Genome-wide screening for genes involved in the epigenetic basis of fragile X syndrome

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SUMMARY

Fragile X syndrome (FXS), the most prevalent heritable form of intellectual disability, is caused by the transcriptional silencing of the FMR1 gene. The epigenetic factors responsible for FMR1 inactivation are largely unknown. Here, we initially demonstrated the feasibility of FMR1 reactivation by targeting a single epigenetic factor, DNMT1. Next, we established a model system for FMR1 silencing using a construct containing the FXS-related mutation upstream to a reporter gene. This construct was methylated in vitro and introduced into a genome-wide loss-of-function (LOF) library established in haploid human pluripotent stem cells (PSCs), allowing the identification of genes whose functional loss reversed the methylation-induced silencing of the FMR1 reporter. Selected candidate genes were further analyzed in haploid- and FXS-patient-derived PSCs, highlighting the epigenetic and metabolic pathways involved in FMR1 regulation. Our work sheds light on the mechanisms responsible for CGG-expansion-mediated FMR1 inactivation and offers novel targets for therapeutic FMR1 reactivation.

INTRODUCTION

Since the identification of the CGG repeat expansion in the fragile X mental retardation 1 (FMR1) gene as the causative mutation in fragile X syndrome (FXS), many efforts have been invested in deciphering the epigenetic processes that disrupt FMR1 expression in patients’ cells. Although several studies have characterized the heterochromatic configuration of full mutation alleles (>200 CGG repeats) (Epsztejn-Litman and Eiges, 2019; Kumari and Usdin, 2010), the factors involved in causing and maintaining FMR1 heterochromatinization remain elusive. It is assumed that the recruitment of repressive DNA-binding factors by the expanded CGG repeats mediates the DNA hypermethylation and inactivation of the FMR1 locus, similar to other disease-associated repeat expansions that are characterized by the acquisition of abnormal DNA hypermethylation (Colak et al., 2014; Yanovsky-Dagan et al., 2015).

As the FXS-causing mutation is located in the non-coding region of FMR1, understanding and targeting the mechanisms responsible for FMR1 inactivation might have a therapeutic value. The rare existence of individuals with apparent normal intelligence who carry an unmethylated CGG expansion indicates that the expression of full-mutation alleles produces functional protein and can prevent the neurocognitive manifestations of FXS (Smeets et al., 1995).

Human pluripotent stem cell (hPSC)-based models of FXS allow us to study the mechanisms responsible for FMR1 silencing and to explore novel treatments capable of reactivating FMR1 expression (Vershkov and Benvenisty, 2017; Zhou et al., 2016). Compound screening using FXS-patient-derived induced pluripotent stem cells (iPSCs) that harbor a completely silenced FMR1 locus highlighted the importance of DNA methylation in the maintenance of FMR1 silencing and identified several candidate compounds that are able to target FMR1 heterochromatinization (Vershkov et al., 2019).

In this study, we aimed to perform a comprehensive analysis of the genes and pathways regulating FMR1 silencing and to identify novel targets for FMR1 reactivation. Since our compound-screening study identified DNA methylation as a central mechanism for repressing FMR1 expression, we first analyzed the consequences of the targeted perturbation of DNA methyltransferase 1 (DNMT1) in FXS-iPSCs and further characterized its role in the maintenance of FMR1 inactivation. Next, we aimed to utilize the recent advances in CRISPR-Cas9-based genetic-screening technology to search for novel regulators involved in maintaining FMR1 inactivation. While establishing complete loss-of-function (LOF) phenotypes is somewhat challenging in diploid cells that require the establishment of homozygous mutations, the use of haploid cells, which harbor a single set of chromosomes, increases the chances of conducting a comprehensive functional genetic screen (Yilmaz et al., 2016). Therefore, we generated a tractable model system to study methylation-induced FMR1 silencing in haploid human embryonic stem cells (ESCs) and used it to screen for genes involved in FMR1 inactivation.

RESULTS

CRISPR-Cas9-based disruption of DNMT1 in FXS-iPSCs

In order to test the possibility of FMR1 reactivation using gene targeting of a single epigenetic factor and to further
Figure 1. Analysis of DNMT1 disruption in FXS-iPSCs

(A) RT-PCR analysis of FMR1 expression in FXS-iPSCs 7 and 12 days after the delivery of sgRNA targeting DNMT1 and Cas9, compared with treatment with 5-azadC (5 μM, 4 days). Values represent the average ± SEM of 2 independent experiments with 3 technical replicates. (B) Volcano plot showing the median log-fold expression change (x axis) and -log(FDR) (y axis) for each gene in FXS-iPSCs following DNMT1 perturbation (3 independent experiments), compared with empty vector controls (4 independent experiments).

