Bromodomain inhibitors regulate the *C9ORF72* locus in ALS

Zane Zeier¹; Rustam Esanov¹; Kinsley C. Belle⁵; Claude-Henry Volmar¹; Andrea L. Johnstone¹; Paul Halley¹; Brooke A. DeRosa⁵; Nathalie Khoury¹; Marka van Blitterswijk²; Rosa Rademakers²; Jeffrey Albert³; Shaun P. Brothers¹; Joanne Wu⁴; Derek M. Dykxhoorn⁵; Michael Benatar⁴; Claes Wahlestedt^{1*}

¹Center for Therapeutic Innovation and Department of Psychiatry & Behavioral Sciences, University of Miami Miller School of Medicine, Miami, FL, 33136, USA

²Mayo Clinic, Jacksonville FL

³IntelliSynRD, Montreal Canada

⁴Department of Neurology, University of Miami Miller School of Medicine

⁵John P. Hussman Institute for Human Genomics and the Dr. John T. Macdonald Foundation Department of Human Genetics, University of Miami Miller School of Medicine

*To whom correspondence should be addressed:

Dr. Claes Wahlestedt
Center for Therapeutic Innovation
University of Miami Miller School of Medicine
1501 NW 10th Ave * BRB # 407 (M-860)
Miami, FL 33136, USA
Telephone: +1 305 243 7694
Fax: +1 305 243 2523
E-mail: cwahlestedt@med.miami.edu

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ABSTRACT

A hexanucleotide repeat expansion residing within the *C9ORF72* gene represents the most common known cause of amyotrophic lateral sclerosis (ALS) and places the disease among a growing family of repeat expansion disorders. The presence of RNA foci, repeat-associated translation products, and sequestration of RNA binding proteins suggests that toxic RNA gain-of-function contributes to pathology while *C9ORF72* haploinsufficiency may be an additional pathological factor. One viable therapeutic strategy for treating expansion diseases is the use of small molecule inhibitors of epigenetic modifier proteins to reactivate expanded genetic loci. Indeed, previous studies have established proof of this principle by increasing the drug-induced expression of expanded (and abnormally heterochromatinized) *FMR1*, *FXN* and *C9ORF72* genes in respective patient cells. While epigenetic modifier proteins are increasingly recognized as druggable targets, there have been few screening strategies to address this avenue of drug discovery in the context of expansion diseases. Here we utilize a semi-high-throughput gene expression based screen to identify siRNAs and small molecule inhibitors of epigenetic modifier proteins that regulate *C9ORF72* RNA in patient fibroblasts, lymphocytes and reprogrammed motor neurons. We found that several bromodomain small molecule inhibitors increase the expression of *C9ORF72* mRNA and pre-mRNA without affecting repressive epigenetic signatures of expanded *C9ORF72* alleles. These data suggest that bromodomain inhibition increases the expression of unexpanded *C9ORF72* alleles and may therefore compensate for haploinsufficiency without increasing the production of toxic RNA and protein products, thereby conferring therapeutic value.

2 INTRODUCTION

3 A GGGGCC hexanucleotide repeat expansion located within the non-coding portion of the *C9ORF72* gene
4 was recently identified as the cause of chromosome 9p21-linked ALS and frontotemporal dementia (FTD)^{1,2}. At
5 present, the *C9ORF72* repeat expansion is the most frequently identified cause of familial ALS accounting for
6 an estimated 38% of familial, 6% of apparently sporadic (i.e. ALS in which there is no family history of disease)
7 and >8% of all patients with this devastating disease³. No other significant cause has yet been identified for
8 sporadic ALS. The *C9ORF72* repeat expansion is also the most frequently identified cause of FTD, accounting
9 for ~25% of familial and ~6% of sporadic FTD³. Evidence supports multiple contributors of pathology which
0 include the production of toxic *C9ORF72* RNAs, Repeat Associated Non-ATG Translation (RANT) products
1 and haploinsufficiency due to the reduced *C9ORF72* expression via an epigenetic mechanism^{4,5} (Figure 1).

2
3 It has been proposed that epigenetic alterations contribute to the pathogenesis of several repeat-expansion
4 disorders described to date, including *C9ORF72* related ALS (C9/ALS)⁴⁻⁶. Fragile X Syndrome (FXS), Fragile
5 X associated Tremor/Ataxia Syndrome (FXTAS), and Friedreich's Ataxia (FA) - all intronic-repeat expansion
6 disorders - serve as useful examples. Hypermethylation of the CGG repeat and an upstream CpG island in the
7 *FMR1* promoter, for example, likely contributes to transcriptional silencing of the *FMR1* gene in FXS^{7,8}.
8 Conversely, in FXTAS overexpression of *FMR1* mRNA in premutation carriers is thought to result from
9 hypomethylation of the CGG repeat expansion, which acts in *cis* to create a more open chromatin structure
0 that favors increased transcription⁹. The intronic GAA repeat expansion in FA drives heterochromatin
1 formation over the *FXN* locus resulting in transcriptional repression in a repeat length-dependent manner^{10,11}.

2
3 The evidence that epigenetic perturbations play a role in the pathophysiology of *C9ORF72*-related disease
4 derives, in part, from its analogy to these other intronic repeat expansion disorders and from the observation
5 that expression of the *C9ORF72* gene is reduced. In fact, the levels of all three transcript variants
6 NM_145005.5 (V1), NM_018325.3 (V2), NM_001256054.1 (V3) are reduced, including variant 2 which does
7 not contain the repeat sequence due to alternative transcription start site utilization^{1,4,12}. Furthermore, recent

empirical evidence has shown that expanded *C9ORF72* alleles are associated with repressive epigenetic markers including histone 3 lysine 9 tri-methylation (H3K9me3) and DNA hypermethylation of CpG islands within the promoter and nearby repeat sequence itself^{4,5,13}. Taken together, these observations indicate that the *C9ORF72* expansion event alters the local epigenetic environment such that the rate of transcription from the expanded allele is reduced in patient cells and tissues. Small molecule histone deacetylase (HDAC) inhibitors have been shown to significantly reduce disease phenotype in FA animal models¹⁴⁻¹⁷. This sets a precedent for small molecule epigenetic compounds being potential tools in the treatment of repeat expansion disorders. Moreover, HDAC and DNA methyltransferase (DNMT) inhibitors have been used in cell model systems of C9/ALS and other repeat expansion disorders to reverse anomalous transcription of expanded gene loci^{4,14,15,18-21}, although translation from therapeutic proof-of-concept to clinical trials has been modest²².

