as a positive control for our subsequent screens since it increased MBNL1 to a higher level with a lower EC_{50}. Fluorescent microscopy image analysis of ISOX-treated ZsGreen-MBNL1 cells also showed a significant increase of nuclear ZsGreen signal in compound treated cells (Supplementary Material, Fig. S2B).

We miniaturized the assay to a 384-well plate format and adapted it to a robotic high-throughput flow cytometry narrow fluorescent signal peak, which is readily separated from the background signal in parental HeLa cells (Fig. 1F). The narrow width of the fluorescent signal provided a means to measure shifts indicating either increases or decreases in ZsGreen-MBNL1 expression.

(CUG)_{exp} repeat transcripts sequester ZsGreen-MBNL1 in nuclear foci and disrupt splicing in reporter cells

In DM1, sequestration of MBNL1 protein from its splicing regulatory complexes by the disease-specific (CUG)_{exp} repeats is followed by splicing disruption. This molecular disease mechanism has been reconstituted in multiple cell and animal models and provides important evidence for MBNL1 function in

Figure 1. Site-specific integration of ZsGreen into endogenous MBNL1 locus generates ZsGreen-MBNL1 cells expressing green fluorescent fusion protein. (A) Schematic diagram of the strategy to insert a ZsGreen cassette into the MBNL1 locus (not to scale). The asterisks indicate the position of the single-strand breaks generated by Cas9 nickase/gRNA. The middle diagram shows the donor vector that contains the left and right homologous MBNL1 arms and the ZsGreen reporter. (B) ZsGreen integration in MBNL1 locus is confirmed by PCR followed by agarose gel analysis. Primer sets and PCR products are indicated in the upper diagram. (C) Droplet digital PCR (ddPCR) quantifying MBNL1 and ZsGreen copy number in no-template control (NTC), parental HeLa and ZsGreen-MBNL1 genomic DNA and plotted on the bar graph. (D) Immunoblotting shows MBNL1 and ZsGreen-MBNL1 protein expression in parental HeLa and ZsGreen-MBNL1 cells. MBNL1-silencing siRNA (siMBNL1) and non-silencing siRNA (siNS) control were used to demonstrate MBNL1 antibody specificity. Tubulin was used as a loading control. (E) Fluorescent microscopy images of parental HeLa and clonal ZsGreen-MBNL1 #27 using the same exposure. The green fluorescent images are on the left; the merged images of green and blue (DAPI staining) signal are on the right. Scale bar is 20 μm. (F) Flow cytometry quantification of green fluorescent signal in parental HeLa cells and ZsGreen-MBNL1 clone #27.
screening platform which increased the output to ~8000 compounds/day. Using this screening platform, we expanded the screen to two larger compound libraries, the Pfizer Chemogenomics Library (CGL) and the FDA-approved drug set. The CGL contains 2753 selective small molecules covering over 1000 biological targets. The CGL was created for phenotypic screening with the purpose of expediting target identification. A hit from this set suggests the annotated target(s) of that pharmacological agent may be involved in perturbing the observable phenotype. Multi-parameter optimization was used in the creation of the library to ensure appropriateness of molecules for cell-based screening (including assessments of permeability, solubility, cytotoxicity and selectivity). As CGL compounds were selected based on their potency against their primary annotated target at a concentration equal to or less than 500 nM, we decided to screen the library by treating ZsGreen-MBNL1 cells with 1 μM CGL compounds in duplicate. Since 5 μM ISOX treatment increased the ZsGreen signal close to 100% (Fig. 3A), the percentage change in ZsGreen-MBNL1 levels was calculated by comparing to ISOX treatment (ΔMFI_{compound}/ΔMFI_{ISOX}).

We plotted the fold increases of the top 128 hits from the CGL screen with their primary targeted gene family indicated (Fig. 3D). Seventy of the top 128 hits targeted kinases with 12 compounds targeting aurora kinase B (AURKB) and cyclin-dependent kinase 2 (CDK2).