(C) Percentage of upregulated genes (FDR < 0.05, log(FC) > 1) per chromosome following DNMT1 disruption in FXS-iPSCs. ***hypergeometric p < 0.001.

(D) Positional enrichment analysis in gene set enrichment analysis (GSEA) for upregulated genes upon DNMT1 perturbation.

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explore the maintenance of DNA methylation in the FMR1 locus, we sought to analyze the consequences of targeted DNMT1 perturbation in FXS-iPSCs. To overcome the sensitivity of hPSCs to the loss of DNMT1 (Liao et al., 2015), we transduced FXS-iPSCs with a lentiviral vector containing Cas9 and single guide RNA (sgRNA) targeting DNMT1 and collected the culture following a short selection period. This way, despite the apparent cell death following lentiviral transduction, we were able to collect viable cultures for gene expression analysis. RT-PCR analysis of the mutated samples demonstrated the reactivation of FMR1 expression following DNMT1 disruption to levels comparable with 5'-aza-2'-deoxycytidine (5-azadC) treatment (Figure 1A).

Analysis of an ESC line with a normal range of CGG repeats did not show any effect of DNMT1 disruption on FMR1 expression, validating the specific effect of DNMT1 targeting on full-mutation alleles (Figure S1A). The transcriptional activity of FMR1 in FXS-iPSCs upon DNMT1 perturbation was accompanied by a significant decrease in DNA-methylation levels in the FMR1 promoter (Figure S1B).

Next, we performed a global transcriptional analysis of the DNMT1 mutants and the control FXS-iPSC samples, which identified FMR1 as one of the top significantly upregulated genes following DNMT1 mutagenesis (Figure 1B, false discovery rate [FDR] < 0.001; Table S1). Analysis of the global transcriptional response following DNMT1 perturbation revealed a striking enrichment of genes located in the regions adjacent to the FMR1 locus and around the distal end of the long (q) arm of the X chromosome (Xq27-28), as well as several other loci on the X chromosome and a single region on the Y chromosome (Figures 1C and 1D). The enrichment of X-chromosome genes in the transcriptional response to DNMT1 loss was not explained by the relative abundance of CpG islands in this chromosome, as analyzed by the CpG-island annotation from the UCSC genome browser (CpgIslandExt) (Figure S1C).

Tissue expression analysis of the top upregulated Xq27-28 genes (Figures 1E and 1F, FDR < 0.05), as well as of all genes in the fragile-X-adjacent region (Figure S1D, X chromosome 140–148 Mb), revealed a cluster of testis-specific expressed genes that are mostly silenced in normal hPSCs. Analysis of the genome-wide transcriptional response following DNMT1 disruption also revealed a significant enrichment of testis-specific expressed genes, which were associated with Gene Ontology (GO) terms such as gonadal development, male gamete generation, and oligospermia, without an associated induction of the other three embryonic germ-layer marker genes (Figures 1F, 1G, S2A, and S2B; Table S2). The activation of germ cell genes upon DNA demethylation is in line with the global erasure of DNA methylation during normal development of primordial germ cells (Guo et al., 2015), suggesting a role for the DNA-demethylation process in the activation of germ cell differentiation. The association between Xq27-28 testis-specific gene induction and FMR1 activation might have specific implications on FMR1 regulation, which was previously linked with testicular differentiation (Reyniers et al., 1993; Bakker et al., 2000).

Establishment of a screening protocol for the identification of genes involved in FMR1 silencing

To search for novel regulators involved in maintaining FMR1 inactivation, we next aimed to conduct a LOF genetic screen using a genome-wide CRISPR-Cas9 library established in haploid hESCs. Since haploid hESCs harbor a standard range of CGG repeats and actively express FMR1, we sought to generate a tractable model system to analyze FMR1 regulation in hPSCs without an endogenous CGG expansion. For this aim, we used a reporter plasmid in which the enhanced green fluorescent protein (EGFP) was placed under the control of a human FMR1 minimal promoter (pFMR1), continued by the FMR1 5’ UTR sequence, starting from 112 bp upstream to the CGG repeats to 68 bp downstream the repetitive sequence (Solvsten and Nielsen, 2011). The continuous FMR1 promoter and 5’ UTR fragment with 240 CGG repeats were positioned upstream to the rabbit β-globin intron II followed by the EGFP reporter in a similar position concerning the mutant CGG repetitive tract as the coding sequence of the endogenous FMR1 gene in FXS patients (Figure 2A).

