APPLICATION OF CRISPR-CAS SYSTEM TO GENE REGULATION

Rescue of Fragile X Syndrome Neurons by DNA Methylation Editing of the *FMR1* Gene

X. Shawn Liu,[1] Hao Wu,[1,3] Marine Krzisch,[1] Xuebing Wu,[1] John Graef,[3] Julien Muffat,[1] Denes Hnisz,[1] Charles H. Li,[1,2] Bingbing Yuan,[1] Chuanyun Xu,[1,5] Yun Li,[1] Dan Vershkov,[4] Angela Cacace,[3] Richard A. Young,[1,2] and Rudolf Jaenisch[1,2,6,*]

[1]Whitehead Institute for Biomedical Research, Cambridge, MA 02142, USA

[2]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142, USA

[3]Fulcrum Therapeutics, One Kendall Square, Binney Street b7102, Cambridge, MA 02139, USA

[4]The Hebrew University of Jerusalem, Edmond J. Safra Campus, Givat Ram, Jerusalem 91904, Israel

[5]Present address: Department of Biology, Stanford University, Stanford, CA 94305, USA

[6]Lead Contact

*Correspondence: jaenisch@wi.mit.edu

https://doi.org/10.1016/j.cell.2018.01.012

SUMMARY

Fragile X syndrome (FXS), the most common genetic form of intellectual disability in males, is caused by silencing of the *FMR1* gene associated with hypermethylation of the CGG expansion mutation in the 5′ UTR of *FMR1* in FXS patients. Here, we applied recently developed DNA methylation editing tools to reverse this hypermethylation event. Targeted demethylation of the CGG expansion by dCas9-Tet1/single guide RNA (sgRNA) switched the heterochromatin status of the upstream *FMR1* promoter to an active chromatin state, restoring a persistent expression of *FMR1* in FXS iPSCs. Neurons derived from methylation-edited FXS iPSCs rescued the electrophysiological abnormalities and restored a wild-type phenotype upon the mutant neurons. *FMR1* expression in edited neurons was maintained *in vivo* after engrafting into the mouse brain. Finally, demethylation of the CGG repeats in post-mitotic FXS neurons also reactivated *FMR1*. Our data establish that demethylation of the CGG expansion is sufficient for *FMR1* reactivation, suggesting potential therapeutic strategies for FXS.

BACKGROUND:
Fragile X syndrome
FMR1 5'UTR hypermethylation

DNA editing to reverse hypermethylation

dCas9-TET1 induces active chromatin at FMR1 promoter

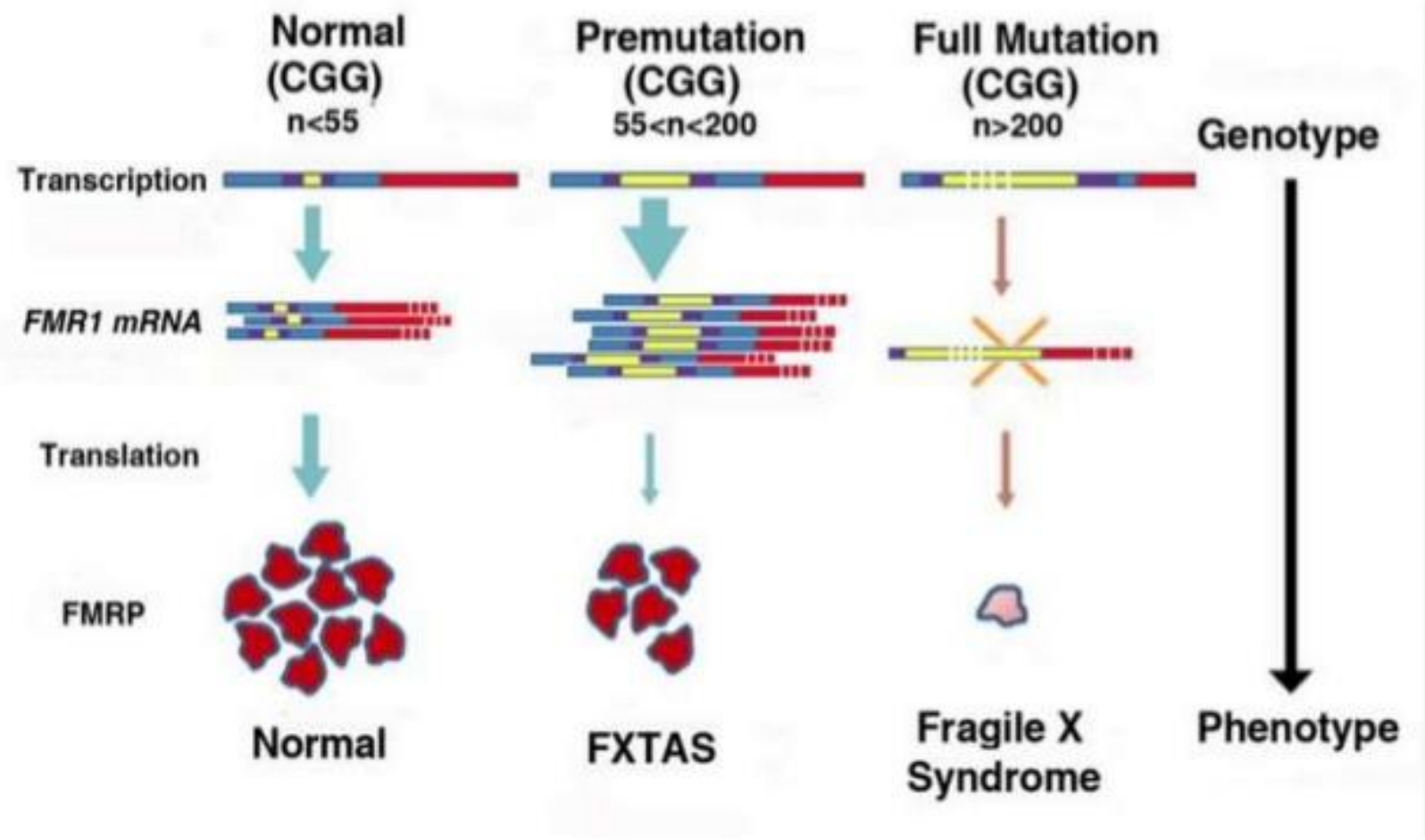
Neurons function

Neurons mantain activity

CONCLUSION

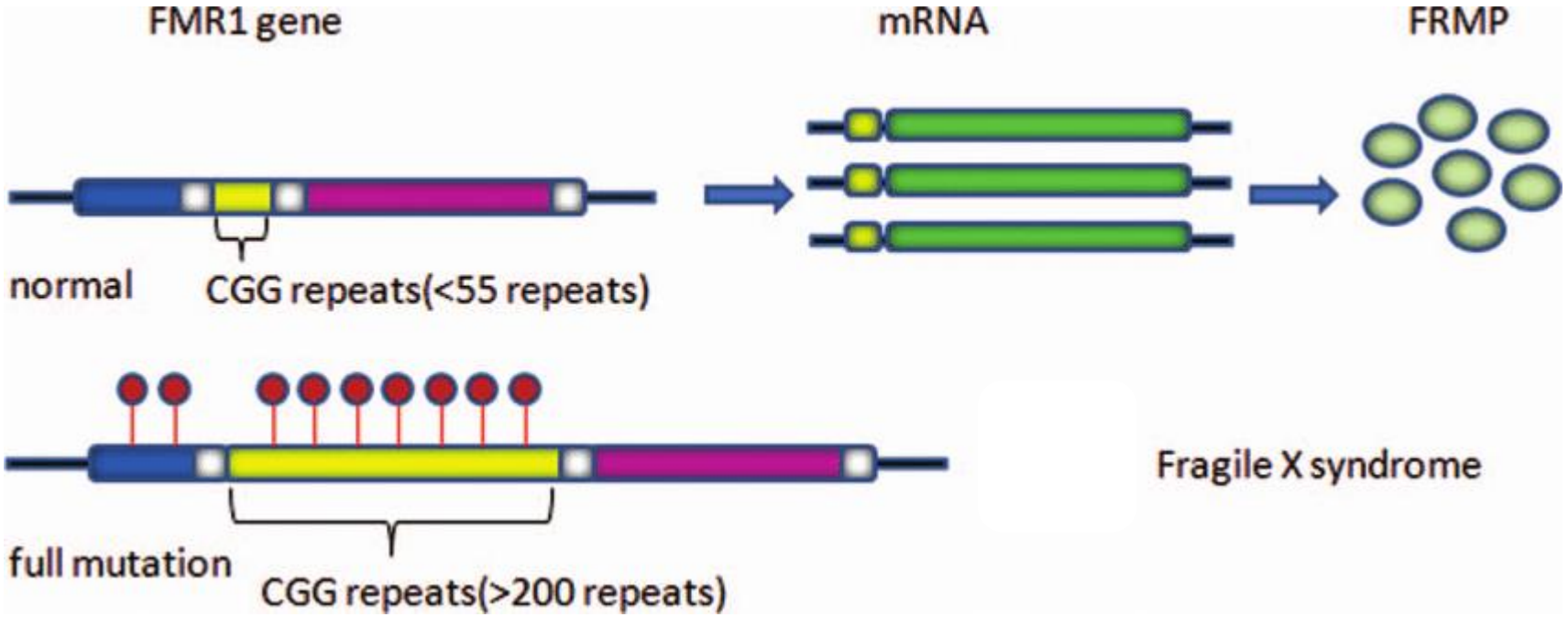
https://youtu.be/afH4-_xFVmY

FMR1 GENE



FMR1 is a gene encoding for FRMP protein

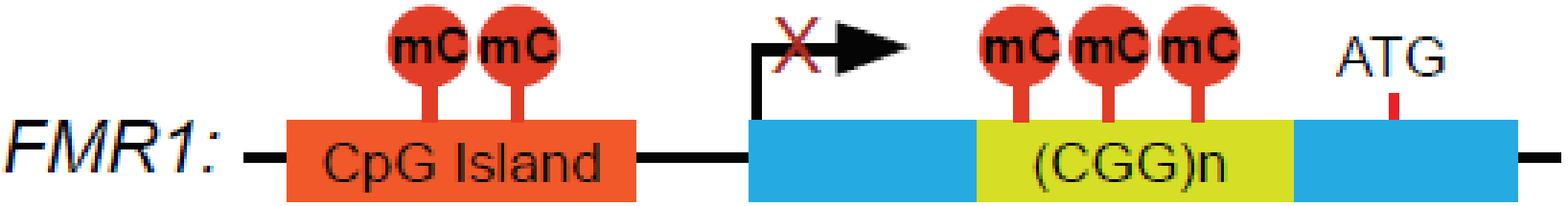
***CGG* expansion is associated with genomic regions hypermethylation and the inibition of FRMP protein**