There have now been remarkable advancements in developing small molecules that target classes of epigenetic proteins other than HDACs or DNMTs²³. One example is the bromodomain-extra terminal (BET) family of bromodomain proteins. While they lack catalytic activity, BETs bind to acetylated histones and function as epigenetic “reader” proteins²⁴. Novel small molecule BET inhibitors effectively displace BET proteins from acetylated histones and elements of the transcriptional machinery²⁵⁻²⁹; they have shown efficacy in mouse models of inflammation, cancer, viral infection and a variety of other indications²⁵⁻³⁰. Examples include JQ1, GSK525762 (I-BET-762) and I-BET151 which exhibit specificity for the BET proteins, particularly BRD2, BRD3 and BRD4 over other bromodomain-containing proteins and epigenetic enzymes²⁵⁻³⁰. Here, we provide data that support a role for BET proteins in regulating the *C9ORF72* locus in ALS and show that the currently available BRD inhibitors may hold therapeutic value for this disease.

RESULTS

Confirmation of expanded *C9ORF72* alleles in patient-derived cells Primary fibroblasts and immortalized lymphocytes derived from the blood of consenting individuals within our patient population were established under strict IRB approved protocols at the University of Miami Miller School of Medicine. To confirm the

presence of expanded *C9ORF72* alleles in patient cells we conducted fragment analysis of repeat-primed PCR products while repeat length was assessed by southern blot analysis as previously described^{1,2,31,32} (Figure 2A). Southern blot analysis of a fibroblast cell line from patient 38 revealed a single 7.4kb band (~850 repeats), indicative of a homogeneous population of cells harboring a long *C9ORF72* repeat expansion mutation. In two other cell lines (fibroblasts and lymphocytes) from a different patient (14) the repeat length was more heterogeneous. Whereas the majority of fibroblasts from this second patient possess a small repeat of approximately 3kb (~120 repeats) in size, a fraction of the cells had longer repeats as indicated by faint high molecular weight bands. Immortalized lymphocytes from the same patient (14) possess long repeats, with at least four visible bands ranging from 5.0 to 9.5 kb (450-1200 repeats) in size (Figure 2A). These data indicate clonal expansion of primary cells *in vitro*, which vary in repeat size due to somatic instability. Such instability has also been observed by southern blot analysis of post-mortem brain tissues from C9/ALS patients, appearing as a high molecular weight smear³². We utilized two fibroblast cell lines harboring the *C9ORF72* expansion (38, 14) as well as an immortalized lymphocyte line from patient 14. Hypermethylation of the *C9ORF72* promoter was observed in cells derived from patient 14 but not 38, which is consistent with previous studies demonstrating that approximately 30% of C9/ALS patients have hypermethylated *C9ORF72* alleles^{5,33-36}. Controls included fibroblasts or lymphocytes with repeat expansions at other gene loci (FMR1, FXN). To extend our findings to cell types affected by *C9ORF72* pathology, we developed induced pluripotent stem cells (iPSCs) from patient fibroblasts (38) that were subsequently used to generate motor neurons. We confirmed that these iPSC-derived neurons maintained the *C9ORF72* repeat expansion using the repeat primed PCR method (Supplementary figure 9).

Cells-to-Ct epigenetic siRNA library screen identifies BRD3 as an epigenetic regulator of C9ORF72 expression We found patient fibroblasts to be refractory to lipid-based transfection necessitating alternative methods of transfection such as electroporation, which is inherently problematic for high-throughput assays and therefore not amenable to our screening studies. In addition, the low level of *C9ORF72* expression in fibroblasts limits the probability of identifying molecules capable of reducing *C9ORF72* expression even further.

0 Given these limitations we utilized a standard immortalized HEK293 cell line in order to screen our custom
1 epigenetic siRNA library. Expression of *C9ORF72* was assessed using an inventoried *Taqman* (Life
2 Technologies) assay that amplifies all three *C9ORF72* RNA variants normalized to an endogenous control,
3 Cyclophilin A, by the relative expression method ($\Delta\Delta C_t$). The siRNA library comprises 789 individual siRNAs
4 targeting 263 epigenetic modifier genes (3 siRNAs per target) (Supplementary table 2). Utilizing this screening
5 strategy, we identified epigenetic modifiers capable of both up and down regulation of *C9ORF72* expression
6 including BRD3, a BET-bromodomain containing protein as a top hit (Supplementary figure 1). Targeted
7 silencing of BRD3 in C9/ALS patient fibroblasts led to increased levels of all three *C9ORF72* variants. The
8 knockdown of either BRD4 (a bromodomain protein closely related to BRD3) or treatment with a non-specific
9 control siRNA had no statistically significant effect on *C9ORF72* expression levels in C9/ALS fibroblasts
0 (Supplementary figure 2).

1

2 ***A small molecule screen identifies several candidate compounds that increased C9ORF72 expression***
3 ***in C9/ALS patient fibroblasts*** Utilizing the Cells-to-Ct method, we interrogated our custom assembled
4 *University of Miami Center for Therapeutic Innovation (CTI)* epigenetics small molecule library in order to
5 identify those compounds capable of altering *C9ORF72* expression levels in patient fibroblasts (38) that harbor
6 the repeat expansion mutation (Supplementary table 1). Given that many of the epigenetic compounds are
7 anti-cancer drugs and are known to be cytotoxic, we monitored overall expression of the endogenous control
8 Cyclophilin A across the various treatments. Any compounds (48) that were found to significantly reduce the
9 amount of Cyclophilin A expression due, presumably, to elevated cell death were removed from the analysis
0 (Supplementary figure 3). Many of the molecules that increased expression of the endogenous control were
1 HDAC inhibitors, indicating a non-selective effect on global transcription. While it was expected that HDAC
2 inhibitors might also increase *C9ORF72* expression - similar to what has been observed in other expansion
3 diseases^{9,14,15,18,21} - in alignment with our siRNA screen, we found that 9 of the top 12 hits were bromodomain
4 inhibitors (Figure 3, Supplementary table 2). Unlike most HDAC inhibitors, the bromodomain inhibitors
5 increased *C9ORF72* expression without altering expression of the endogenous control gene (Supplementary

figure 3).