The FDA drug set contains 1040 small molecules and is a set of FDA-approved marketed and clinical stage compounds. Use of this compound set in screening may rapidly link an early phenotypic assay endpoint to human patient data and provide a direct path for testing in preclinical animal models. To explore all these possibilities, we screened this set of compounds at 10 μM in duplicates. After the compound fluorescence test in HeLa cells, 34 compounds remained positive with a hit rate of 4.7%. Ten compounds increased the ZsGreen-MBNL1 level more than 100% at 10 μM (Supplementary Material, Table S2). A subset of hits from the FDA drug set (12 compounds) was confirmed in ZsGreen reporter cells in a dose-response manner to determine the EC50 and maximum MBNL1 increase. Eleven compounds increased MBNL1 more than 100% and seven of these showed an EC50 in the single digit or below μM range and below (Table 1). Many of these compounds have been used as cancer, hematologic and dermatologic therapies (Table 1).
Figure 3. Cell-based screen identifies small molecules that up-regulate MBNL1 expression. (A, B) Dose response in ZsGreen-MBNL1 cells following ISOX (A) and vorinostat (B) treatment. ZsGreen-MBNL1 and parental HeLa cells were treated with compound and the mean fluorescent intensity (MFI) of the cells was quantified by flow cytometry 48 h after treatment. MFI_{HeLa} was subtracted from MFI_{ZsGreen-MBNL1} and the resulting MFI relative to DMSO treatment is plotted. (C) Box plot of the ZsGreen-MBNL1 signal changes in CGL screen. Each compound was screened at 1 μM in replicates. The percentage increase of ZsGreen-MBNL1 after compound treatment was calculated as follows: (MFI_{compound} - MFI_{DMSO})/(MFI_{ISOX} - MFI_{DMSO})×100% and plotted on Y axis. The CGL compounds are divided into gene families (listed on X axis) based on each compound’s primary annotated target activity (at 500 nM or less). The black line in each box represents the mean MBNL1 percentage change, each box indicates the distribution of 50% of compounds around the mean, the whiskers indicate the next 25%, and the outliers are plotted as dots. (D) The percentage increase of ZsGreen-MBNL1 signal after the treatment of top 128 compounds. ZsGreen-MBNL1 and parental HeLa cells were treated with each compound at 1 μM followed by flow cytometry quantifications. The MFI increases were calculated by subtracting MFI_{HeLa} from MFI_{ZsGreen}, and then the percentage increases were calculated and plotted to its primary targeting gene family. Each dot represents one compound. Negative control (0.1% DMSO) did not change MBNL1 levels (indicated as 0% baseline) and the positive control (5 μM ISOX) increased MBNL1 by 100% (indicated by the red line) on the plot C and D.
Since the CGL contains the original 61 epigenetic compounds from the pilot screen, we evaluated the reliability of our system by comparing the results from these two independent screens (initial 96-well format versus automated 384-well format). We re-identified 8 of the 9 epigenetic hits in the CGL screen performed at 10 \( \mu \)M. Two of the HDAC inhibitors showed a significant increase in ZsGreen-MBNL1 signal from the CGL 1 \( \mu \)M screen which is consistent with the fact that most of these compounds possess EC\(_{50}\)s in the >1 \( \mu \)M range (Supplementary Material, Table S1). Box plot analysis revealed that the compounds targeting histone deacetylases showed a distribution shift toward MBNL1 up-regulation (Fig. 3C). These data suggested the high reproducibility of our screening system and highlight the regulation of MBNL1 expression by HDAC enzymes.

Small molecule HDAC inhibitors increase MBNL1 levels and reverse the splicing defect caused by (CUG)\(_{exp}\) repeats

We further characterized two of the HDAC inhibitors identified in the pilot screen that increased ZsGreen-MBNL1 signal approximately two-times: vorinostat and ISOX. ISOX is reported to inhibit HDAC6 at low nM concentrations, but also inhibits HDAC1 and other HDACs at high nM concentrations. Vorinostat (suberoylanilide hydroxamic acid, SAHA) is a potent, reversible, pan-HDAC inhibitor (class I and class II HDACs) altering gene transcription and inducing cell cycle arrest and/or apoptosis in a wide variety of cells. Vorinostat was the first HDAC inhibitor approved by the FDA for treatment of cutaneous T cell lymphoma (CTCL) (48). To test whether vorinostat and ISOX can increase endogenous MBNL1 expression in DM1 cells, we treated normal and DM1 patient fibroblasts with vorinostat or ISOX for 2 days, then quantified the MBNL1 level by immunoblot using an antibody against MBNL1 (Fig. 4A). Following normalization to tubulin, both vorinostat and ISOX treatments increased MBNL1 levels in a statistically significant manner as compared with DMSO-treated controls in both normal and DM1 patient fibroblasts (Fig. 4B).