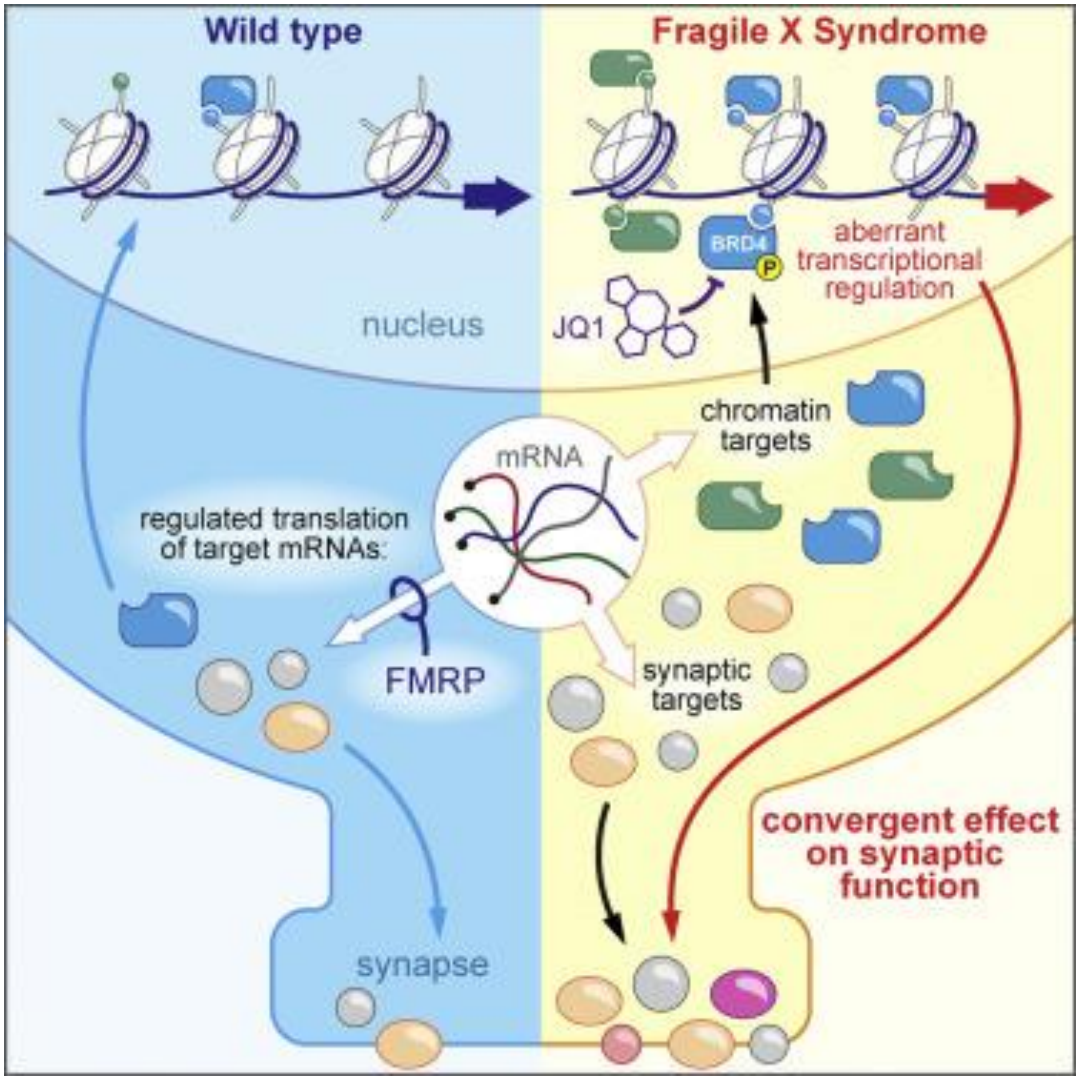
Fragile X syndrome (FXS) is the most frequent form of intellectual disability and autism spectrum disorder.

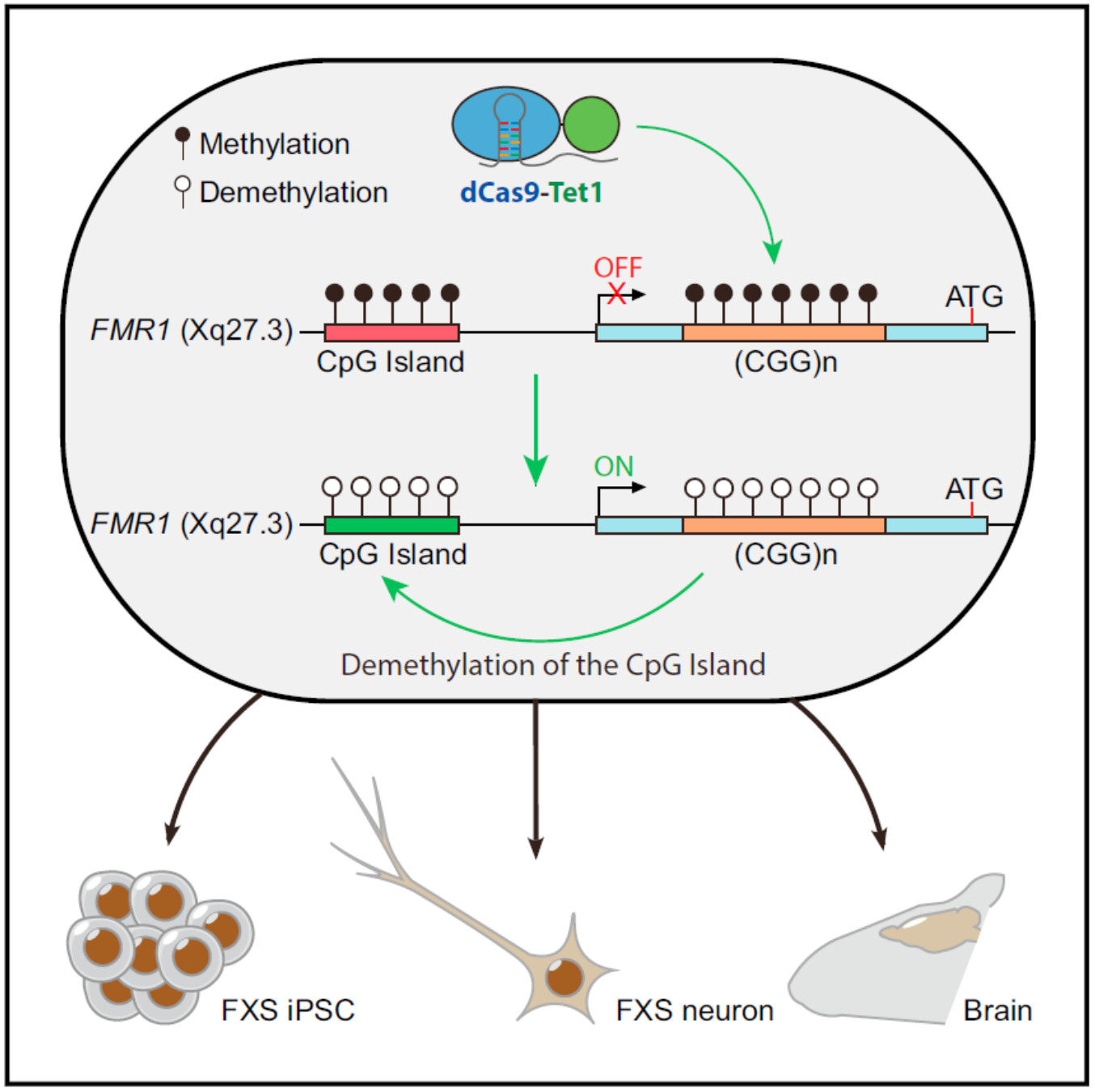
Most cases of FXS are caused by a *CGG* trinucleotide repeat expansion in the promoter region of the FMR1 gene classified by the American College of Medical Genetics guidelines as follows:
normal alleles have between 6 and 44 *CGG* repeats, gray zone alleles have between 45 and 54 repeats, premutation alleles have between 55 and 200 repeats, and full mutation alleles have more than 200 repeats.

A CGG trinucleotide repeat (> 200) expansion mutation at the 5′ UTR of FMR1, accompanied by DNA hypermethylation, was thought to result in heterochromatin formation at the FMR1 promoter and subsequent silencing of FMR1 expression in FXS, but the molecular mechanisms are not fully understood.



FMRP (fragile X mental retardation protein)
is a an RNA binding protein in neurons and has been shown to be a molecular brake for local protein synthesis at developing synapses and, hence, is essential for the maintenance of normal synaptic plasticity

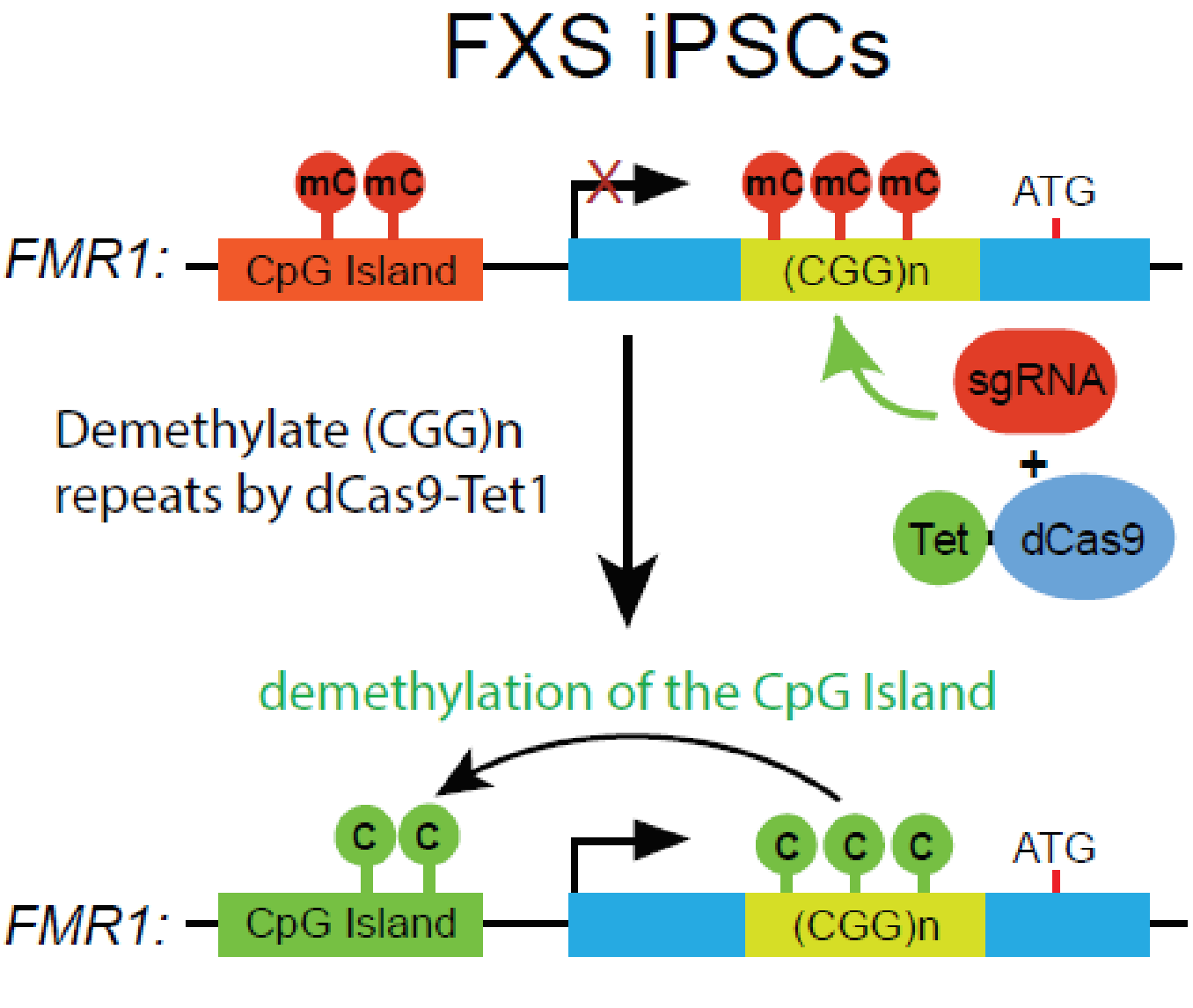



Methylation
Demethylation
dCas9-Tet1
OFF
ATG
FMR1 (Xq27.3)
CpG Island
(CGG)n
ON
ATG
FMR1 (Xq27.3)
CpG Island
(CGG)n
Demethylation of the CpG Island
FXS iPSC
FXS neuron
Brain