While the DNMT inhibitor 5-azadeoxycytidine (5AZA) increased *C9ORF72* expression, it was not a top hit despite several studies demonstrating its ability to reverse gene repression caused by expansion mutations, including *C9ORF72*^{4,19,20}. This was an expected finding since the patient cell line used in the screen (38) does not harbor an allele with a hypermethylated *C9ORF72* promoter. Furthermore, 5AZA is a nucleoside with a unique mechanism of action requiring cell cycle progression during which inhibition of maintenance DNA methylation results in daughter cells with depleted levels of DNA methylation³⁷. Thus it requires protracted periods of drug treatment in order to generate enough daughter cells in which gene rescue can be observed. Since our screen was performed using slow-replicating fibroblasts seeded near confluence and treated for only 48hrs, it is unlikely that de-methylated daughter cells contributed significantly to the aggregate *C9ORF72* expression level.

Notably, we observed that the distribution of *C9ORF72* expression values across the data set was biased toward upregulation with minimal down regulation of *C9ORF72* gene expression in the presence of any compound (Figure 3). This is likely due to the relatively low basal expression of *C9ORF72* in fibroblasts as compared to other cell types¹², such that a further reduction is difficult to achieve. Nevertheless, this afforded us the opportunity to identify compounds that may compensate for haploinsufficiency by increasing *C9ORF72* expression levels.

Assessment of small molecule performance in upregulating expression of C9ORF72 levels Dose response studies were conducted on three bromodomain inhibitors – two commercially available and well characterized inhibitors (IBET-151 and JQ1) and a novel compound developed by Epigenetix (EP72) (Figure 4A). All three of these inhibitors had similar dose response curves when analyzed for increased *C9ORF72* expression levels. To ensure the specificity of this response for *C9ORF72*, dose response curves of IBET-151 were generated for induction of *C9ORF72* expression, as well as, two additional well validated expansion

repeat associated loci - FMR1 and FXN – in the C9/ALS fibroblasts. Only expression of *C9ORF72* was elevated in a dose response manner in these cells, while FMR1 and FXN levels showed no significant change in expression (Figure 4B). However, neither the FMR1 nor the FXN loci contain expanded repeats in these C9/ALS fibroblasts. Therefore, we tested the impact of treatment with IBET-151 on fibroblasts derived from FA and FXS patients bearing the repeats in their respective genes. In both the FA (Figure 4 C) and the FXS fibroblasts (Figure 4 D), expression of *C9ORF72* was elevated in a dose –dependent manner while the BRD inhibitor IBET-151 had no statistically significant effect on the expression levels of *FMR1* or *FXN* in either fibroblasts. These results show that the effect of the BRD inhibitors is specific for the *C9ORF72* repeat rather than a more generalized effect on expanded repeats.

BRD inhibitors increase nascent C9ORF72 pre-mRNA expression levels Since the gene expression studies utilized primer/probe sets that span a *C9ORF72* intron, and therefore detect mature *C9ORF72* mRNA, it is possible that increased expression levels that we observed in response to BRD inhibitors could result from a perturbation of RNA processing such that pools of mutant *C9ORF72* pre-mRNA are freed from repression by an unknown mechanism. This would result in a surge of processed mRNA production independent of increased transcription. Therefore, we tested whether BRD inhibitors increase *C9ORF72* expression through an epigenetic mechanism, requiring new transcription, or if a downstream mechanism such as increased RNA processing efficiency could explain the observed increase in *C9ORF72* expression. To that end, intron-nested primers were designed that anneal near to the *C9ORF72* expansion to detect pre-mRNA and quantitative real-time quantitative PCR (QPCR) analysis was conducted. Efforts were taken to remove potential contamination by genomic DNA, which was monitored using no-reverse transcriptase controls (see methods). We observed a clear and statistically significant increase in *C9ORF72* pre-mRNA expression in both patient fibroblasts and lymphocytes in response to IBET-151 treatment (Figure 5A). These results suggest that the enhanced *C9ORF72* expression level seen upon treatment with BRD inhibitors results from an epigenetically regulated transcription-level mechanism of action.

Cell cycle arrest does not effect C9ORF72 expression Evidence suggests that bromodomain inhibitors may be used as chemotherapeutic agents that block cell cycle progression²⁵ through inhibition of BRD4^{38,39}. Therefore, we sought to determine if increased C9ORF72 expression by IBET-151 is an indirect effect caused by cell cycle arrest or if the effect is independent of cell cycle progression. Using a commonly employed double thymidine block⁴⁰, patient fibroblasts were arrested in the G1-S phase, analogous to arrest induced by BRD4 inhibition³⁹. No significant change in C9ORF72 expression was seen upon blockage of the cell cycle as measured by QPCR, indicating that cell-cycle arrest cannot explain the effects of IBET-151 on C9ORF72 transcription rates (Supplementary figure 4). Importantly, the fact that the positive effect of BRD inhibitors on C9ORF72 expression is independent of cell cycle progression suggests that these inhibitors may be an effective approach to increase C9ORF72 expression in non-dividing, terminally differentiated neurons, a key cell type in ALS pathogenesis.

BRD treatment is well tolerated in patient fibroblasts Since inhibition of BRD4 has been shown to induce apoptosis in immortalized, rapidly-dividing cell lines³⁹ we sought to determine the level of toxicity in patient fibroblasts exposed to increasing doses of IBET-151. An ATP luminescence assay was used to analyze cell viability. Any loss of cell number due to treatment with IBET-151 would lead to decreased levels of ATP in the cultured cells. Linear regression analysis demonstrated a high correlation ($r^2=0.97$) between luminescence and cell number (Supplementary figure 5). The treatment of C9/ALS fibroblasts with increasing concentrations of IBET-151 did not substantially reduce fibroblast viability, with a high concentration (10 μ M) resulting in only a 20% loss of ATP luminescence. The observed resilience of fibroblasts to IBET-151 as compared to cancer cell lines is likely due to their relative quiescent state. We conclude that IBET-151 is well tolerated in fibroblast cultures and likely other non-dividing cell types such as neurons.