To test if vorinostat and ISOX treatment can rescue the splicing defect caused by DM1 repeats, we characterized SERCA1 exon 22 splicing in ZsGreen-MBNL1 cells transfected with (CUG)\(_{400}\) repeat expressing constructs. ISOX treatment increased the exon 22 inclusion in repeat-expressing cells from 66 to 70%, and vorinostat treatment significantly recovered the

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### Table 1. The top 12 hits from the FDA drug set screen with their EC\(_{50}\) and maximum percentage increase in MBNL1 over a negative control

| Compound Name                     | EC\(_{50}\) (\( \mu \)M) | Maximum Percentage Increase | FDA-approved Therapeutic Area                  |
|-----------------------------------|--------------------------|----------------------------|-----------------------------------------------|
| Mycophenolic acid                 | 1.3                      | 119                        | Antiarthritic drugs                            |
| Chlormethine                      | 1.9                      | 158                        | Lymphoma therapy                               |
| Melphalan                         | 4.0                      | 150                        | Cancer therapy                                 |
| Cytarabine                        | 3.1                      | 189                        | Lymphoma therapy                               |
| Thiotepa                          | 12.2                     | 159                        | Hematologic agents                             |
| Monobenzone                       | 50.0                     | 123                        | Depigmenting agent                             |
| Bleomycin                         | 34.0                     | 137                        | Cancer therapy                                 |
| Chlorambucil                      | 17.0                     | 262                        | Lymphoma therapy                               |
| Floxuridine                       | 0.001                    | 164                        | Cancer therapy                                 |
| Mitomycin                         | 3.4                      | 150                        | Cancer therapy                                 |
| Methylthioninium chloride         | 3.2                      | 120                        | Alzheimer’s dementia treatment                 |
| Triamcinolone Acetonide           | 0.008                    | 71                         | Analgesic drugs                                |

DMSO (0.1%) and ISOX (5 \( \mu \)M) treatments were used as inter-plate negative and positive controls. The percentage increase in ZsGreen-MBNL1 levels were calculated by comparing to 5 \( \mu \)M ISOX treatment (100%) and 0.1% DMSO treatment (0%).
exon 22 inclusion to 78%, which is similar to the percentage of untreated cells without the overexpressed repeats (77%) (Supplementary Material, Fig. S3A and B). Interestingly, a similar splicing pattern shift (77–81% after ISOX treatment and 77–87% after vorinostat treatment) was observed in cells without the transfected repeats (Supplementary Material, Fig. S3A and B). To translate our findings to a more disease relevant model, we characterized the SERCA1 exon 22 and INSR exon 11 splicing in normal and DM1 patient-derived fibroblasts. These DM1 fibroblasts express DMPK transcripts containing ~1000 CUG repeats. In normal fibroblasts, 87% of SERCA1 transcripts had exon 22 while only 58% of SERCA1 transcripts contained this exon in DM1 fibroblasts. Treatment of DM1 fibroblasts with either ISOX or vorinostat increased SERCA1 exon 22 inclusion from 58 to 76% and 74%, respectively (Fig. 5A and B). Likewise, exon 11 was included in 78% of normal fibroblast INSR transcripts versus 47% of DM1 patient fibroblast transcripts. ISOX and vorinostat treatment increased INSR exon 11 inclusions from 47 to 53% and 56%, respectively (Fig. 5A and C). In normal fibroblasts, ISOX and vorinostat treatment increase SERCA1 exon 22 inclusion to more than 96% and INSR exon 11 inclusions to more than 89%. All of the changes were statistically significant (Fig. 5B and C).