Highlights

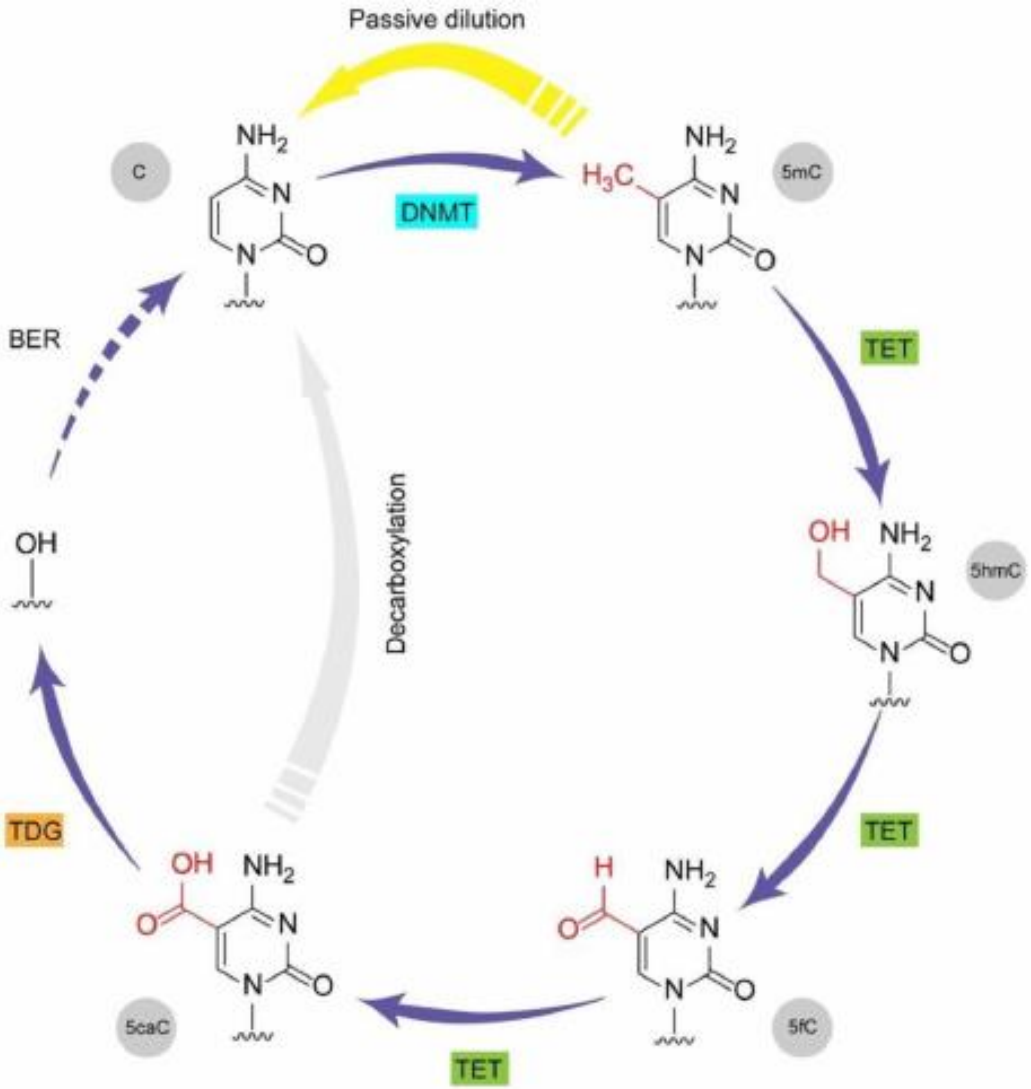
- Targeted demethylation of CGG repeats by dCas9-Tet1 reactivates *FMR1* in FXS cells
- Demethylation of CGG repeats induces an active chromatin status for *FMR1* promoter
- Methylation-edited FXS neurons behave similarly as wild-type neurons
- *FMR1* reactivation by dCas9-Tet1 is sustainable in a human/mouse chimeric model

Targeted demethylation of CGG repeats by dCas9-Tet1 reactivates FMR1 in FXS cells

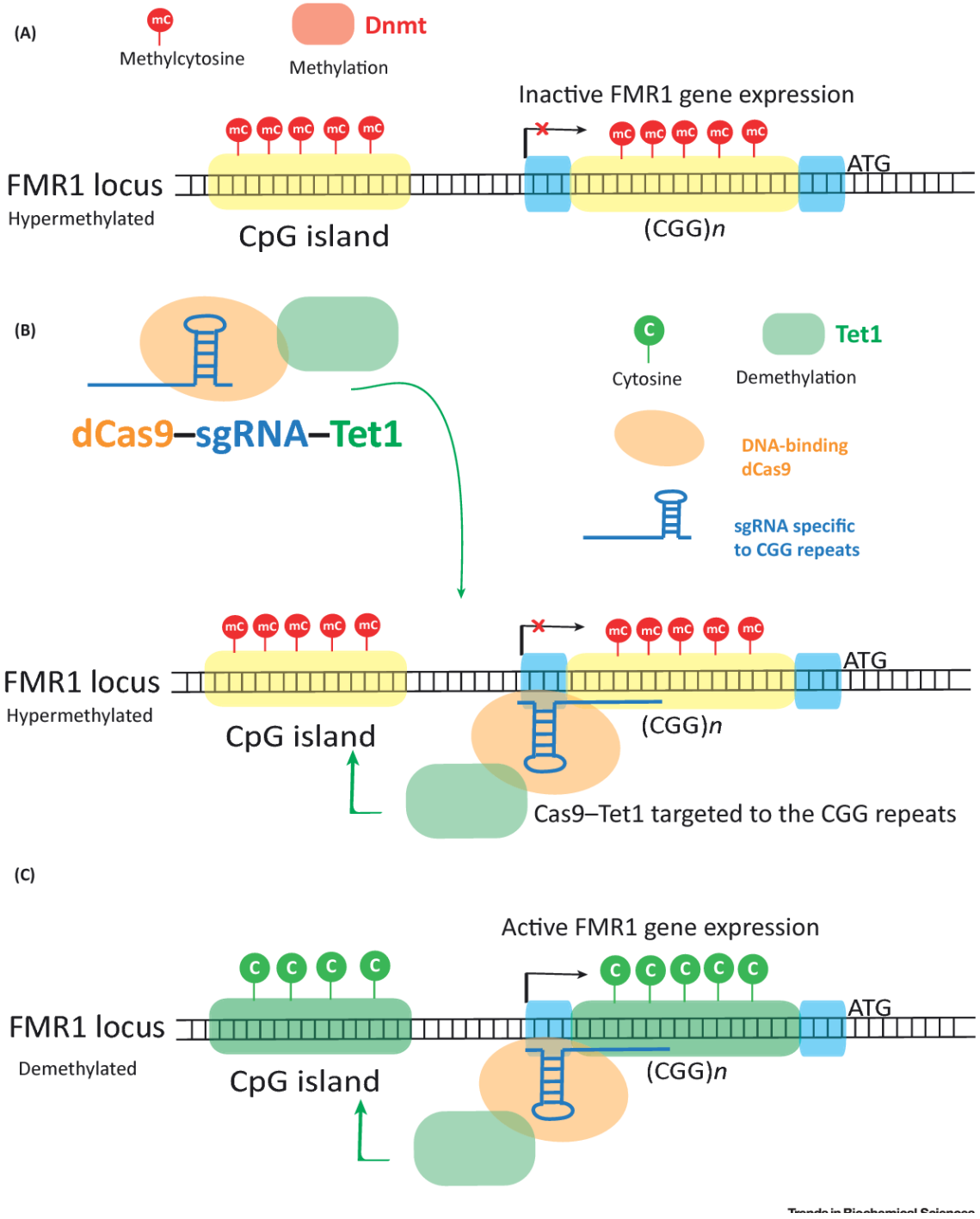


Cycle of DNA methylation and demethylation.

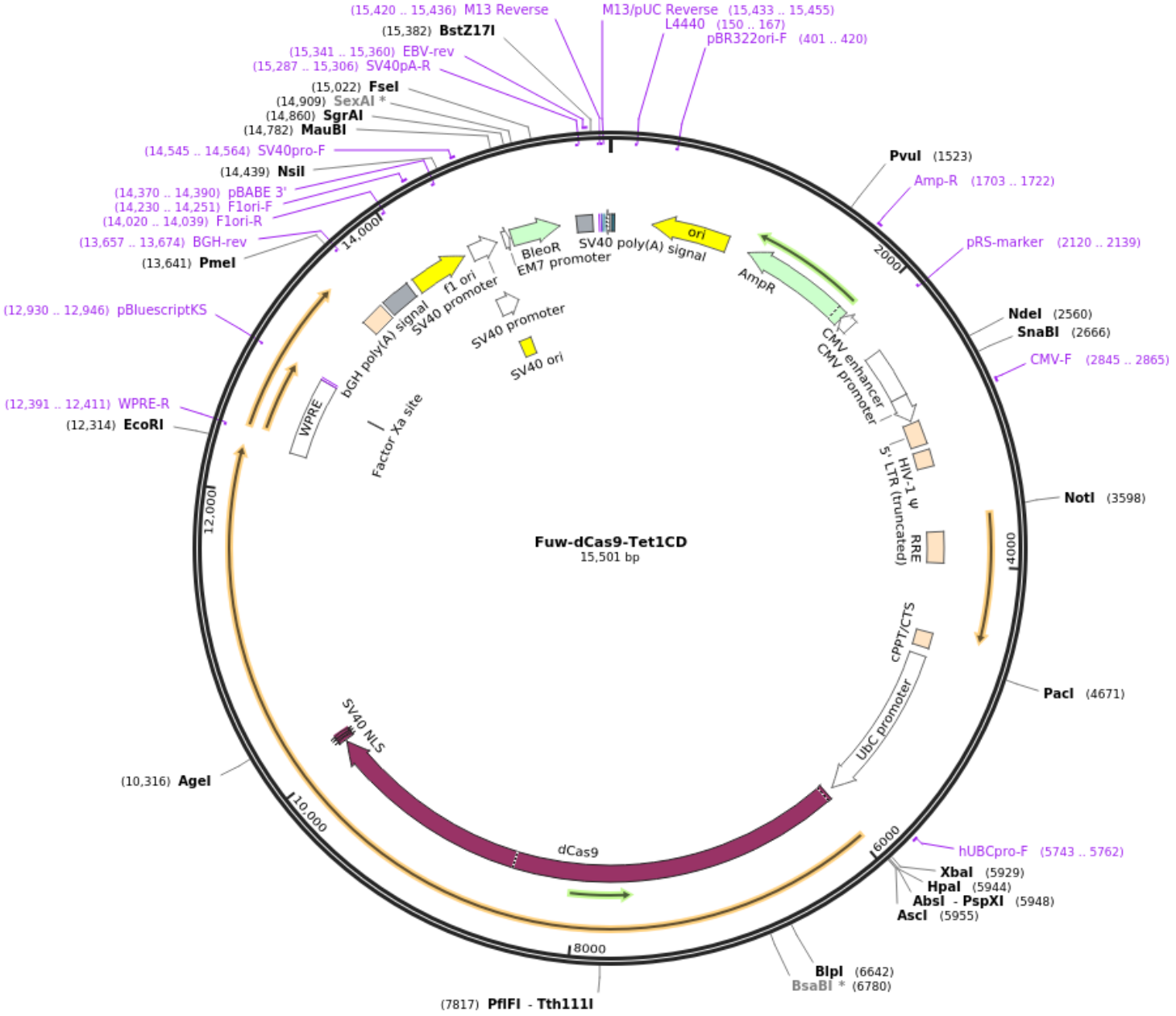
Ten eleven translocation, TET dioxygenanses oxidize 5-methylcytosines (5mCs) and promote locus-specific reversal of DNA methylation



Active de- methylation is achieved by iterative oxidation of the methyl group of 5mC by Tet dioxygenases and restoration of unmodified cyto- sines (C). The latter is thought to occur by either repli- cation-dependent dilution (not shown) or TDG glycosylase-initiated base excision repair. Of note, TDG can recognize and excise both 5fC and 5caC. An alternative direct mechanism is feasible (grey arrow), but an enzyme responsible for 5caC decarboxylation remains to be identified.


(A)
mC
Methylcytosine
Dnmt
Methylation
Inactive FMR1 gene expression
FMR1 locus
Hypermethylated
CpG island
(CGG)n
ATG
(B)
dCas9–sgRNA–Tet1
C
Cytosine
Tet1
Demethylation
DNA-binding dCas9
sgRNA specific to CGG repeats
FMR1 locus
Hypermethylated
CpG island
(CGG)n
ATG
Cas9–Tet1 targeted to the CGG repeats
(C)
Active FMR1 gene expression
FMR1 locus
Demethylated
CpG island
(CGG)n
ATG

https://www.addgene.org/84475/sequences/



All the FXS iPSC lines were derived from male patients.
The CGG repeat expansion mutations were verified by Claritas Genomics Inc with Asuragen AmplideX mPCR approach and the mycoplasma test was negative.