BRD inhibitors do not effect C9ORF72 promoter DNA hypermethylation or H3K9me3 levels C9ORF72 expansion mutations are associated with reduced gene expression levels^{1,2,4,12}, multiple repressive chromatin markers⁴ and, in a subset of individuals, hypermethylation of CpG islands within the gene promoter^{5,33}. These

4 studies suggest a cis-effect whereby the expanded allele triggers heterochromatinization and reduced
5 *C9ORF72* expression, while the unexpanded allele is expressed normally. De-repression of the expanded
6 *C9ORF72* allele can be achieved using a nucleoside DNA methyltransferase inhibitor, 5AZA⁴. However, it is
7 likely that such de-repression would exacerbate pathology associated with toxic RNA species produced by the
8 expanded, hypermethylated allele³⁴. Therefore, increasing expression levels from the unexpanded *C9ORF72*
9 allele is a preferable strategy to compensate for the effects of haploinsufficiency. In order to determine if IBET-
0 151 decreases *C9ORF72* promoter DNA methylation in a hypermethylated C9/ALS patient cell line, we utilized
1 a methylation sensitive restriction digest followed by QPCR amplification of the *C9ORF72* upstream CpG
2 island, similar to previously published methods⁵. Validation of this method using commercially available
3 standard controls (see methods) demonstrated accurate assessment of DNA methylation with a correlation
4 coefficient (r^2) value of 0.97 (Supplementary figure 6). While treatment with 5AZA significantly decreased the
5 level of *C9ORF72* promoter DNA methylation in hypermethylated C9/ALS lymphocytes, treatment with IBET-
6 151 did not (Figure 5C). As expected, neither 5AZA nor IBET-151 treatment altered the level of *C9ORF72* DNA
7 methylation in lymphocytes from an unaffected control individual (Figure 5D). These data indicate that BRD
8 inhibitors likely increase transcription rates of the unexpanded allele, without affecting the epigenetic status of
9 the expanded, hypermethylated allele and are thus unlikely to increase toxic RNAs or RANT products.

0
1 DNA methylation is a relatively stable repressive epigenetic modification as opposed to histone methylation,
2 which tends to be more dynamic and reversible. In addition, DNA methylation typically corresponds with
3 silencing of expanded FMR1 alleles⁴¹, although there are rare cases where expression of methylated FMR1
4 alleles occurs⁴² likely due to cryptic DNA methylation mosaicism⁴³. In order to determine if BRD inhibitors de-
5 repress the expanded *C9ORF72* allele in a DNA methylation independent manner, we measured the levels of
6 H3K9me3, a repressive histone methylation marker shown to be associated with expanded *C9ORF72* alleles⁴
7 in C9/ALS lymphocytes. Control experiments demonstrated that anti-H3K9me3 immunoprecipitations were
8 highly enriched for histone H3 - as determined by immunoblot - and genomic DNA encoding the inactive SAT2
9 gene relative to the constitutively active GAPDH housekeeping gene, as determined by QPCR (Supplementary

figure 7). Having optimized the chromatin immunoprecipitation (ChIP) protocol, we examined the *C9ORF72* locus and corroborated earlier observations⁴ that the gene is enriched for H3K9me3 in C9/ALS lymphocytes compared to control unaffected cells. Finally, we assessed *C9ORF72* H3K9me3 enrichment following treatment of patient lymphocytes with IBET-151 and observed no change in histone methylation levels as measured by 3 primer sets annealing near the C9 expansion (C9.A-C) (Figure 5B). Consistent with our analysis of DNA methylation, these data provide further evidence that BRD inhibitors increase expression of the unexpanded *C9ORF72* allele without affecting expanded alleles which are enriched for the repressive H3K9me3 mark.

BRD inhibitors show only modest changes in the global transcriptional profile in human neurons While increasing the expression of *C9ORF72* in order to compensate for haploinsufficiency may be therapeutically relevant, our epigenetic strategy is not locus specific. Thus, we sought to examine what effects bromodomain inhibitors, specifically IBET-151, has on the global cellular transcriptional program in human neurons. We treated HCN-2 human cortical neurons (ATCC), with either IBET-151 (1 μ M) or vehicle control (0.01% DMSO) for 24 hours and conducted a microarray gene expression study. Consistent with previous reports, IBET-151 affected the expression of a relatively small subset of genes rather than inducing global transcriptional dysregulation^{26,28}. Using a Benjamini-Hochberg test to adjust for a liberal false discovery rate of 20% no genes exhibited a significant difference between IBET-151 and vehicle treatment. While using a threshold of $p < 0.05$ and fold change > 2.0 or < -2.0 , there were only 57 genes down-regulated and 21 genes up-regulated in response to IBET-151 treatment (Supplementary table 3). Functional analysis of these genes suggested the majority are involved in transcription and translational regulation, cell structure, signaling, and homeostasis, and inflammatory responses (Supplementary figure 8). Notably, interleukin 6 (IL6), a cytokine whose production is inhibited by IBET-151²⁸, was down-regulated over 4-fold in our analysis with IBET-151 treated human cortical neurons. In contrast, cell cycle genes such as BCL2, which have shown to be down-regulated by IBET-151 treatment in cancer cells²⁸, were not affected in the cortical neurons. This suggests that while some targets of bromodomain inhibitors may be conserved across cell types, others exhibit cell and/or context

specificity.

While the overall effect of BRD inhibition on transcription is not specific to *C9ORF72*, its expression levels were significantly up-regulated ($p=0.041$; fold change 1.3) in our microarray analysis (Supplementary table 4). These findings corroborate our QPCR gene expression studies in patient fibroblasts and lymphocytes using an unbiased method of assessing gene expression. Future studies will need to be performed in order to thoroughly examine the therapeutic efficacy and safety profile of bromodomain inhibitors in animal models of C9/ALS. We remain optimistic that BRD inhibition is a viable therapeutic strategy, as other anti-cancer drugs - which can be highly toxic and alter global transcription profiles *in vitro* - are surprisingly well tolerated *in vivo*.

Bromodomain inhibitors increase C9ORF72 expression in disease relevant, affected cell types We observed that bromodomain inhibitors increased *C9ORF72* expression in two patient fibroblast cell lines (38, 14) with expanded alleles (Figure 2C). Cells from patient 14 harbor hypermethylated alleles at CpG sites within the *C9ORF72* promoter while cells from patient 38 do not. Our data further demonstrate that BRD inhibitors increased expression in unaffected cells including HEK293 cancer cells and primary human cortical neurons. We next sought to determine whether this effect extends to disease relevant cell types. To test this, we generated induced pluripotent stem cells (iPSC) from patient fibroblasts (38) and differentiated them along a motorneuron lineage. The resulting neuronal cultures stained positive for the immature neuronal marker Tuj1, and also the motor neuron marker ISL1, which was localized to the perinucleus (Figure 6A). These neuronal cultures were treated with a set of 13 different small molecule BRD inhibitors. Although the increase in *C9ORF72* expression was variable across treatments, all of the BRD inhibitors led to elevated levels of *C9ORF72* transcripts compared to the control treatment (DMSO) (Figure 6B).