To avoid the effect of transgene-integration-site variability, we tested the expression of the pFMR1-CGG-EGFP construct using transient transfection in haploid hESCs (Figure 2A). As expected, the occurrence of a full-mutation length (n = 240) CGG repeat tract per se was not sufficient for the transcriptional inactivation of EGFP expression (Figure S3A). This observation is in line with previous studies showing that unmethylated CGG expansions are expressed in FXS ESCs without acquiring de novo DNA hypermethylation (Vershkov and Benvenisty, 2017).

(E) Heatmap of expression levels (Z score transcript per million [TPM]) across tissues (data from the GTEx study) of the significantly upregulated Xq27-28 genes (FDR < 0.05) in FXS-iPSCs following DNMT1 disruption.
(F) Enriched GO and Human Phenotype Ontology terms (analyzed using GSEA, FDR q < 0.05) among the upregulated genes in FXS-iPSCs following DNMT1 disruption.
(G) Activation of testis-specific marker genes involved in transcriptional regulation upon DNMT1 disruption (mean ± SEM values, n = 3 DNMT1 perturbation, n = 4 control FXS-iPSCs).
Figure 2. Establishment of a screening protocol for the identification of genes involved in FMR1 silencing

(A) Schematic illustration depicting the LOF genetic screening experimental setup. Haploid hESCs transduced with the lentiviral CRISPR-Cas9 sgRNA library were transfected with the methylated pFMR1-(240)CGG-EGFP construct in 4 independent replicates. The least (~30%) and most (3%–4%) GFP-fluorescent cells were sorted 48 h following transfection, and DNA sequencing of the sgRNA segment was used to analyze the distribution of sgRNAs within the GFP(+) and GFP(-) populations.

(B) DNA methylation analysis of the pFMR1-(240)CGG-EGFP construct using the McrBC restriction enzyme, which cleaves DNA containing methylcytosine on one or both strands. From left to right: 1 – ladder, 2 – control plasmid with one McrBC site, 3 – unmethylated construct, and 4 – methylated construct.

(C) Pyrosequencing analysis of DNA methylation of the FMR1 promoter sequence (in 11 CpG positions) in the methylated pFMR1-CGG(240)-EGFP plasmid, and the corresponding genomic CpG positions in FXS- and WT-iPSCs (positions (-456) to (-409) from the start site of FMR1 translation).

(D and E) Transient transfection of haploid human ESCs with either an unmethylated (bottom middle panel) or methylated (bottom right panel) construct, followed by flow cytometry analysis 48 h post transfection.
Therefore, to induce the epigenetic repression of the pFMR1-(240)CGG-EGFP construct, we used *in vitro* methylation using the recombinant CpG methyltransferase M.SssI. DNA methylation of the construct was validated by its digestion with the methylation-sensitive McrBC restriction enzyme and by bisulfite-pyrosequencing analysis (Figures 2B and 2C). *In vitro* methylation using M.SssI efficiently silenced pFMR1-(240)CGG-EGFP expression following transient transfection, with >10-fold enrichment of the GFP-positive cell fraction between the cultures transfected with unmethylated and methylated constructs (Figures 2D and 2E). Interestingly, pre-treatment with the demethylating agent 5-azadC was associated with higher GFP fluorescence upon transfection with the methylated construct, suggesting that the depletion of DNA-methylation machinery interferes with the epigenetic silencing of the reporter plasmid (Figure S3B).

**Using an LOF genome-wide library to screen for genes involved in FMR1 inactivation**

Next, we applied our assay to the CRISPR-Cas9 haploid hESCs library, which contains 178,896 different gRNA constructs, targeting 18,166 genes (Yilmaz et al., 2018). Forty-eight hours following transfection with the methylated pFMR1-(240)CGG-EGFP construct, library cells were harvested and sorted to GFP(+) and GFP(-) populations (Figure 2A). The abundance of different gRNAs represented in both populations was assessed by the amplification of the sgRNA-containing genomic DNA segment and high-throughput sequencing. Following the mapping of the reads to the sgRNA sequences, an enrichment score was assigned to each gene by calculating the log2 fold change (FC) of its sgRNA counts between GFP(+) and GFP(-) populations (n = 4 and n = 3) to allow for the identification of genes predicted to be involved in silencing the expression of the methylated pFMR1-(240)CGG-EGFP.