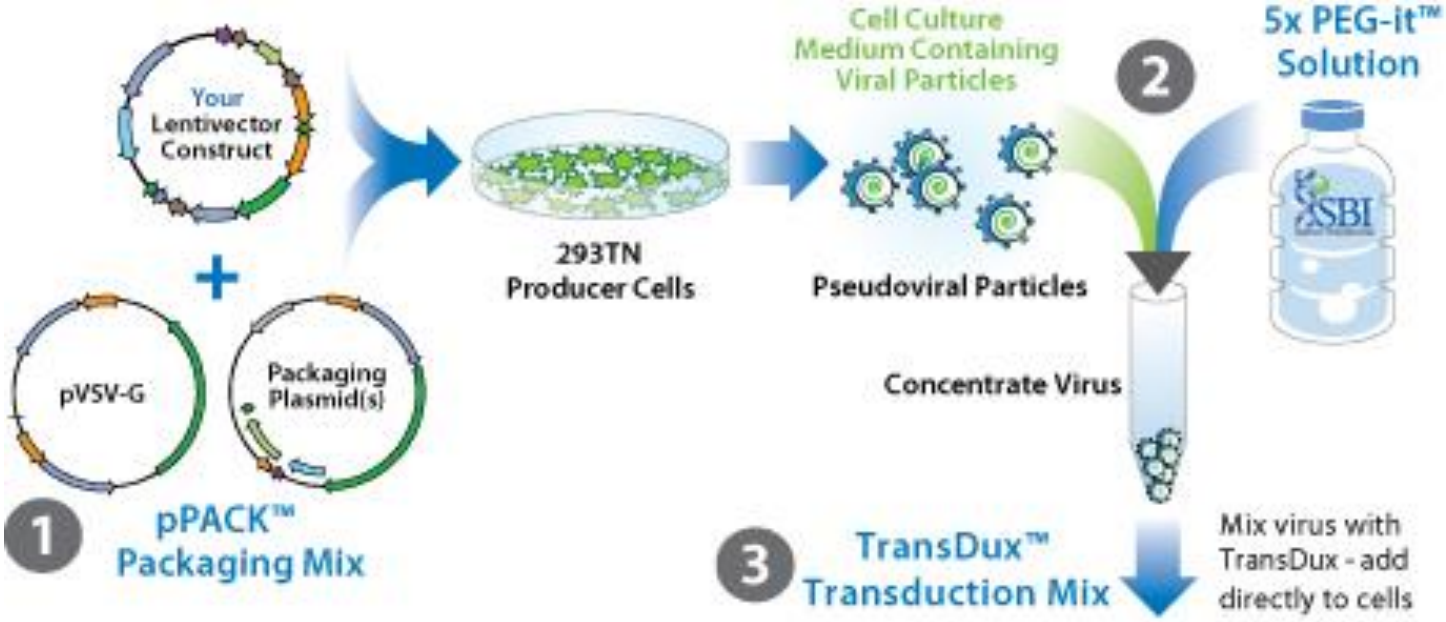
Samples

FXS iPSCs were cultured either with mTeSR1 medium (STEMCELL, #85850) or on irradiated mouse embryonic fibroblasts (MEFs) with standard hESCs medium.

Primary cell lines

dCas9-Tet1-P2A-BFP, sgRNAs, and AcrIIA4 were produced by transfecting HEK293T cells with FUW constructs or pgRNA constructs together with standard packaging vectors (pCMV-dR8.74 and pCMV-VSVG) followed by ultra-centrifugation-based concentration.

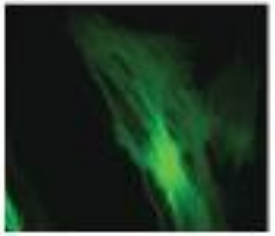
Preparation of viral particles



LentiStarter Kit (cat# LV050A-1)

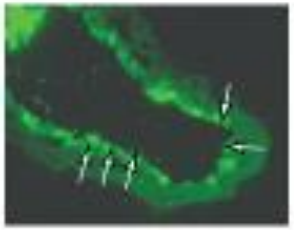
	Component	Amount
1	pPACKH1-Plamid Packaging Mix	40 µl
2	PEG-it	5 ml
3	TransDux (200x)	50 µl

Human Astrocytes (GFP)



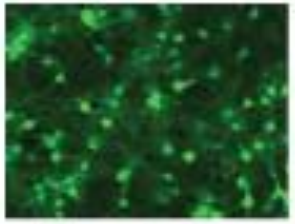
Human embryonic kidney cells (RFP)

Animal Models



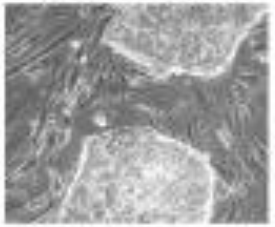
Mouse Carotid Artery (GFP)

Primary Cells

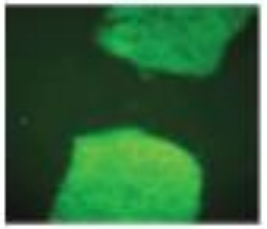


Human Primary Neurons (GFP)

Phase contrast



GFP



Human Embryonic H9 Cells

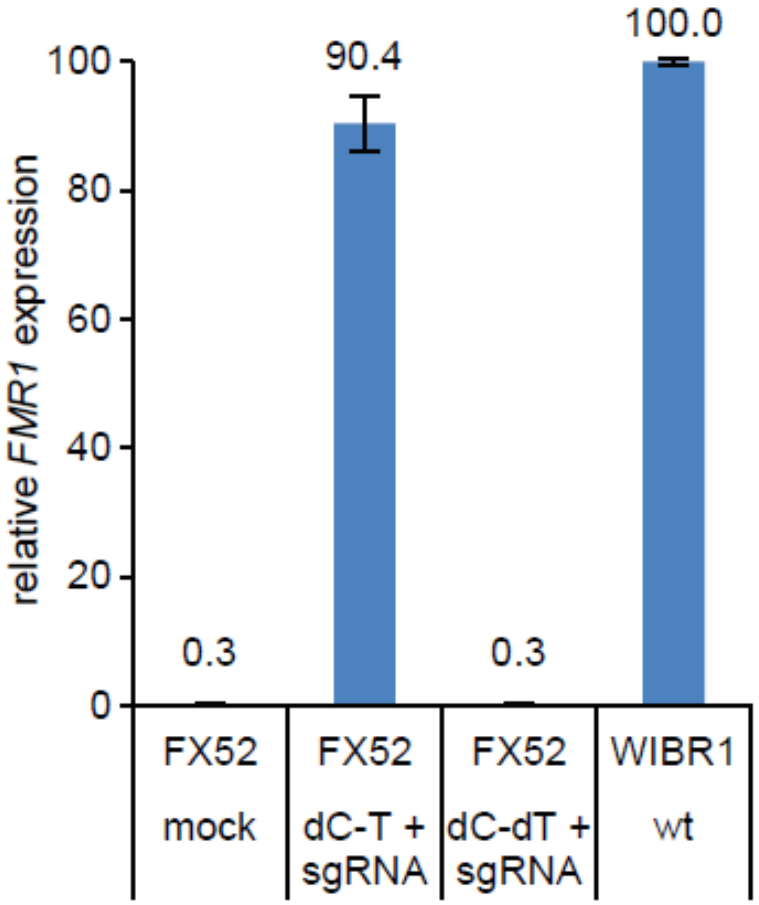
CONTRUCTS USED IN THE EXPERIMENT

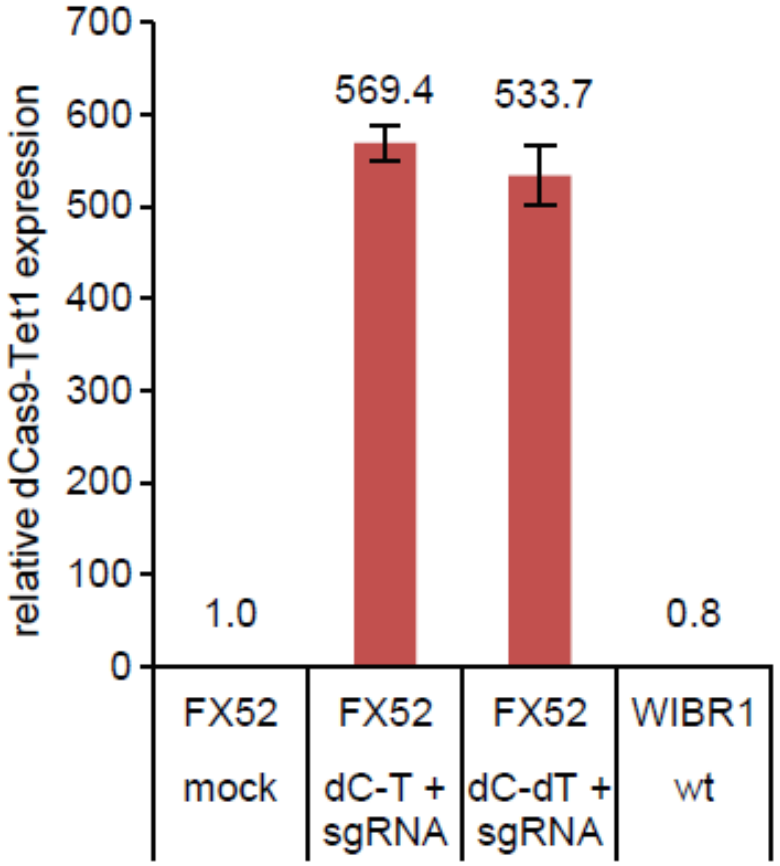
dCas9-Tet1-P2A-BFP **(dC-T)** with an mCherry-expressing sgRNA targeting the CGG repeats GGCGGCGGCGGCGGCGGCGGNGG (CGG sgRNA)

dCas9 fused with a catalytically dead Tet1 **(dC-dT)** with the same sgRNA.