This suggests that the compounds ISOX and vorinostat not only increased MBNL1 expression but also partially rescued the splicing pattern that is disrupted by sequestration of MBNL1 at repeat transcripts. These results confirm the utility of our screening system which helps provide confidence in future validation experiments for the targets identified in the CGL and FDA drug set screen.

As ISOX and vorinostat are generic HDAC inhibitors, in addition to increasing MBNL1 expression, they could directly affect SERCA1, INSR and DMPK transcription as well as directly affect toxic RNA nuclear aggregation. To evaluate these possibilities, we compared the steady state SERCA1, INSR and DMPK mRNA level in ISOX or vorinostat treated fibroblasts with control DMSO treated cells by RT-qPCR. In both normal and DM1 fibroblasts, no significant change of SERCA1, INSR and DMPK mRNA was observed after compound treatment with the exception of a small reduction of INSR mRNA in DM1 fibroblasts after ISOX treatment (Supplementary Material, Fig. S4). These data suggest that ISOX and vorinostat had no effect on SERCA1 and DMPK transcription. To test if ISOX and vorinostat changed toxic RNA nuclear aggregation, we quantified (CUG)$_{1000}$ foci in DM1 fibroblast nuclei by FISH staining. ISOX and vorinostat did not
change either the average number or size of foci (Supplementary Material, Fig. S5A and B) while only slightly decreasing the signal intensity at the foci (Supplementary Material, Fig. S5C). Since the DMPK transcript level was not affected (Supplementary Material, Fig. S4C), it is possible that the slight reduction of signal intensity was caused by increasing the overall MBNL1 level in cells thereby blocking the probe accessibility at (CUG)_{exp} foci. These results suggest that ISOX and vorinostat have a very minimal effect on the toxic transcripts and SERCA1/INSR transcription. MBNL1 upregulation is the main mechanism for splicing rescue.

**Discussion**

As a multi-systemic disease with complicated pathological mechanisms, DM is a challenge to treat. The toxic transcripts and MBNL ribonucleoprotein particle (RNP) aggregates are the common phenomenon in various tissues and both subtype of DMs (49–52). The disrupted splicing caused by MBNL1 sequestration plays central role in DM pathology. Most therapeutic strategies have been focused on degrading toxic (CUG)_{exp}-RNA or disrupting (CUG)_{exp}-RNP formation (53–59). So far, the most advanced therapeutic candidate for DM1 has been the antisense oligonucleotide (ASO) targeting the toxic repeat transcripts (55). Preclinically, small molecules have been identified which block the toxic RNA and MBNL protein interaction (60–62). A small molecule modality remains attractive for a number of reasons: safety with limited or no immune response, ease of administration and tissue delivery, CNS penetration and favorable manufacturing cost. MBNL1 upregulation is a valuable independent approach and can potentially be combined with RNA targeting strategies. Previous work from Kanadia et al. showed two-fold increase in MBNL1 levels by AAV-mediated MBNL1 overexpression is sufficient to reverse myotonia in a mouse model of DM1 (43). While preparing this manuscript, a study demonstrated that the non-steroidal anti-inflammatory drug (NSAID) phenylbutazone (PBZ) increased MBNL1 expression 1.3-fold both in vitro and in vivo to partially rescue aberrant splicing and decrease abnormal central nuclei in muscle fibers (63). These data suggest even a small increase of MBNL1 level can be beneficial to DM1 disease phenotypes. A transgenic MBNL1 mouse model using a ubiquitous promoter for constitutively high expression demonstrated that both early and long-term increase of MBNL1 levels are well-tolerated suggesting this therapeutic strategy is viable (44). Theoretically, this approach of MBNL1 up-regulation could provide two additional benefits in that: 1) solely increasing MBNL1 should not disrupt the (CUG)_{exp}-MBNL aggregates which prevents the toxic transcripts from exiting the nucleus for RAN translation; 2) increasing MBNL1 levels could also functionally compensate for the loss of MBNL2 in CNS since MBNL family proteins are very similar in structure and may be functionally interchangeable (30).