DISCUSSION

In order to identify epigenetic regulators of *C9ORF72* expression, we conducted gene-expression based screens to interrogate custom small molecule and siRNA libraries targeting all major classes of epigenetic

modifier proteins. Our data demonstrate that siRNAs against the BRD3 protein and several small molecule bromodomain inhibitors (including selective BET inhibitors) increase expression of *C9ORF72* in patient and non-affected fibroblasts. In patient cells, *C9ORF72* pre-mRNA and all three mRNA variants were increased without affecting heterochromatin markers of expanded *C9ORF72* alleles, namely H3K9me3 and hypermethylation of CpG islands within the gene promoter^{4,5}. These findings suggest an effect that is specific to unexpanded *C9ORF72* alleles, reducing the likelihood that they can exacerbate pathology associated with accumulation of toxic products from expanded heterochromatinized alleles. In addition, bromodomain inhibitors did not increase expression of expanded FMR1 or FXN alleles in fibroblasts taken from Fragile X syndrome or Freiderich's ataxia patients indicating a *C9ORF72*-specific effect. We conclude that an epigenetic strategy targeting the unexpanded *C9ORF72* allele may be useful for rescuing *C9ORF72* haploinsufficiency. Future studies will determine: 1) if hypermethylation of the promoter or repeat sequence itself blocks the upregulation of expanded alleles and 2) whether BRD inhibitors increase levels of poly-dipeptides or number of RNA foci, two hallmarks of cells that harbor *C9ORF72* expansions.

While toxic RNA gain-of-function is expected to be one primary pathological mechanism underlying C9/ALS, there is substantial evidence to support a role for haploinsufficiency as a contributing factor. First, the *C9ORF72* gene product is related to DENN-like Rab GTPases involved in vesicular trafficking during autophagic processing^{44,45}, a mechanism closely associated with neurodegenerative disease. Second, it has been well established that lymphocytes, iPSC-derived neurons and post mortem cortical tissues from mutation carriers are enriched for heterochromatin markers and have reduced expression rates of *C9ORF72*^{1,2,5,46-49}. Third, motor deficits were observed in a zebrafish model of *C9ORF72* loss-of-function⁴⁷. Finally, studies have shown that cells with the *C9ORF72* expansion produce RAN products^{49,50} and that *C9ORF72* insufficiency renders cells more vulnerable to glutamate toxicity¹² and autophagic stress⁴⁶. Therefore, it is reasonable to hypothesize that a loss of *C9ORF72* function renders cells more vulnerable to toxicity perpetrated by RNA or protein products of the expanded allele itself. In such a scenario, upregulation of the unexpanded *C9ORF72* allele may increase the resilience of cells harboring these mutations. Nevertheless, there is also evidence that

diminishes the putative role of *C9ORF72* haploinsufficiency. For example, homozygous individuals with expansion mutations in both *C9ORF72* alleles, do not present with a more drastic phenotype as would be expected if loss-of-function were a major pathological factor⁵¹. In addition, antisense oligonucleotide-mediated knockdown of *C9ORF72* did not exacerbate toxicity in iPSC-derived neurons but rather abrogated markers of disease¹². The generation of *C9ORF72* knockout mice may shed light on the role of haploinsufficiency but will need to be characterized in the proper disease context such that haploinsufficiency and production of toxic products are concurrent. Taken together, further investigations are needed in order to fully realize the contribution of *C9ORF72* haploinsufficiency in C9/ALS pathology and if therapeutic value can be gained by compensating for the deficit.

Currently there are 47 known bromodomain-containing proteins in humans that typically regulate gene transcription by recognizing acetylated histone lysine residues and recruiting transcriptional and/or chromatin remodeling complexes^{39,52,53}. Efforts to identify small molecule inhibitors of bromodomains have primarily focused on four structurally related BET proteins BRD2, BRD3, BRD4, and BRDT^{24,25}. This subclass of bromodomain proteins are amenable to regulation by small molecules and play critical roles in cellular processes underlying human disease²⁴. The various mechanisms by which BET proteins mediate their gene regulatory function are only beginning to be elucidated⁵⁴. The best studied BET protein, BRD4, regulates transcriptional elongation, binds histone modifiers and nucleosome remodeling enzymes⁵⁵⁻⁵⁸. Others have reported that BRD4 binds directly to several transcription factors and is highly enriched in super-enhancer regions^{59,60}. Genome-wide BRD4 occupancy has been investigated using chromatin immunoprecipitation followed by DNA sequencing revealing that BRD4 associates with active promoters in a context and cell-type specific manner⁶⁰⁻⁶². Interestingly, BRD4 inhibits the DNA damage response which has been linked to the stability of repeat expansion mutations^{63,64}. While the role of BRD4 in oncogenesis has now been investigated for several years, the role of the other BET proteins is much less defined.

The BET inhibitors JQ1²⁵ and IBET-762 (GSK525762)^{26,65} are the two most extensively studied BET inhibitors.

4 JQ1 is a high affinity, selective BET inhibitor that has shown robust activity in a variety of disease models
5 including pulmonary fibrosis, viral infection and several forms of cancer. JQ1 has been safely administered to
6 mice at doses as high as 50 mg/kg BID for 3 months with no overt side effects ⁶⁶ but suffers from high
7 microsomal clearance (151 μ L/min/mg), ⁶⁷ a limiting factor for clinical utility. IBET-762 is also a high affinity and
8 selective BET inhibitor that has shown robust activity in a number of preclinical disease models prompting two
9 phase 1 clinical trials (clinical trials ID: NCT01587703 & NCT01587703). Unlike JQ1, IBET-762 has low
0 microsomal clearance (12 μ L/min/mg), however, it has very low cell permeability, very high efflux but zero
1 measurable brain penetration; limiting its utility in the CNS. Another BET inhibitor RVX-208 has been evaluated
2 for cardiovascular disease in multiple clinical trials sponsored by the Resverologix Corp, but may also have
3 potential for treating neurodegenerative disease. Despite progression to clinical trials there are no known FDA
4 approved BET inhibitors, however, benzodiazepines – particularly Alprazolam (Xanax; BRD4 K_d=2.46 μ M) -
5 have weak BET inhibitor activity ⁶⁸. Clearly the role of BET inhibitors in the nervous system has only recently
6 begun to be investigated. Thus the microarray data reported here confirm the effect of BRD inhibition on
7 C9ORF72 expression and help to define the genome-wide transcriptional response in human neurons.