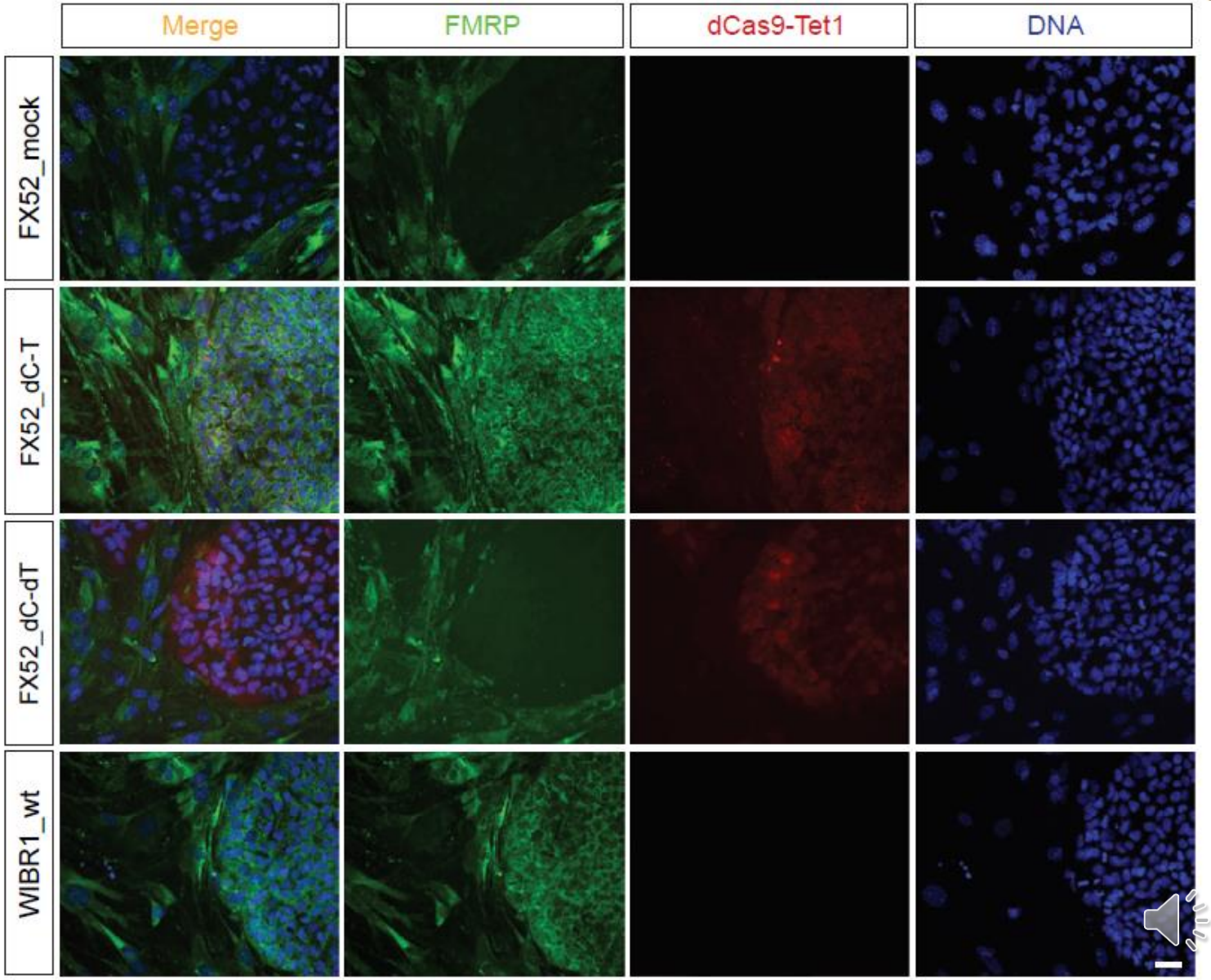
FMR1 and dCas9-TET1 expression

the expression level of FMR1 mRNA in cells with dC-T/CGG sgRNA was restored to 90% of the one in **wild-type WIBR1** human embryonic stem cells (hESCs)

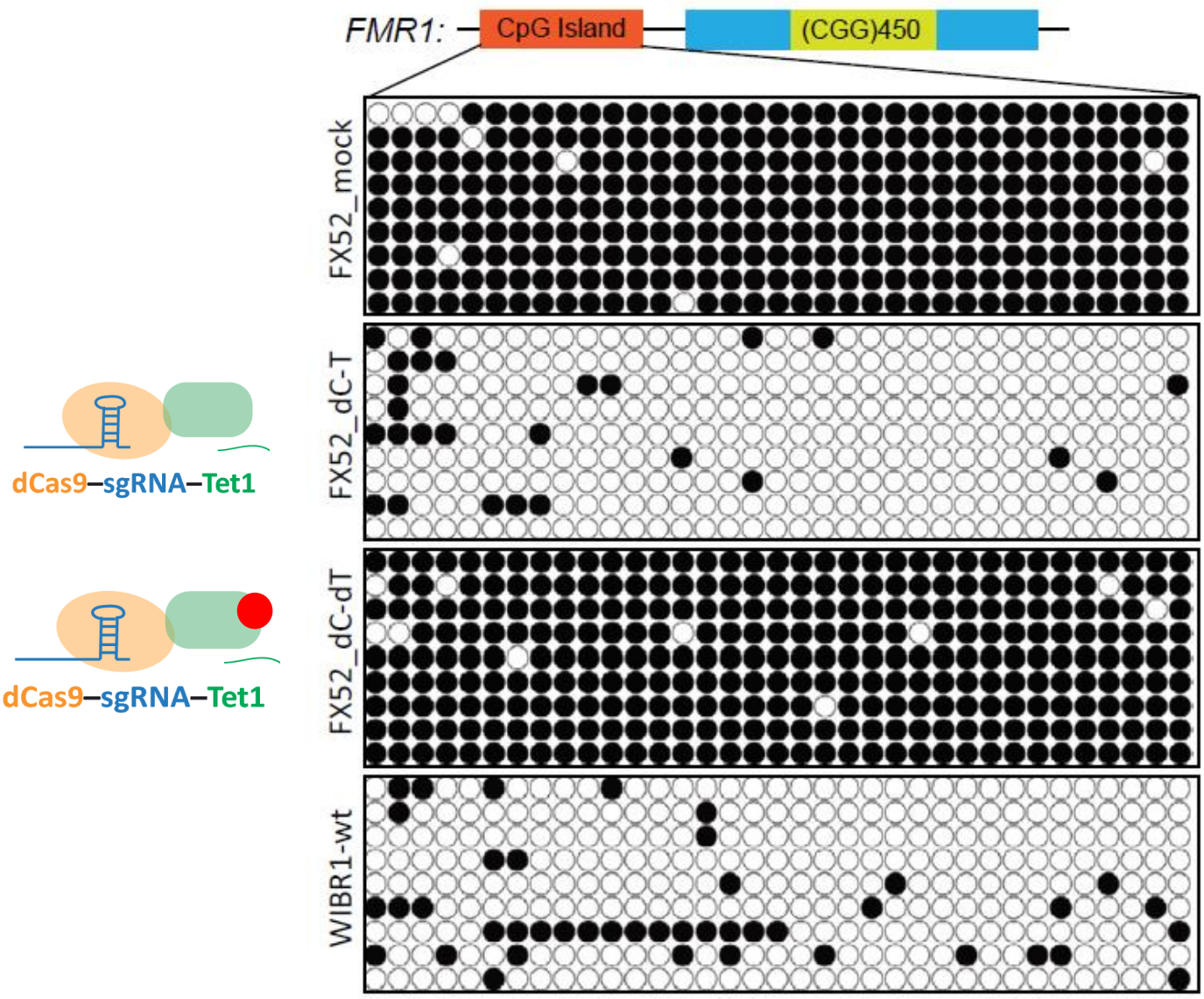




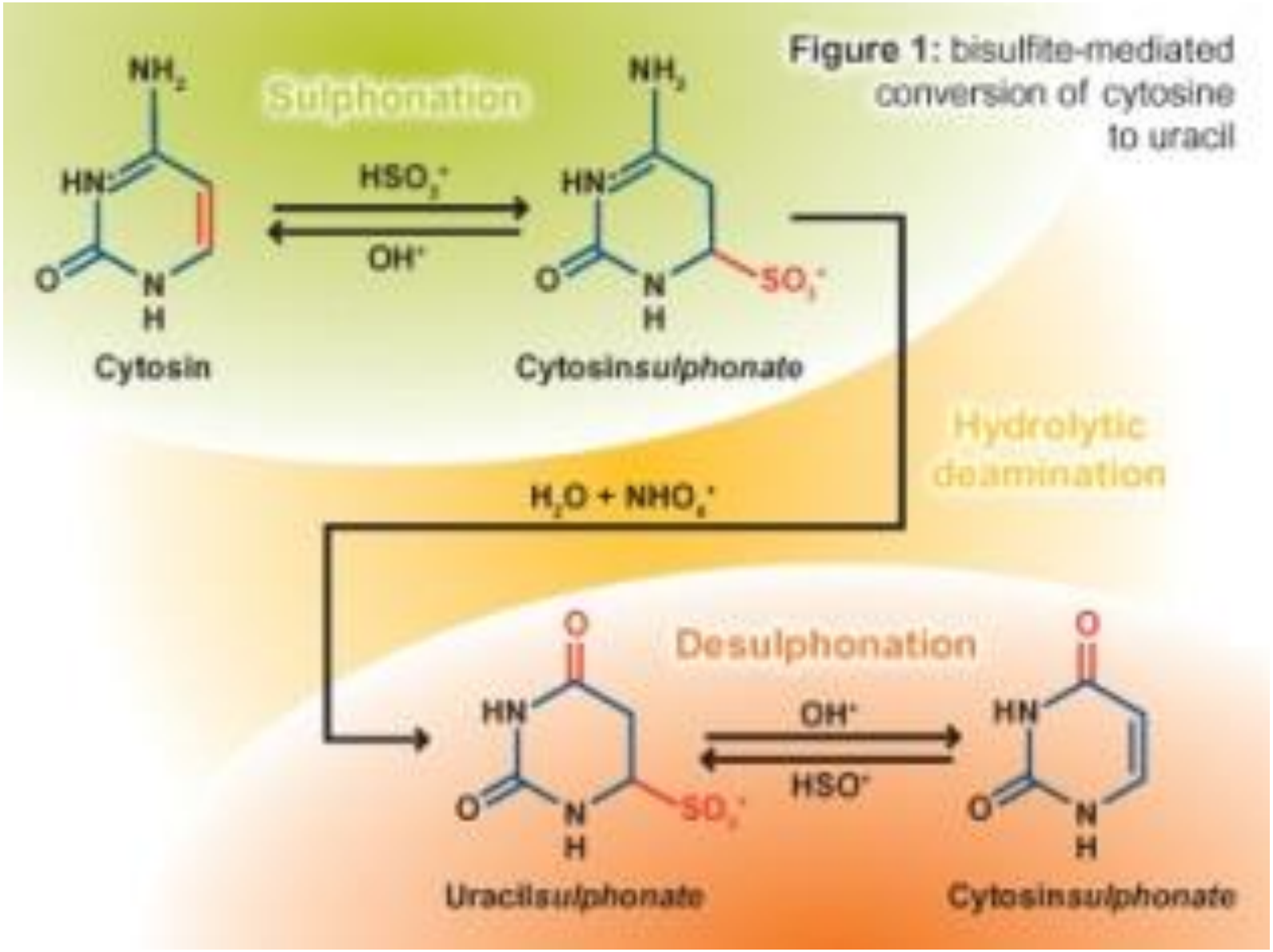
FMRP expression was restored in dC-T/CGG sgRNA-expressing FX52 iPSCs to 73% of the wild-type level in WIBR1 cells, as shown by immunofluorescence staining



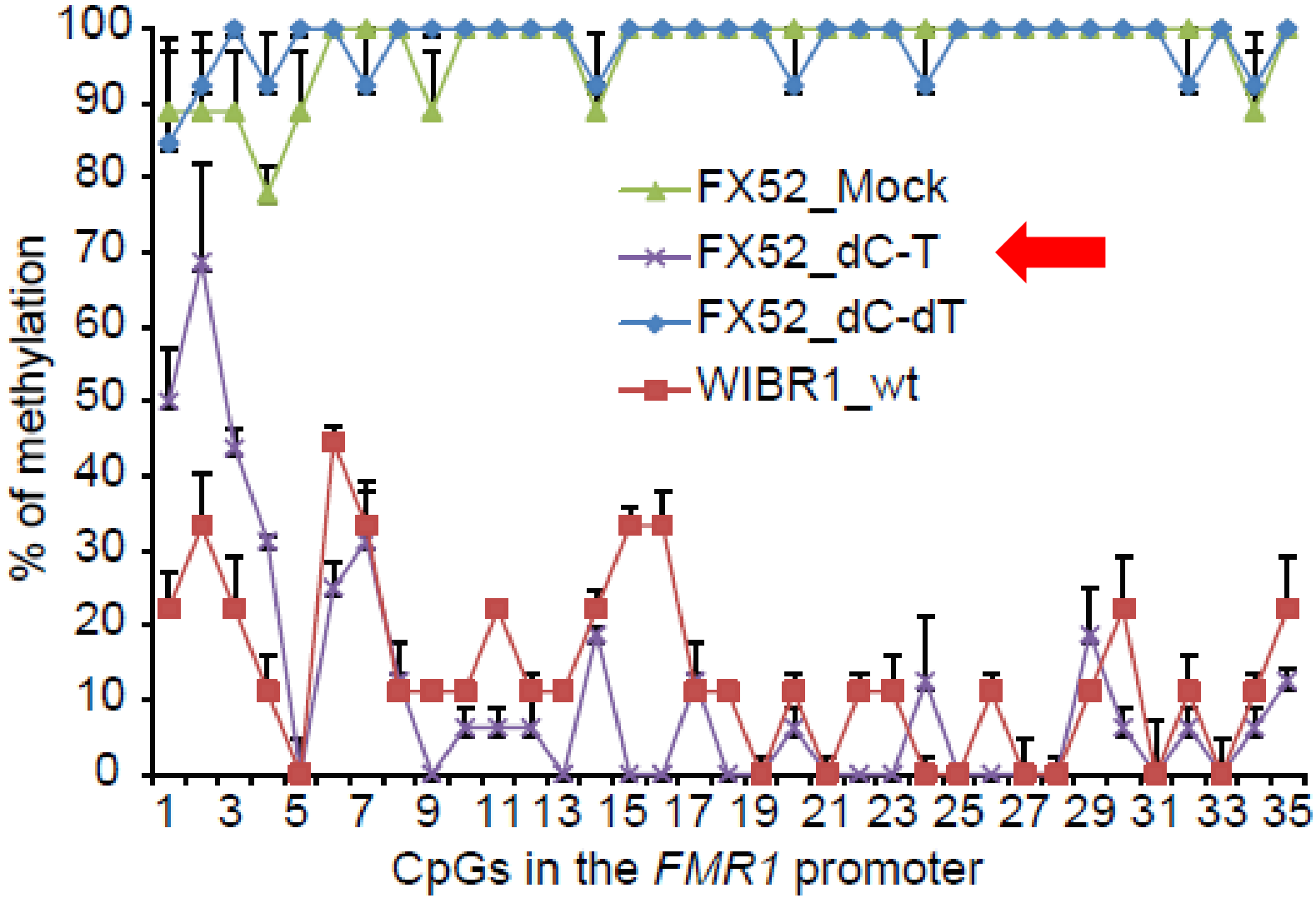
dCas9-TET1 reduced methylation at CpG island
Methylation levels of the CGG repeats in the FMR1 locus by bisulfite sequencing