Analysis of the significantly enriched genes revealed several functionally related gene groups (Figures 3A–3C; Tables S3 and S4): First, a subset of the candidate genes was related to chromatin regulation and transcriptional repression, identified either by the Epifactors database (Medvedeva et al., 2015), as being listed in databases of transcription factors or as associated with chromatin-related GO annotations (Lambert et al., 2018). Functional-annotation analysis revealed a significant association of the top enriched genes with GO terms related to chromatin regulation (Figure 3C; Table S2), and a canonical-pathway analysis demonstrated a significant association with the reactome of RNA polymerase II (FDR <0.0001). Specifically, genes included in the Epifactor database were significantly enriched in the candidate list compared with their representation in the library (7.6% versus 3.8%, p value = 0.02 using Fisher’s exact test; Figure 3D). Interestingly, another subset of the enriched genes was related to several metabolic pathways, specifically the mitochondrial respiration pathway, including four different subunits that assemble the succinate dehydrogenase complex (Figures 3E and 3F). Finally, some enriched genes were categorized as growth-restricting genes in hPSCs (Figure 3A) (Yilmaz et al., 2018). Although cell-cycle-related genes may influence the epigenetic landscape, these genes might be overrepresented in the screen because their disruption confers a growth advantage under selection pressure. Other enriched categories might be related to confounding factors as transfection efficiency (e.g., the internalization and intracellular trafficking of the plasmid DNA). To filter out genes that were overrepresented due to selection pressure, we excluded genes identified as growth restricting in hESCs (FDR <0.05, CRISPR score >1). This led to the establishment of a candidate list of 155 genes predicted to be involved in maintaining gene repression, 28 of which...
were previously shown to have a role in chromatin regulation (Figure 3E; Table S1). Among these genes were transcriptional co-repressors (e.g., ZNF217, ZFP90, CTBP2), chromatin remodeling factors (e.g., SMARCD1), and RNA polymerase II transcription-initiation factors (e.g., TAF8) (Figure 3G). The enrichment of both epigenetic and mitochondrial factors among the GFP-positive population was not correlated with their association with growth restriction in a previous essentiality screen in haploid hPSCs (Figures 3E and 3F) (Yilmaz et al., 2018).

Characterization of genes predicted to be involved in FMR1 epigenetic silencing

Next, we aimed to validate the effect of disrupting selected candidate genes on the expression of the methylated pFMR1-(240)CGG-EGFP construct. Using transduction with CRISPR-Cas9 and two sgRNAs per gene, the selected genes were mutated in haploid hESCs. Mutant cultures were then transfected with both unmethylated and methylated pFMR1-(240)CGG-EGFP constructs, and the GFP fluorescent population was compared between the two cultures (Figure 4A). This way, we could isolate the epigenetic effect of candidate-gene disruption and exclude confounding factors as transfection efficiency or translational control. Haploid hESCs infected with lentiviral vectors targeting the transcriptional regulators SMARCD1 and ZNF217, as well as the metabolic factor C6orf57, showed the highest levels of methylated-construct expression compared with control haploid hESCs, demonstrating a significant increase in relative GFP fluorescence upon methylated-construct transfection, reflecting the disinhibition of DNA-methylation-mediated silencing (Figure 4B).