8

9 Significant progress has been made in developing HDAC inhibitors with the potential to rescue gene
0 expression and ameliorate pathological loss-of-function that arise from repeat expansions ^{22,23,69}. For example,
1 narrow-spectrum HDAC inhibitors such as splitomycin, which specifically inhibits SIRT1, induce gene
2 reactivation and partial reversal of the heterochromatin state of the *FMR1* locus in patient-derived lymphoblasts
3 ¹⁸ as does the DNA methyltransferase inhibitor 5AZA ^{19,20}. Similarly, a specific class of pimelic o-
4 aminobenzamide HDAC inhibitors, analogs of the BML-210 compound, have been shown to achieve gene
5 reactivation in mouse models of FA and to reverse key pathological features ^{14,15,21}. In addition, repressive
6 epigenetic markers associated with expanded *C9ORF72* alleles were reduced by exposure to 5AZA ⁴. While
7 these previous ‘transcriptional therapy’ efforts - primarily focused on HDAC inhibitors - have not yet provided
8 clinical utility for repeat expansion disorders, only recently has the field of epigenetic therapy matured to a point
9 where drug discovery efforts can begin in earnest ²³. The proliferation of epigenetic small molecules is being

driven by the increasing number of epigenetic modifier proteins whose structure, function, and drugability are sufficiently well defined. Our data show that BET proteins may be one - of potentially many - epigenetic targets that could have therapeutic efficacy in repeat expansion disorders. Finally, while our data indicate that BET inhibitors did not affect FMR1 or FXN expression in patient fibroblasts, epigenetic regulation is often cell-type specific. Thus these inhibitors may yet have therapeutic potential for these disorders which should be evaluated in more appropriate cell-types such as reprogrammed neurons from Fragile X and FA patients.

MATERIALS AND METHODS

Collection and maintenance of patient fibroblasts Dermal biopsies were collected from consenting patients using established IRB protocols at the University of Miami. Fibroblasts were dissociated from tissue using collagenase type II then isolated by filtration (70µm) and centrifugation (300rcf). Cells were maintained at 37°C and 5% CO₂ in standard tissue culture treated flasks with *Advanced* DMEM (Life Technologies) supplemented with 15% fetal bovine serum and human fibroblast growth factor.

siRNA library screen Three siRNAs targeting each of the 263 epigenetic modifier proteins represented in the siRNA library were pooled and utilized at a 90nM final concentration (30nM per siRNA). In a final volume of 60uL, 15,000 cells/well were transfected using 0.2% Lipofectamine 2000. Transfection mixes were prepared in OptiMEM and added to Cell-Bind 384-well plates seeded with HEK293 cells using the reverse-transfection strategy. In order to assess transfection efficiency we utilized a siRNA targeting *C9ORF72* and a universal negative control siRNA that has no known target (Ambion). The performance of these experimental controls in the Cells-to-Ct siRNA screen was assessed by *C9ORF72* expression change in wells with the *C9ORF72* siRNA compared to those with the inert negative control siRNA as quantified using two statistical analyses, the student's t-test (p) and z-test (Z). Efficient transfection and assay performance were considered to be acceptable when $p < 0.0001$ and $Z > 0.5$.

Epigenetic library screen We adapted the manufacturer's protocol (Ambion) by miniaturization in order to

utilize the Cells-to-Ct kit for analyzing gene expression in a 384 well format. Patient fibroblasts (15,000 cells in 25 μ L volume) were plated on *Cell-Bind* (Corning) 384 well plates and allowed to adhere and recover overnight. The following day, compound was dissolved in 5 μ L of HBSS and applied in a total volume of 30 μ L at 10 μ M final concentration then incubated for 48hrs. Gene expression was assessed utilizing the Cell-to-Ct kit and inventoried *Taqman*TM assays for *C9ORF72* and the endogenous control, Cyclophilin A. A list of all primers used in the study can be found in Supplementary table 5. Raw data were collected using the 7900HT real-time-PCR machine.

QPCR assessment of *C9ORF72* pre-mRNA Using SYBR green universal master mix (Life Technologies) and primers (Supplementary table 5) that hybridize within the first intron of *C9ORF72* RNA, the unspliced transcript was amplified using RNA purified from 6-well dishes of patient fibroblasts treated with IBET-151 for 48hrs. Trizol (Invitrogen) reagent was used for cell lysis and initial isolation of RNA. Following addition of chloroform and centrifugation, the aqueous phase containing RNA was semi-precipitated by adding 1 volume of 80% ethanol and applied to RNeasy columns (Qiagen) with on-column DNase treatment according to the product instructions. Eluents were treated for a second time with DNase in-solution, and then re-purified with the RNeasy mini elute kit (Qiagen). Reverse transcription (RT) was performed using the Qanta Biosciences Supermix containing both oligo-dT and random priming. Inclusion of no-RT controls in QPCR analysis were included to ensure complete removal of genomic DNA as determined by a lack of detectable amplification after 40 cycles.

Cell cycle arrest Fibroblasts were arrested in the G1 phase using a standard double thymidine block protocol⁴⁰. Briefly, synchronization was achieved by treating cells with 2mM thymidine for 18 hours, then released by media replacement for 9 hours and blocked again by exposure to drug for 17 hours.

Cell viability Patient fibroblasts were seeded in white tissue culture treated 384-well plates (20,000 cells/well) and allowed to recover overnight. The following day, cells were treated with drug diluted in HBSS using a 6-

point dose response protocol with 1:3 serial dilutions of compound starting at 10 μ M maximum final concentration in quadruplicate wells. Final DMSO concentration was maintained at 0.2% for all wells. After 48 hours, cell viability was assessed using an ATP assay (Promega) according to the manufacturer's instructions. Data were collected using a luminescent detection protocol on the *EnVision® Multilabel* Reader (Perkin-Elmer) plate reader platform.