To monitor the endogenous MBNL1 expression, we generated a reporter cell line expressing ZsGreen tagged MBNL1 protein from its native locus. The green fluorescent signal from the fusion protein represents the level of endogenous MBNL1 in cells. Importantly, the ZsGreen-MBNL1 fusion protein functions as the wild type MBNL1 in binding to (CUG)_{exp} transcripts and forming nuclear aggregates. Therefore, we envision that this reporter cell line could also be used to monitor the nuclear foci formation and identify compounds or targets that disrupt foci or block repeat binding, or to track MBNL1 localization in cells or to dissect the RNP complex by performing pull-down experiments.

To identify novel MBNL1 modulators, we developed a cell-based phenotypic screen approach using the ZsGreen-MBN1 reporter cells. Phenotypic screens do not rely on prior knowledge of a molecular mechanism and have the potential to uncover previously unknown mechanisms of disease state modulation. A cell-based screen has the advantage of analyzing responses to compound treatment in a more physiologically-relevant system than cell-free system. This reporter cell system showed a low variation in the observed fluorescent signal from the ZsGreen tagged endogenous MBNL1 proteins. Although high-content imaging is also compatible with the fluorescent signal quantification of these reporter cells, flow cytometry quantification provided a quick and sensitive way to reliably measure a modest 30% significant increase in whole cell MBNL1 levels. Miniaturization of the cellular system to a 384-well format was successful with ~90% reproducibility of initial format. Importantly, we confirmed two of the hits (ISOX and vorinostat) increased endogenous MBNL1 levels in DM1 patient-derived fibroblasts and partially rescued the aberrant splicing. The HDAC inhibitors did not change the DMPK transcript level, which could alter the amounts of sequestered MBNL1, nor did these compounds affect the steady state of the SERCA1 and INSR mRNA transcripts. Furthermore, there was no change in the number and size of nuclear foci in compound treated DM1 cells except for a slight decrease of intensity. Collectively, these data suggest these compounds had a minimal effect on toxic RNA aggregates. These results demonstrated that our medium throughput-screening platform is highly reliable with the required sensitivity to detect modest increases in MBNL1 levels that translate to modulating the pathological phenotype of DM1 patient cells.

The identification of several HDAC inhibitors that increased MBNL1 suggests a regulatory role of HDACs in MBNL1 expression. However, we recognize that the observed activity at the effective concentration ~1μM was probably due to inhibition of multiple HDAC family members rather than one sub-type. Furthermore, global inhibition of HDAC activity has a complicated effect on gene transcription which can lead to cell cycle arrest, DNA repair inhibition, apoptosis induction and non-histone protein acetylation; all of these events can lead to downstream alterations in gene expression (64). While we noticed a cell growth inhibition by ISOX and vorinostat in our ZsGreen-MBNL1 reporter HeLa cells, there was a minimal toxic effect on the slower dividing DM1 fibroblasts (data not shown) and the MBNL1 increase was still observed. It is also possible that ISOX and vorinostat may increase MBNL1 level through mechanisms other than inhibiting HDACs.

To reveal additional regulatory pathways, we expanded the screen to two expanded compound libraries (CGL and an FDA drug set) and identified 160 small molecule hits of which 34 compounds increased MBNL1 more than 100%. Although preliminary, the CGL screen identified putative regulative pathways for MBNL1 expression including the kinase family proteins AURKB and CDK2. Interestingly, previous reports have shown that the CDK inhibitors C16 and C51 can reduce nuclear (CUG)_{exp} aggregation and reverse mis-splicing in DM1 (65). In addition, a selective PDE10 inhibitor increased MBNL1 levels close to 2 fold in the ZsGreen reporter cells suggesting this phosphodiesterase may modulate MBNL1 expression. These pilot screens have suggested several novel regulatory mechanisms of MBNL1 expression with the goal of modulating MBNL1 sufficiently to ameliorate DM1 pathology. With the potential of expanding to the larger compound library and/or knockdown screens, it enables future detailed mechanism studies of MBNL1 expression and possible new therapeutic avenues for DM.
Materials and Methods