https://www.jove.com/video/3170/dna-methylation-bisulphite-modification-and-analysis



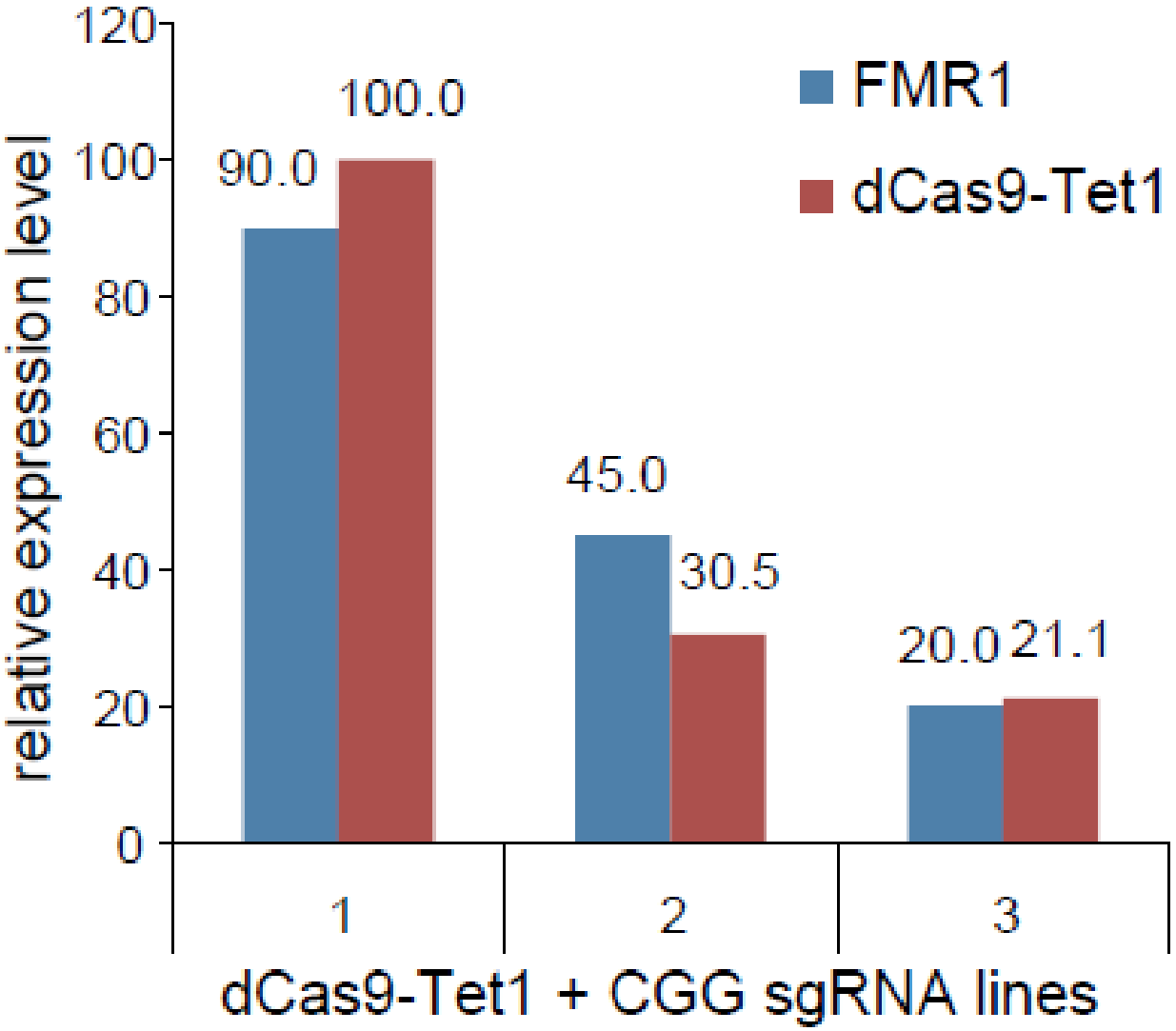
dCas9-TET1 reduced methylation at CpG island
Methylation levels of the CGG repeats in the FMR1 locus



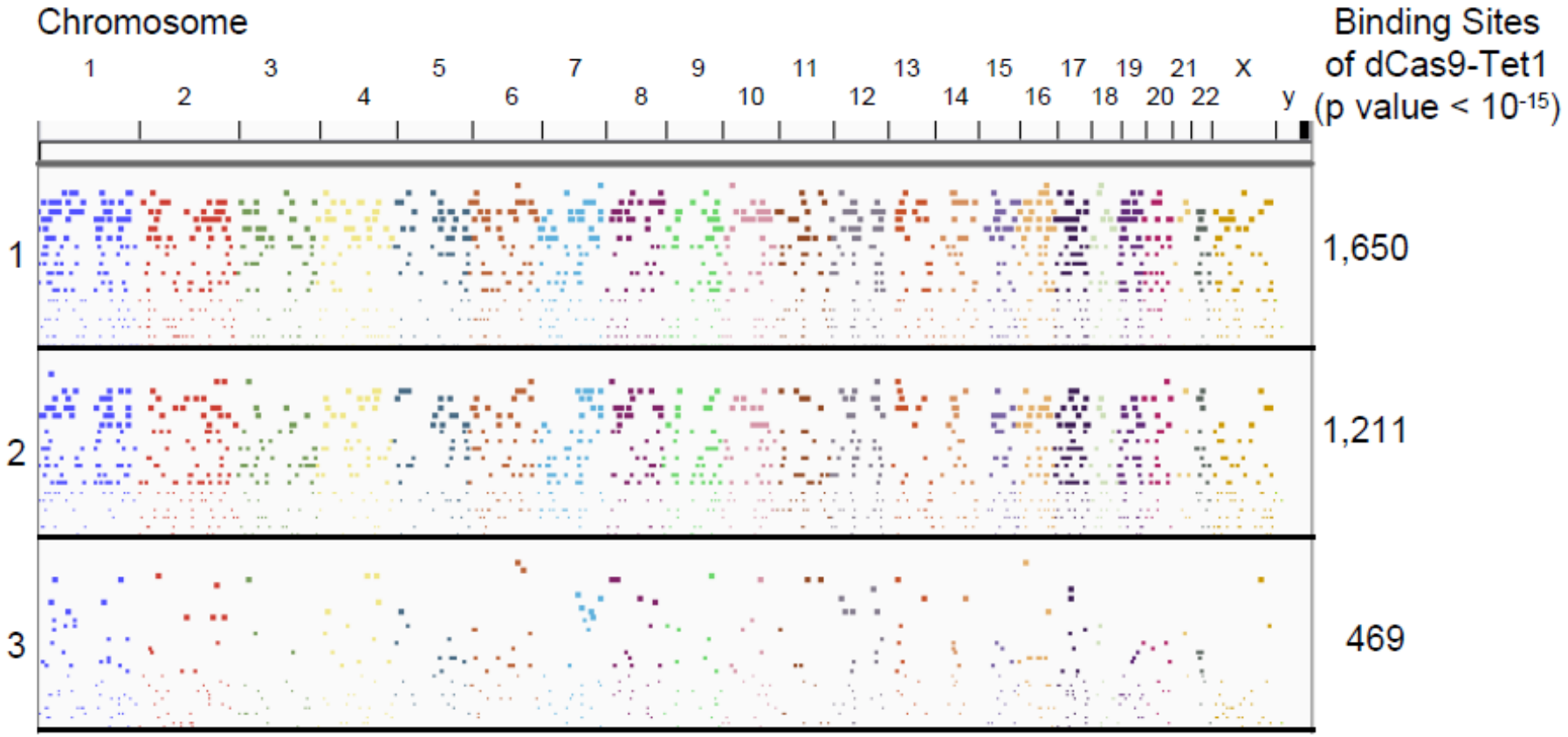
Off-Target Effects of dCas9-Tet1/CGG sgRNA

The presence of the CGG sgRNA targeting sequence GGCGGCGGCGGCGGCGGCGGNGG in other genomic loci raises concerns regarding off-target effects of the dCas9-Tet1/sgRNA system used.

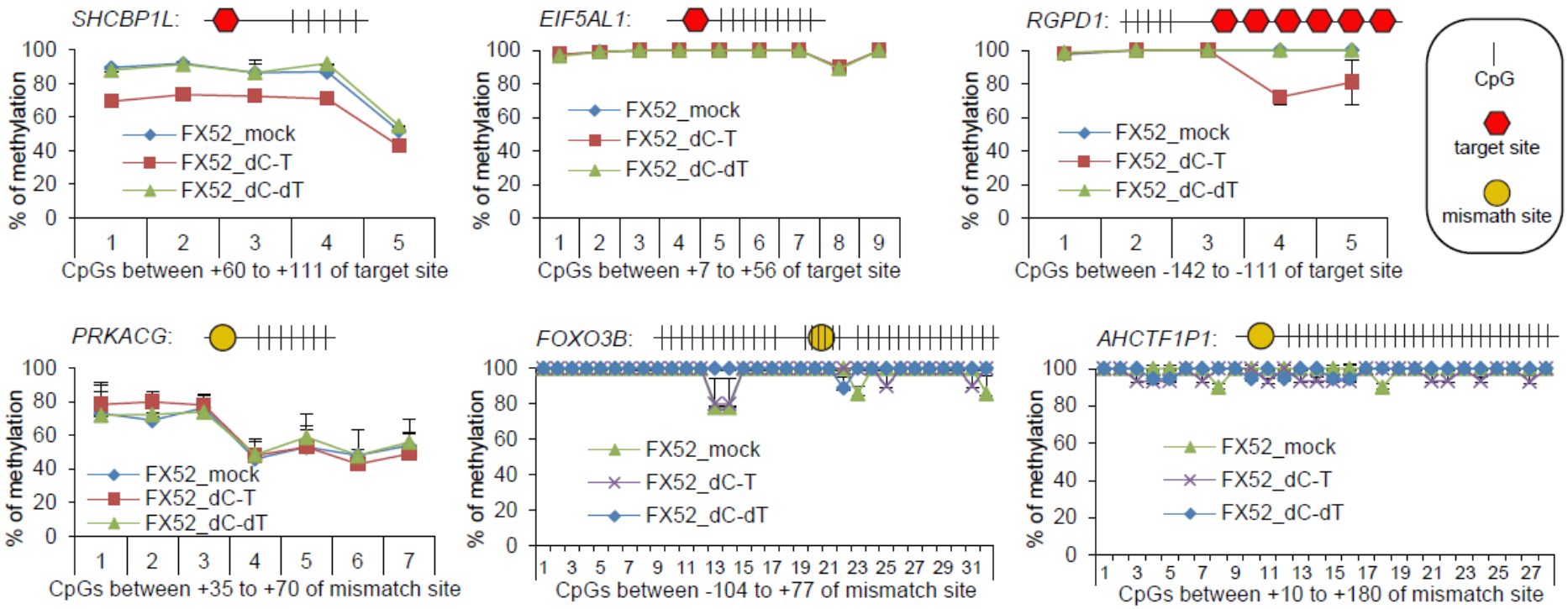
qRT-PCR analysis of three FX52 iPSC lines with different expression levels of dCas9-Tet1 and different restoration levels of FMR1 normalized to wild-type WIBR1 hESCs.