C9ORF72 CpG methylation Methylation of cytosine residues within the *C9ORF72* promoter was assessed using a methylation-sensitive restriction digest and subsequent QPCR amplification protocol similar to a previous report, but with several modifications⁵. Genomic DNA from patient fibroblasts was isolated using the Qiaamp mini kit (Qiagen) according to the kit instructions. Restriction digest was performed on 100ng of DNA using 4 units (4U) of the methylation sensitive Hha1 enzyme (NEB) at 37°C for 90 minutes followed by heat inactivation at 95°C for 10 minutes. For each sample, a control digest excluding the addition of restriction enzyme was performed (0U) (Supplementary figure 6c). Restriction digestion products were analyzed by SYBR green QPCR using two primer sets, one set flanked two Hha1 restriction sites (C9me F/R) and another set nearby, that did not flank any Hha1 restriction sites (C9ec F/R) (Supplementary figure 6a). Normalized relative quantification values (nRQ) were calculated where $dCT = C9me - C9ec$; $ddCT = dCT_{treatment} - dCT_{control}$; $RQ = 2^{-ddCT}$; $nRQ = RQ_{4U} / RQ_{0U}$. Validation of the methylation assessment protocol was performed using pre-mixed calibration standard DNA (EpigenDX Inc.) (Supplementary figure 6b). Bisulfite pyrosequencing of the *C9ORF72* promoter was performed by EpigenDx Inc. using the PSQTM96HS system and custom designed primers developed by EpigenDx (ADS3232-FS1). The assay covers 8 CpG dinucleotides within the promoter and 5' untranslated region of *C9ORF72* from -55 to +19 relative to the transcriptional start site of the human genomic sequence (GRCh38:CM000671.2/Chr9(-))

Chromatin immunoprecipitation Chromatin immunoprecipitation was performed using standard techniques similar to what was previously reported⁴. Briefly, 1 million patient fibroblasts were cross-linked in 1%

formaldehyde for 10 minutes at room temperature, then quenched with glycine (0.125M) for 5 minutes. Cells were washed in PBS containing protease inhibitors and sonicated 3 times for 5 minutes using the Bioruptor UCD200 (Diagenode, Denville NJ) sonication system set to the high setting. Following centrifugation at 12,000 rcf, and one freeze/thaw cycle, lysates were transferred to a tube containing G-protein Dynabeads (Invitrogen) complexed with an anti-H3K9me3 antibody (Abcam ab8898), or control IgG antibody, and allowed to incubate overnight at 4°C with rotation. The following day, stringency washes were performed as follows: 2 times in low salt buffer (0.1% SDS, 1% Triton X, 2mM EDTA, 20mM Tris-HCL pH 8.1, 150mM NaCl), 1 time in high salt buffer (0.1% SDS, 1% TritonX, 2mM EDTA, 20mM Tris-HCL pH 8.1, 500mM NaCl), one time in lithium chloride buffer (0.25M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1mM EDTA, 10mM Tris-HCL pH8.1), and two times in TE buffer (10mM Tris-HCL, pH 8.1, 1mM EDTA). Precipitated complexes were resuspended in 150µL de-crosslinking buffer (1% SDS, 0.1M NaHCO₃, 0.2M NaCl) and incubated at 65°C for 4 hours, followed by the addition of 50µL proteinase K digestion buffer (final concentration: 0.05M Tris-HCL pH 8.1, 0.1M EDTA, proteinase K) and incubated at 42°C for 2 hours. De-crosslinked genomic DNA was isolated using the QiaQuick PCR purification kit according to the manufacturer's instructions.

Microarray analysis Human cortical neurons (HCN-2; ATCC) were cultured under conditions similar to those previously described⁷⁰ in order to promote neuronal maturation. Briefly, six well tissue culture dishes were first prepared by coating with 100 µg/mL poly-D-lysine (Sigma) for 4 hours at room temperature, followed by an overnight coating at 4°C with 5 µg/mL laminin (Cultrex, Trevigen). After washing the plates, HCN-2 neurons were plated at an average density of 160,000 cells/well. The cells were grown at 37°C, 5% CO₂ in a defined, serum-free medium⁷¹ freshly supplemented with 5 nM forskolin (Sigma) as well as 50 ng/mL of each of the following neurotrophic factors: BDNF, GDNF, NGF, and CNTF (Peprotech). Twice per week, half the media volume was replaced with fresh media. After 14 days *in vitro*, the cells were treated with 1 µM IBET-151 (Tocris) or vehicle control (0.01% DMSO). RNA was harvested after 24 hours of treatment using a standard phenol:chloroform extraction. Human Gene ST 1.0 Arrays were performed by the Scripps Genomics and Cell

Based Screening Core (Jupiter, FL) using standard protocols. Probe set intensities were quantified using GeneChip Command Console (AGCC) and analyzed with RMA normalization using Genome Console software (Affymetrix). HCN-2 cells cultured from separate ATCC stocks at different times were used to generate three replicates each for vehicle controls and IBET-151 treated samples. Genes that exhibited a significant response to IBET-151 treatment were identified using a two-tailed t-test ($p < 0.05$) as well as a 2.0 fold change cutoff. Functional annotations were determined using GeneGo Metacore Pathway Analysis and DAVID Functional Annotation Tool.

Generation of induced pluripotent stem cells and neuronal differentiation Primary dermal fibroblasts were reprogrammed using a sendai viral vector system to express the Yamanaka factors according to the manufacturer's recommendations (Cytotune 2.0, Life Technologies). Transduced fibroblasts were seeded onto mouse embryonic fibroblasts and emerging iPSC colonies were subsequently transferred onto feeder-free plates coated with MatrigelTM in mTeSR1TM medium for at least 10 passages. The expression of pluripotency markers was confirmed by RT-PCR (LIN28 and NANOG) and immunocytochemistry using several antibodies (Oct4, Sox2, NANOG, SSEA4, TRA-1-60, TRA-1-81; Cell Signalling, cat. 9656S) according to previously published methods⁴⁶. The iPSCs were then transferred into flasks coated with Poly(2-hydroxyethyl methacrylate) (poly-HEMA, Sigma, cat. P3932) and grown in suspension as neurosphere precursor cells using neural stem cell medium consisting of neurobasal medium (Life technologies, cat. 21103-049) supplemented with 2%B27 with no vitamin A (Life technologies, cat. 12587-010), human fibroblast growth factor (bFGF – 100ng/ul), epidermal growth factor (EGF – 100ng/ul,) and heparin (5ug/ul).⁷² Terminal differentiation of motor neurons was carried out for 4 weeks in neuronal differentiation medium consisting of DMEM/F12 supplemented with 1%N2, 2%B27, 1% Non-essential amino acids, heparin 2µg/ml, 1% antibiotic/antimycotic, 0.1µM Retinoic acid, 1 µM purmorphamine, 1µM cAMP, 200ng/ml ascorbic acid (AA), 10ng/ml glia-derived neurotrophic factor (GDNF) and 10ng/ml brain-derived neurotrophic factor (BDNF).⁷³ on poly-L-ornithine/laminin coated dishes. To confirm efficient motor neuron differentiation cells were fixed with 4%