Generation of CRISPR knock-in constructs

The two CRISPR gRNA sequences (5’-GACGACATGGTAGATTTTAC and 5’-GACGACATTCGGGACACAAA) 11bp upstream and 13bp downstream of MBNL1 start codon were synthesized and cloned into Cas9 nickase vector pZGB-RNG5_D10A (ZGENEBIO) to produce pZGB-D10A-MBNL1-Lg and pZGB-D10A-MBNL1-Rg constructs. pUC19-ZsGreen-MBNL1 knock-in constructs were generated by infusion-cloning 3 fragments together. The ZsGreen sequences were amplified from pZsGreen1-Vector (Clontech, 632473) using PCR primers (forward: 5’-CTGTTAAATCTAAACAGCCCCAGTC CGAAGCC; reverse: 5’-TACATCCAGAACCACCCGGGCGAACCGGAGCG) and CloneAMP HiFi PCR Premix (Clontech, 639298). The genomic sequences spanning translation start codon of human MBNL1 (chr3:152299748–152300851) were synthesized as two DNA fragments (chr3:152299748–152300190 and chr3:152300194–152300851). The three fragments were cloned into pUC19 vector using InFusion HD Cloning Kit (Clontech, 638909) following manufacturer’s instructions. ZsGreen was assembled in-frame with MBNL1 before the ATG start codon and flanked by 454bp upstream and 658bp downstream.

Cell culture and stable cell line generation

All cells were maintained at 37°C with 5% CO₂. HeLa cells (ATCC, CCL-2) were cultured in DMEM growth media (Gibco, 10569-010) with 10% fetal bovine serum (FBS) (Sigma, F2442) and 20 mM HEPES (Gibco, 15630-080). pZGB-D10A-MBNL1-Lg (1μg), pZGB-D10A-MBNL1-Rg (1μg) and pUC19-ZsGreen-MBNL1 (3μg) were co-transfected into 3 x 10⁶ HeLa cells in 6-well plate using Lipofectamine® 2000 Transfection Reagent (Thermo Fisher Scientific, 11668019) as per manufacturer’s instructions. Forty-eight hours post-transfection, cells were expanded and harvested using 0.05% Trypsin (Invitrogen, 25300-054) and growth media. Dissociated cells were resuspended at 3x10⁶ cells/ml in sorting buffer which consisted of DPBS (Invitrogen, 14190-144) with 2% FBS (Invitrogen, 16140-071), 20 μg/ml of gentamicin (Invitrogen, 15710-064), and 2 μg/ml of propidium iodide (Invitrogen, P1304MP). A BD SORP Aria II (Becton Dickinson) and an 85 micron nozzle were used for the cell sorts. Cells were first gated through a forward side scatter gate to exclude aggregates followed by a side scatter select a population of events apart from background and debris. A third gate using the 561 laser followed by a side scatter served a population of events apart from background.

PCR and ddPCR validate ZsGreen integration

Genomic DNAs were isolated from colonized cells using QIAamp blood DNA mini kit (Qiagen, 51104). 100 ng gDNA was used as a template for the PCR amplification with Herculase II Fusion DNA Polymerases (Agilent Genomics, 600675) and the following primers: FZ038, 5’-TCTGCCAGGAAATCAAGGAG; FZ039, 5’-ACACCGTGTAACAAGGGAGT; FZ040, 5’-TACGACCCGGCCCACTTTTT; FZ041, 5’-ACACCGTGTAACAGGACCG; FZ042, 5’-ACGCCGTAGAACTTGGACTC; FZ043, 5’-ACACCGTGTAACAAGGGAGT; FZ044, 5’-TCTGCCAGGAAATCAAGGAG. PCR amplification was performed as follows: 95°C for 30 s; and final extension at 72°C for 30 s. The PCR products were analyzed by electrophoresis with 1% agarose gels.

Small molecule libraries

A total of three compound libraries were generated and screened. The Epigenetic Modulators Library contains 61 small molecules that target a series of epigenetic modulators. The Chemogenomic Library (CGL) consisted of 2753 small molecule pharmacological compounds that cover over 1000 biological targets. The FDA-approved Drug Set consists of 1040 drugs.
Compound treatment

Cells were aliquot into multi-well plate and cultured at 37°C with 5% CO2 overnight. Small molecule compounds were dissolved in DMSO to generate a 1000× stock and then diluted with the growth media to the final concentration. Cells were incubated with the compound containing media for 2 days. DMSO was used as the negative control in each plate. The Epigenetic Modulators Libraries were screened in 96-well plate format at three concentrations (10 μM, 1 μM, and 100 nM). CGL was screened at 1 μM and FDA-Drug Set was screened at 10 μM in 384-well plate format, and each compound was screened in duplicate. ISOX (5 μM) was used as the inter-plate positive control. Secondary validation screens were performed over an 11-point dilution range with a half-log dilution from 10 μM to 100 pM.