Finally, as transiently transfected promoters might not be subjected to the same regulatory mechanisms that operate on the endogenous FMR1 promoter, we infected FXS-iPSCs with lentiviral constructs containing Cas9 and sgRNAs targeting the candidate genes. RT-PCR analysis of the mutated samples revealed some increase above basal FMR1 transcription levels following the disruption of SMARCD1, ZNF217, and C6orf57 (Figure 4C). However, the effect was lower than the reactivation effects observed with DNMT1 perturbation or demethylating treatment. Although none of the mutated samples reached the FMR1 expression levels associated with DNMT1 perturbation, the relative increase in FMR1 mRNA in the mutant samples suggests an interaction of these genes with the endogenously silenced FMR1 locus. DNA-methylation analysis of the FMR1 promoter in the mutated samples identified an overall decrease in DNA methylation in the C6orf57 mutant, but it did not reach statistical significance (p = 0.09; Figure S3C). This could reflect the lower sensitivity of the pyrosequencing assay in a heterogeneous population. To further characterize the regulatory effect of the identified genes, we performed a global transcriptional analysis of the ZNF217- and SMARCD1-mutated samples. Upon comparison of up- and downregulated genes (p < 0.001 and |FC| > 1), the mutated samples showed a bias toward a positive effect on gene expression, supporting the repressive function of these genes (Figure 4D). Analysis of the enriched gene sets among the upregulated genes following SMARCD1 or ZNF217 disruption identified overlapping regulators of both gene groups, pointing to common regulatory pathways for these target genes (Figure 4E).

DISCUSSION

In this study, we have used genome-wide CRISPR-Cas9 screening to identify genes involved in FMR1 inactivation. The characterization of the transcriptional outcomes of DNMT1 disruption in FXS-iPSCs revealed FMR1 as one of the most upregulated genes in the mutated samples and the FMR1-adjacent genomic region as the most enriched locus in the transcriptional response to the loss of DNMT1. The ability of DNMT1 disruption alone to fully reproduce the effect size of the demethylating agent 5-azadC, which binds the DNA methyltransferases DNMT1, DNMT3A, and DNMT3B (Oka et al., 2005), shows that the reactivating effect of this compound is mediated primarily through DNMT1, with a lesser or no role for DNMT3A and DNMT3B in maintaining FMR1 inactivation in iPSCs. As the cytotoxic effect of 5-azadC was previously shown to be mediated primarily by DNMT3A and DNMT3B (Oka et al., 2005), this finding calls for the use of selective DNMT1 inhibitors that will be able to provide robust FMR1 reactivation with reduced toxicity compared with 5-azadC.

The substantial overexpression of genes located in Xq27-28 following DNMT1 disruption reflects the inherent epigenetic features of this chromosomal region, which appears to be tightly regulated by cellular DNA-methylation levels. The association of FMR1 activation with the induction of the adjacent testis-specific genes is of particular interest: first, FMR1 itself is known to be particularly highly expressed in both adult and fetal testis (Bakker et al., 2000). Second, in carriers of full-mutation FMR1 alleles, the CGG repeats become unstable during spermatogenesis, leading to active, pre-mutation-length FMR1 alleles in the sperm of fragile X patients (Reyniers et al., 1993). The co-activation of the expanded FMR1 locus and the adjacent male gamete-specific expression program might thus point to the link between male gamete differentiation and FMR1 epigenetic regulation. The activation of testis-specific regions during spermatogenesis may be associated with the recruitment of transcriptional activators and chromatin remodeling enzymes, therefore altering the epigenetic landscape of the Xq27-28 chromosomal region. These results
call for the analysis of the epigenetic status of full-mutation alleles during spermatogenesis.

As DNMT1 disruption resulted in only partial reactivation of FMR1 expression, to levels comparable with 5-azadC treatment, we turned to search for additional mechanisms that contribute to FMR1 inactivation. For this aim, we developed a genetic screening platform that provides a traceable readout of the transcriptional output.

Figure 4. Verification of candidate hit genes predicted to be involved in FMR1 inactivation

(A) Experimental workflow. Mutant haploid hESCs were transfected with either a methylated or unmethylated pFMR1-(240)CGG-GFP construct and analyzed by flow cytometry.

(B) Analysis of the mutants of candidate genes reveals higher relative GFP fluorescence following transfection with methylated pFMR1-(240)CGG-GFP, relative to samples infected with Cas9 without sgRNA. Bars indicate the normalized ratio of GFP fluorescence between the cell cultures transfected with methylated and unmethylated constructs. Data are presented as mean values ± SEM, and statistical tests were performed with 3 independent experiments.

(C) Analysis of heterogeneous mutant populations of FXS-iPSCs for candidate genes SMARCD1, C6orf57, ZNF217, ZFP90, SATB2, QRICH1, and TAF8. Statistical tests were performed from at least 3 independent experiments. Error bars represent SEM.

(D) Bar plot showing the number of the significantly up- (blue) and downregulated (red) genes for the DNMT1, SMARCD1, and ZNF217 samples (p < 0.001, |log FC| > 1).