ChIP-Seq against dCas9-TET1
To identify the aspecific binding in three cell lines



Methylation analysis of genes with dCas9-TET1 binding

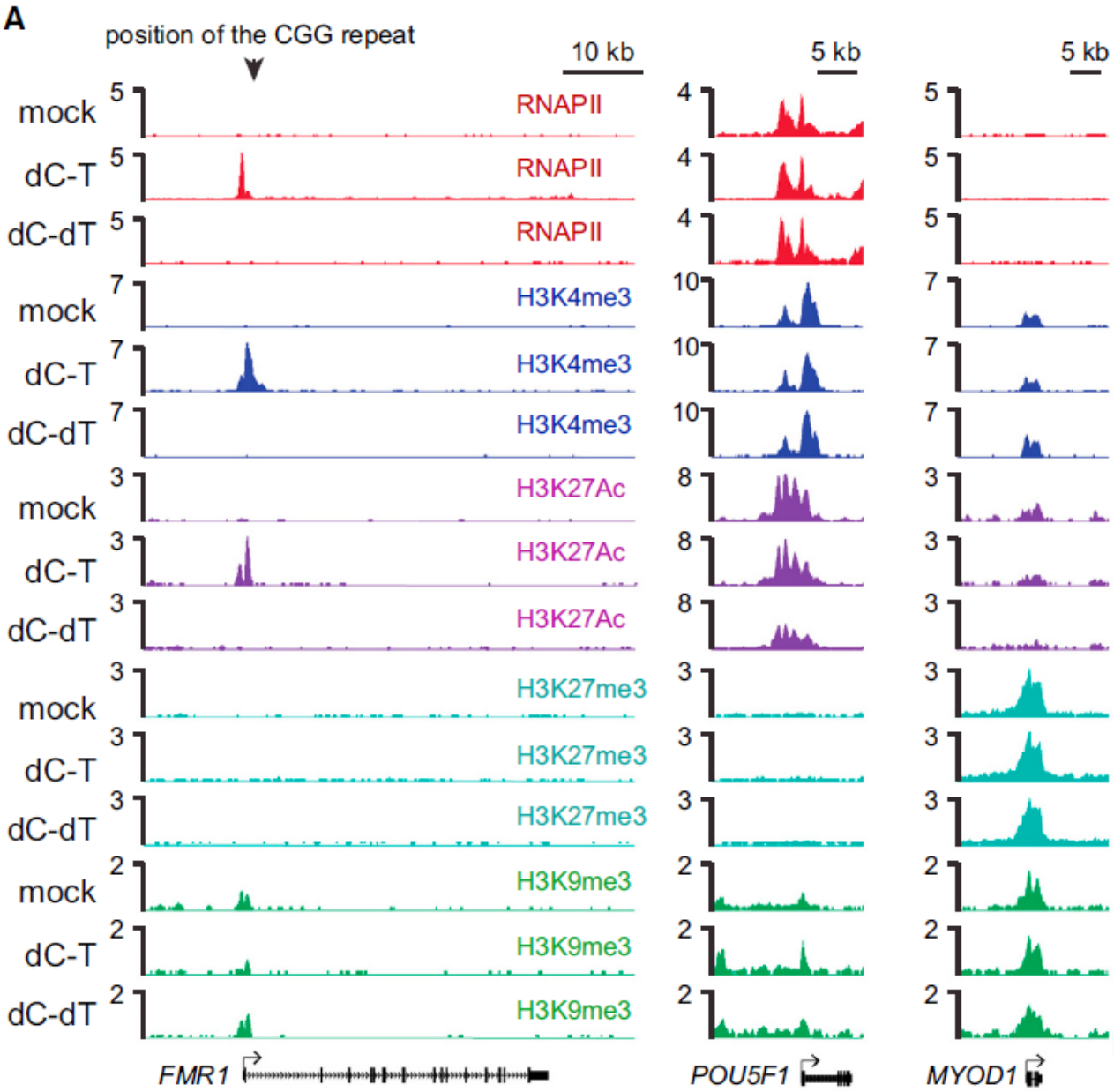


BS-seq showed a 20% and 30% reduction of methylation levels for SHCBP1L with one CGG sgRNA targeting site and RGPD1 with 6 targeting sites, respectively, but no detectable methylation changes for the other four genes.

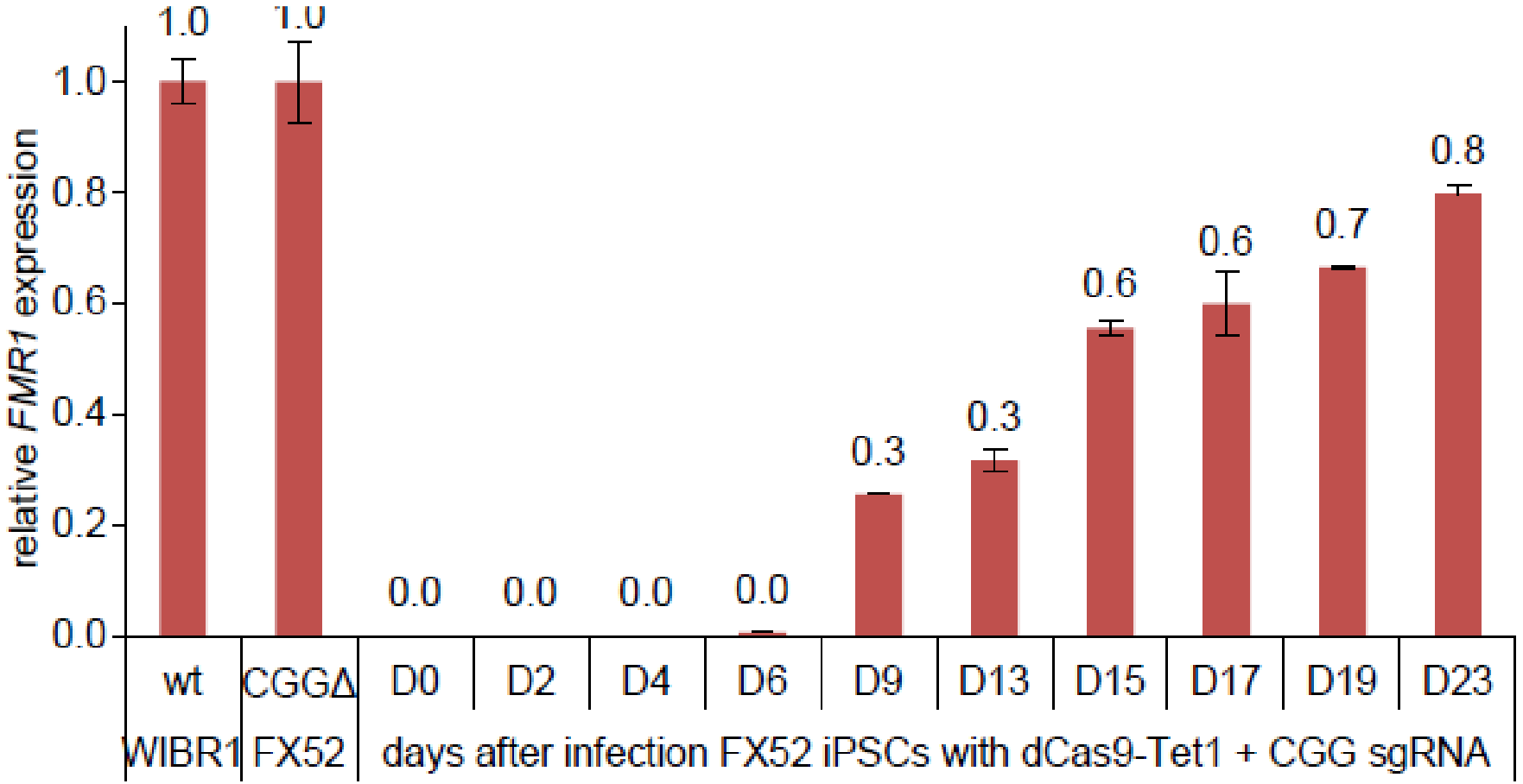
Chromatin Conformation of the Reactivated FMR1 Promoter