Fluorescent flow cytometry analysis of cells

Cells in 384-well plate were suspended in 10% FBS. Cells were pelleted at 300 x g for 5 min, washed with 50 μL DPBS and re-suspended in 10 μL Alignflow beads solution (Life Technologies, A-16501, 25 drop/100 mL DPBS). The liquid handling was performed on a shaker in the dark. Last, the coverslips were transferred quickly to ice. Equal volume of hybridization buffer (4 × SSC Buffer, 2 μg/μL BSA, 40 μM Vanadyl and 20% dextran sulfate) were added into probe mixture, mixed and aliquoted 25 μL to parafilm and overlayed with coverslips. Hybridization was then performed in a pre-warmed light-proof humidified chamber for 2–3 h at 37°C. Following hybridization, cells were washed for 30 min at 37°C in a solution of 40% formamide and 1× SSC, then 30 min at 37°C in 20% formamide and 2× SSC, and 3× 10 min at room temperature in 1× SSC. All the washes were performed on a shaker in the dark. Last, the coverslips were rinsed in 1× PBS/SmM MgCl2 and mounted with ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific, P36962). The fluorescent cell images were acquired by confocal microscopy and analyzed by the software CellProfiler.

Immunostaining

Cells grown on coverslips were washed with 1× PBS, fixed with 4% paraformaldehyde/1× PBS for 15 min and washed in 1× PBS for 3× 5 min each at room temperature. Then blocked in Blocking Buffer (1× PBS/5% Normal Goat Serum/0.3% Triton X-100) for 1 h, and incubated with Mouse anti-MBNL1 (Sigma, M3320, 1:1000) in Antibody Dilution Buffer (1× PBS/5% Normal Goat Serum/2% BSA/0.3% Triton X-100) at 4°C overnight. Antibody was washed off with 3× 5 min 1× PBS washes and incubated in Alexa Fluor594 donkey anti-mouse secondary antibody (Life Technologies, A-21203) diluted in Antibody Dilution Buffer for 1 h. The cells are washed with 1× PBS 3× 5 min and mounted with ProLong Diamond Antifade Mountant with DAPI.

Rt-PCR

Total RNA was prepared from cells with RNeasy Plus Mini Kit (Qiagen, 74136) as per manufacturer’s instructions. The first strand cDNAs were synthesized from 1g total RNA using Oligo(dT)20 primer or random hexamer and SuperScriptIII reverse transcriptase (Invitrogen, 18080-093) following the manufacturer’s protocol. For alternative splicing quantification, 1/20 of cDNA was used as a template for the PCR amplification with Hercule II Fusion DNA Polymerases (Agilent Genomics, 600675) and the following primers: SERCA1_exon22 forward -5’- GCTCATGGTCTCCTAAAGATCTCAC and reverse – 5’.
AGCTCTGCTGAAGATGTGTCAC, INSR_exon11 forward -5’ CCA AAGACAGACTCTTCGAT and reverse 5’ AACATCGCCAAGGGA CCTGC. PCR amplification was performed as follows: 95 °C for 1 min; followed by 30 cycles of 95 °C for 20 s, 55 °C for 20 s, 68 °C for 1 min and final extension at 68 °C for 4 min. The PCR products were analyzed by electrophoresis with 2.2% agarose gels and the density of bands was quantified using ImageJ. To quantify SERCA1, INSR, DMPK mRNA and 18S rRNA, quantitative PCR were performed per manufacturer’s instructions using TaqMan® Gene Expression Assay (ThermoFisher, Hs01092295, Hs00961557, Hs01094329 and Hs99999901).

**Supplementary Material**

Supplementary Material is available at HMG online.

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