(E) Enriched gene sets among the genes upregulated following either SMARCD1 or ZNF217 disruption.

(F) Schematic representation summarizing a proposed model of FMR1 epigenetic regulation following CGG repeat expansion. *p < 0.05.
of an exogenous methylated FMR1 promoter followed by an expanded CGG repeat tract. This platform allowed us to utilize haploid hPSCs for the study of FMR1 silencing, although these cells harbor a normal range of CGG repeats.

Transient transfection of reporter plasmids was previously used for studying the relationship between CGG repeat length and the regulation of gene expression (Solvsten and Nielsen, 2011). As exogenous plasmid DNA is assembled into nucleosome structures and becomes associated with the transcription machinery (Smith and Hager, 1997), this system allows for the analysis of an interplay of different layers of transcriptional regulation that might be relevant to FXS pathophysiology. Although the transcriptional regulation of episomal vectors is different than their endogenous counterparts, this system presented several important advantages: first, since DNA methylation of CpG sites is known to be affected by the methylation status of adjacent sequences, transient transfection allows us to avoid the locus-specific effects of random genomic integration. Second, transient vectors rarely carry mammalian replication origins, which allowed us to test the epigenetic regulation of FMR1 independently of cell replication, which is of interest because neurons, the disease-relevant cell type in FXS, do not proliferate.

While transfection with a plasmid containing full-mutation-length CGG repeats was not sufficient to repress EGFP expression, in vitro methylation of the construct efficiently silenced the reporter gene, establishing a clear and traceable phenotype suitable for large-scale screening. Using our screen, we established a list of 155 candidate genes potentially important for FMR1 regulation. As expected, among the enriched genes, there was a substantial fraction of epigenetic regulators. ZNF217, an enriched gene selected for further validation, is a member of the LSD-CoREST complex, which is known to repress the expression from promoters by the recruitment of C-terminal binding proteins (CtBP1), one of which was also enriched in our screen (CTBP2). SMARCD1, another candidate gene, is a subunit of the SWI/SNF complex, which is involved in transcriptional regulation by chromatin remodeling. In hESCs, SMARCD1 knockdown resulted in chromatin de-condensation with reduced heterochromatin foci (Alajem et al., 2015). Moreover, SMARCD1 knockdown led to an aberrant gene expression profile, including failure in silencing the pluripotency network upon differentiation. We also note that one of the enriched transcriptional regulators, ZBTB14, was previously found to repress the transcription of CGG-repeat-containing elements (Orlov et al., 2007).

Besides the enrichment of epigenetic factors, analysis of the candidate genes list revealed enrichment of several metabolic pathways. This observation may be explained by the well-known influence of metabolic enzymes on the epigenome, mainly by catalyzing the production and degradation of metabolites that function as substrates, cofactors, or inhibitors of chromatin-modifying enzymes (Kaelin and McKnight, 2013). C6orf57 plays a role in the assembly of succinate dehydrogenase (SDH) complex. Besides C6orf57, the SDH complex subunits SDHC, SDHA, and SDHAF2 were also found to be enriched in our screen. Inactivating SDH mutations were observed in several types of tumors, leading to succinate accumulation and inhibition of 2-oxoglutarate-dependent dioxygenases that are responsible for histone demethylation (Cervera et al., 2009). That SDH disruption led to enhanced expression of the methylated FMR1 promoter construct points to the importance of epigenetic regulation by this metabolic pathway and may reflect its differential influence on both histone and DNA methylation.

Overall, our results suggest a complex model of epigenetic regulation that merges different levels of cellular organization: factors involved directly in transcription regulation and nucleosome assembly but also non-nuclear factors that determine cellular metabolism (Figure 4F). This model suggests that the FMR1 locus regulation might be tightly linked to developmental time point, cell identity, and metabolic state. In addition, the increased sensitivity of Xq27-28 genes to DNMT1 disruption highlights the contribution of regional and cell-type-specific epigenetic features in FXS pathogenesis.