7 paraformaldehyde and stained with antibodies for Islet-1 (ISL1, Millipore AB4326) and beta III tubulin (TUJ1,
8 Sigma T8578).

9
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FIGURES LEGENDS

Figure 1 – Putative mechanisms of toxicity caused by the GGGGCC repeat expansion in C9/ALS. Epigenetic markers of heterochromatin such as H3K9me3 and in about 30% of cases DNA methylation, are enriched at the expanded allele. These changes are consistent with the observed reduction of transcription rates and evidence of haploinsufficiency. Production of expanded RNA products lead to markers of disease such as RNA foci and repeat associated non-ATG (RAN) translation products. Both the gain-of-function, and loss-of-function, aspects of disease which perturb protein and RNA processing pathways, renders affected cells vulnerable.

Figure 2 – Description and characterization of cells models. A) Southern blot analysis of gDNA from two patients (38 and 14) confirms the presence of both expanded and wild type (wt) alleles in fibroblasts (FB) and lymphocytes (LYM). B) Table of cell lines used for each experiment in the study and the rationale for their use. C) Heat map illustrating the quantification of CpG methylation near the transcriptional start site of C9ORF72 using bisulfite pyrosequencing.

Figure 3 – Epigenetic compound library screen in C9/ALS fibroblasts (38). The Y axis indicates relative quantification (RQ) of C9ORF72 mRNA normalized to an endogenous control gene (Cycophilin A) and scaled to a positive (3% DMSO, EC100) and negative (0.2% DMSO, EC0) control; Z=0.612. Open circles indicate bromodomain inhibitors.

Figure 4 – Compound dose response. Three different BRD inhibitors increase C9ORF72 expression in C9/ALS cells (A). IBET-151 increases C9ORF72 expression but not FMR1 or FXN in C9/ALS (B), FA (C) or FXS patient fibroblasts (D) while neither FMR1 nor FXN are affected in any of the cell lines. Y axis: relative quantification (RQ) of gene expression. X axis: concentration of drug (1:3 dilution series 10 μ M -41nM represented as Log units). Orange traces indicate expression of a gene in cells where that gene is expanded.

Figure 5 – The BRD inhibitor IBET-151 regulates the C9ORF72 locus. A) C9ORF72 pre-mRNA expression is increased ($p<0.001$) by IBET-151 in C9/ALS fibroblasts and lymphocytes from a patient with hypermethylated C9ORF72 allele (14) (N=3, error bars are SEM, $*p<0.05$, $***p<0.001$). B) H3K9me3 immunoprecipitants from C9/ALS lymphocytes (14) were amplified using three C9ORF72 primer sets (A,B,C) and a control (CTL) primer set. Y axis is relative quantification (RQ) calculated by normalizing immunoprecipitants (IP) to the input (IN) and an endogenous control gene (GAPDH). N=3 error bars are SEM. C) DNA methylation sensitive QPCR analysis of the CpG island near the promoter of the C9ORF72 gene. Hypermethylation in a C9/ALS fibroblast line (14) was reduced by exposure to the demethylating positive control compound 5AZA, but not IBET-151 nor a DMSO negative control. D) In contrast, neither 5AZA nor IBET-151 reduced methylation in a non-methylated control cell line. Note the difference in scale where control cells have approximately 100 fold less methylation as compared to the C9/ALS cell line (14), see methods for calculation of RQ. (N=3, error bars are SEM, $*p<0.05$).

Figure 6 – Small molecule bromodomain inhibitors increase C9ORF72 expression in C9/ALS motor neurons A) Immunocytochemistry confirms expression of neuronal markers (ISL, Tuj1) by iPSC-derived C9/ALS motor neurons. B) Y axis is relative quantification (RQ) of C9ORF72 expression to GAPDH, normalized to a DMSO control (error bars are SEM, N=2, $*p<0.05$ student's t-test).

Figure 1

Figure 2

B

Cell line	C9ORF72 promoter status	Experiment	Rational
38 fibroblast	unmethylated	small molecule screen (Fig 3); dose response validation (Fig 4)	adherent patient cells amenable to small scale chemical screens
14 fibroblast	hypermethylated	dose response validation (Fig 4)	hypermethylated patient cells
14 lymphocyte	hypermethylated	ChIP and DNA methylation (Fig 5)	highly proliferative patient cells
HEK293	unmethylated (no expansion)	siRNA screen (Supplementary Fig 1)	scalable and highly transfectable
38 iPSC neurons	undetermined	validation of BRD inhibitors (Fig 6);	neuronal patient cell type

C

Sample ID	CpG 1	CpG 2	CpG 3	CpG 4	CpG 5	CpG 6	CpG 7	CpG 8	Mean
38 fibroblast	2.23	3.00	0.00	2.53	3.55	2.28	7.66	2.18	2.9
14 fibroblast	30.97	32.38	26.58	4.83	24.73	26.06	29.55	30.25	25.7
14 lymphocyte	33.44	39.53	7.35	6.70	46.23	10.90	8.27	2.08	19.3
Low Control	4.2	3.5	2.1	0.0	0.0	0.0	0.0	0.0	1.2
Medium Control	46.9	49.7	44.9	33.2	44.7	36.3	45.8	41.7	42.9
High Control	91.6	93.0	88.0	78.8	90.8	83.6	81.6	86.5	86.7
CpG (From TSS)	-55	-45	-36	-26	-20	2	11	19	

Figure 3

Figure 4

Figure 5

Figure 6