The analysis of the consequences of candidate-gene disruption on the methylated pFMR1-(240)CGG-EGFP expression demonstrated the disinhibition of the methylated construct in selected mutants. Targeting of SMARCD1, ZNF217, and C6orf57 in FXS-iPSCs was associated with some increase above the basal levels of FMR1 expression, suggesting the interaction of these genes with the inactive FMR1 locus. The fact that disruption of no single factor besides DNMT1 resulted in robust FMR1 expression and that even DNMT1 mutagenesis induced only partial levels of FMR1 mRNA may reflect the robustness of endogenous FMR1 silencing compared with the exogenous construct, possibly due to functional redundancy of different repressive mechanisms. It is possible that two or more factors should be targeted to achieve robust FMR1 expression or that the screen was not fully comprehensive in mimicking the physiological context of FMR1 inactivation.

Collectively, this work demonstrates the utility of LOF genetic screening in the study of genetic and epigenetic diseases. We use a system of an exogenous pFMR1-(240)CGG-EGFP reporter as a model for FMR1 silencing and highlight gene networks associated with transcriptional regulation of FMR1 expression. Our results prompt further investigation regarding the involvement of epigenetic and metabolic factors in the induction and maintenance of FMR1 inactivation and present novel targets for FMR1 reactivating therapy.
EXPERIMENTAL PROCEDURES

Cell lines
Throughout the study, we used the following cell lines: the FXS-iPSCs male cell line AS2 (Vershkov et al., 2019) and haploid hPESCs - hPES10 (Sagi et al., 2016). CSES7 and CSES9 hESCs and their derivatives were used as a reference for wild-type expression (Biancotti et al., 2010). 293T cells from R. Weinberg (Whitehead Institute) were used for the construction of lentiviral constructs.

In vitro DNA methylation
The pFMR1-n(CGG)-EGFP construct (Sølvsten and Nielsen, 2011) was methylated using an M.SssI CpG methyltransferase (NEB) according to the manufacturer's recommendations. The S-adenosylmethionine (SAM) concentration was adjusted to 640 μM. DNA was extracted using ethanol precipitation.

CRISPR-Cas9 LOF library
For the genome-wide screen, we used a CRISPR-Cas9-based genome-wide LOF library of haploid hESCs previously established in our lab (Yilmaz et al., 2018). Briefly, haploid-enriched ESCs cultures of the hPES10 cell line were infected with a lentivirus CRISPR-Cas9 genome-wide library at a multiplicity of infection of 0.3. Infected cells were then selected with puromycin (Sigma) and cultured for about fifteen doublings before harvesting and freezing. The library was then thawed and cultured at 37°C with 5% CO2 in feeder-free conditions using matrigel-coated plates (Corning) and mTeSR1 medium (STEMCELL Technologies) supplemented with 10 μM ROCK inhibitor (Y27632, Stemgent) for 1 day after thawing or splitting.

Library transfection
Ten μM ROCK inhibitor (Y27632) was added 1 h prior to trypsini-zation and transfection. Library cells were harvested using TrypLE Select Enzyme solution (Thermo Fisher Scientific, cat no. 12563029) into a single-cell state. Following the first centrifugation, cells were re-suspended twice with DMEM/F12 and centrifuged again before incubation with the plasmid DNA. Transfection of the methylated pFMR1-CGG(240)-EGFP construct was performed using the Xtreme Gene9 reagent (Roche) using standard conditions. Forty-eight h following transfection, cells were washed with PBS, harvested using TrypLE Select, re-suspended in PBS supplemented with 15% fetal bovine serum (FBS), filtered through a 70 μm cell strainer (Corning), and sorted using BD FACDAria III to GFP(+) and GFP(-) populations. Transfection efficiency was assessed using the simultaneous transfection of control haploid hESCs with an unmethylated pFMR1-CGG(240)-EGFP construct.

Data and code availability
The accession number for the CRISPR-Cas9 library sequencing data reported in this paper is in GEO: GSE182391. The accession number for the RNA sequencing (RNA-seq) data reported in this paper is in GEO: GSE182851. The accession number for the CRISPR-Cas9 library sequencing data reported in this paper is in GEO: GSE182391.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2022.03.011.

AUTHOR CONTRIBUTIONS
D.V. and N.B. designed the experiments and interpreted the data. D.V. performed the experiments with a significant contribution from O.Y. and analyzed the data. A.L.N. provided the pFMR1-(240)CGG-EGFP plasmid. A.Y. assisted with the establishment of the LOF library in haploid hESCs. D.V. and N.B. wrote the manuscript with input from all other authors. N.B. supervised the study.

CONFLICT OF INTERESTS
N.B. is CSO of NewStem, Ltd.

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