cC-T

cC-dT

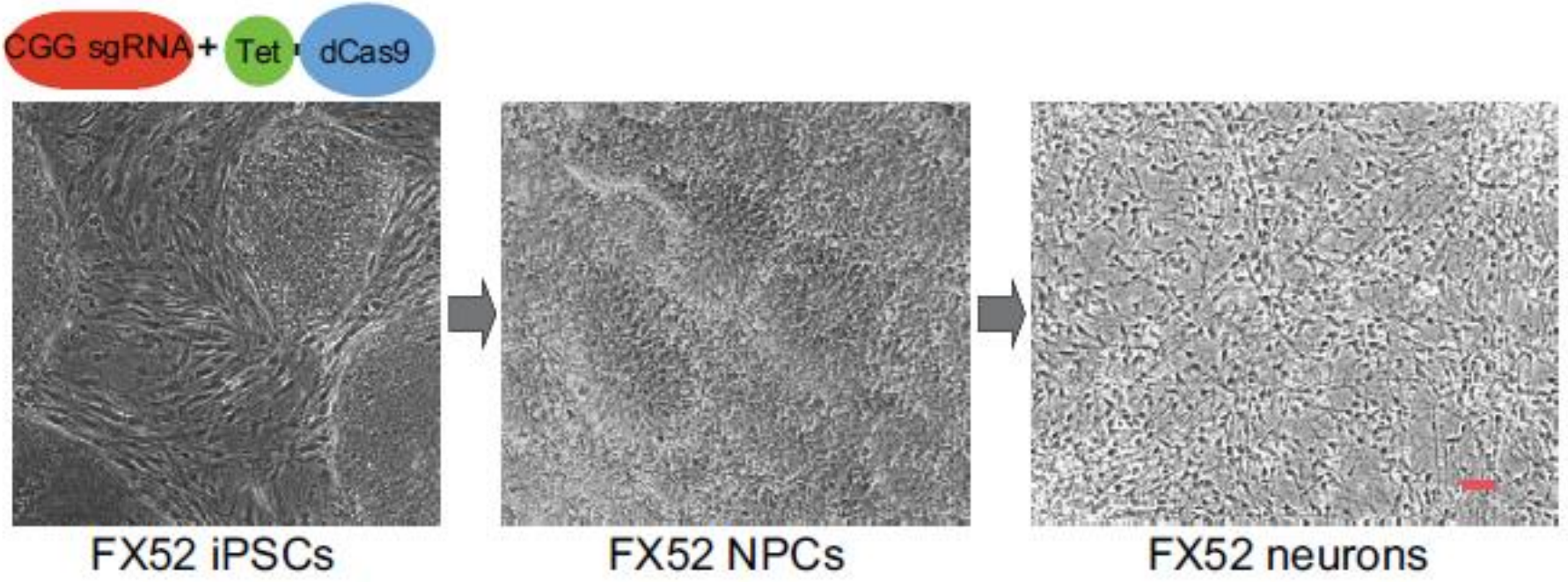


The Kinetics and Persistence of Methylation Editing

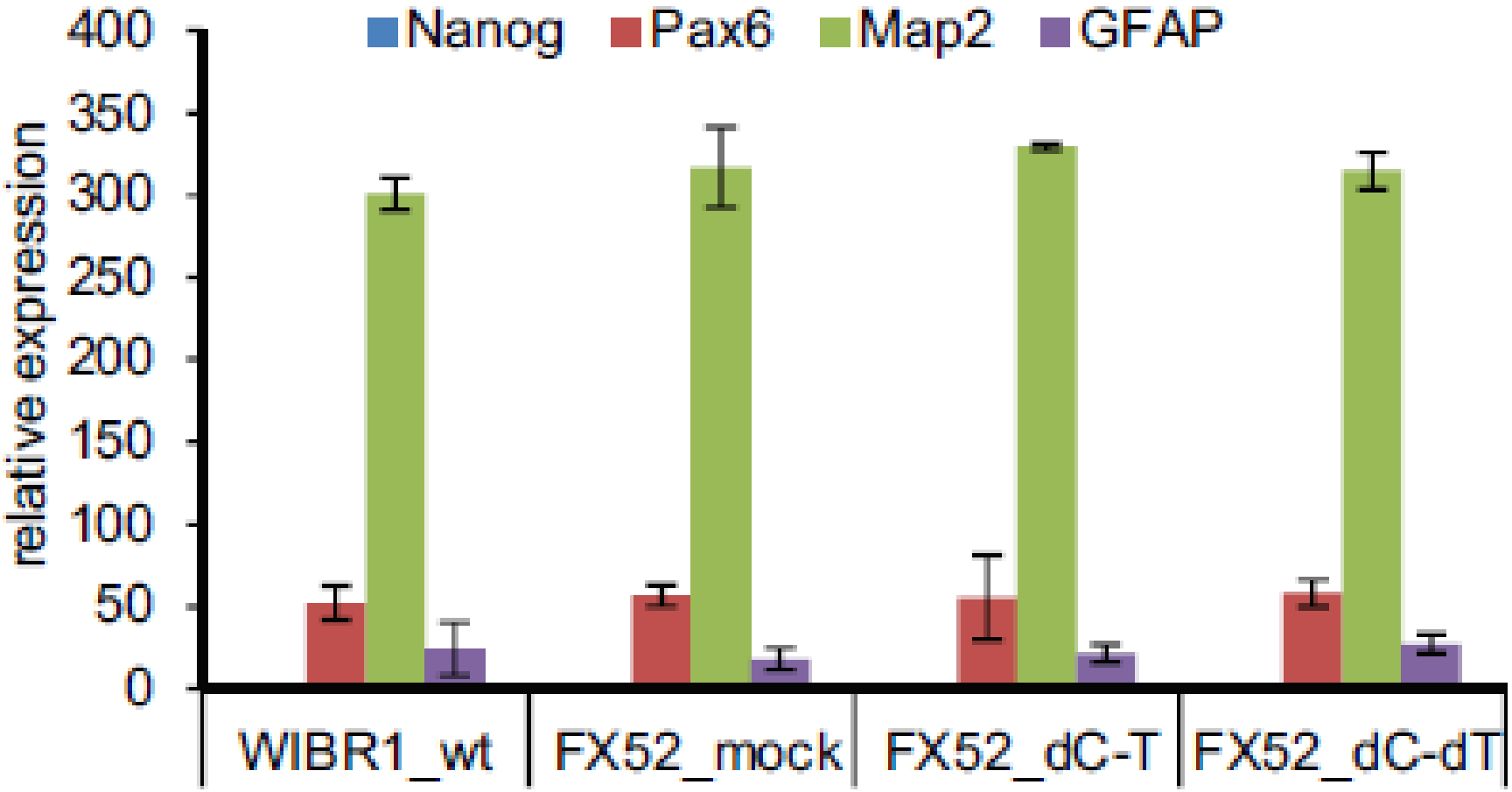


Phenotypical Rescue of FXS-Related Cellular Deficits

FMR1 reactivation on the rescue of FXS-related cellular phenotypes post-mitotic neurons were derived from the methylation-edited FX52 iPSCs

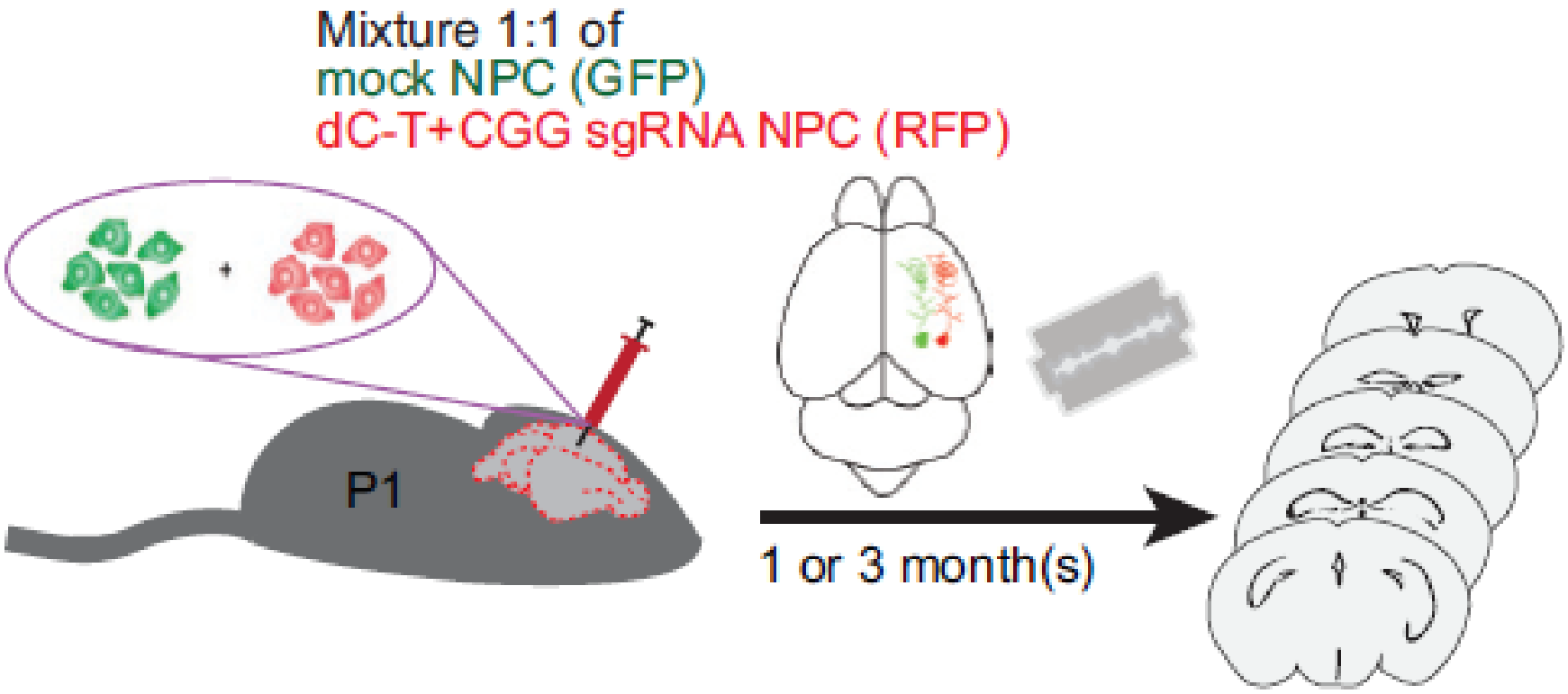


Gene expression analysis of lineage-specific markers suggested comparable differentiation states between wild-type and mutant neural cultures

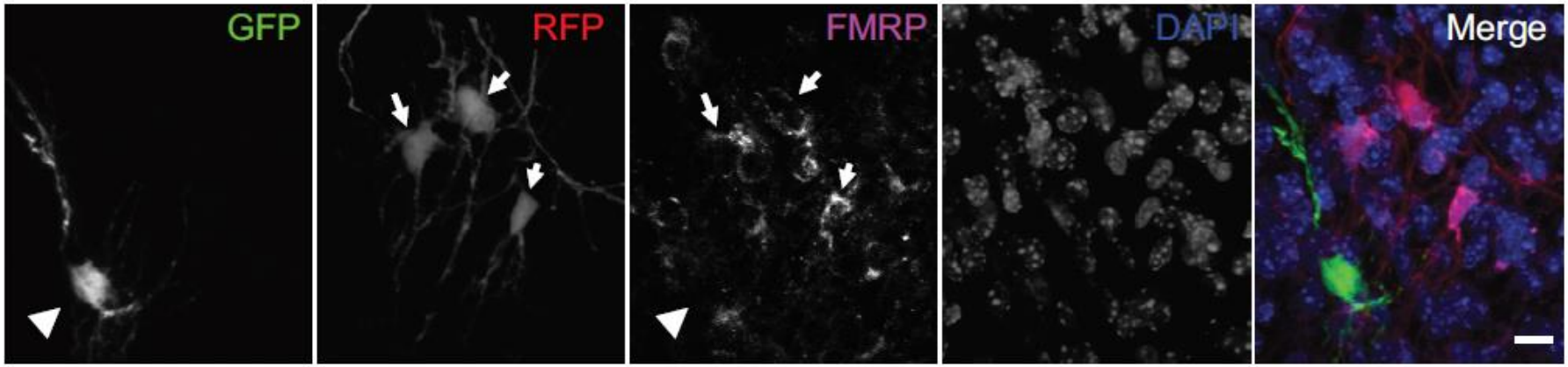


FMR1 Reactivation in Edited FXS Neurons Is Sustained after Engrafting into Mouse Brains

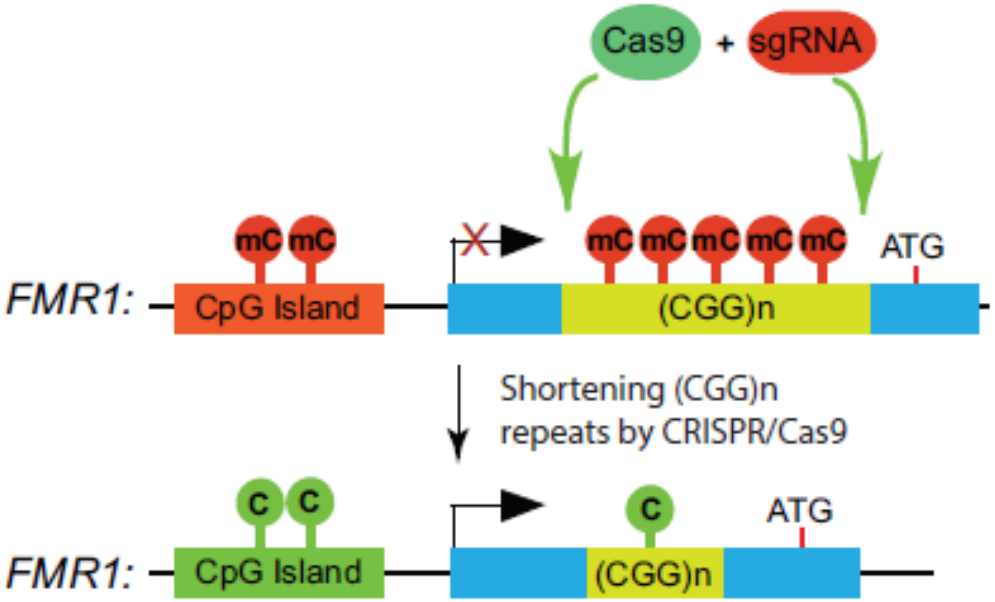
To test whether the reactivation of FMR1 in methylation-edited FXS cells is sustainable in vivo, FX52 mock- or methylation-edited neuronal precursor cells (NPCs) were labeled with GFP or red fluorescent protein (RFP) lentiviruses (dC-T+CGGsgRNA), respectively, and then the mixture of these two types of NPCs was injected into the P1 mouse brain for subsequent analysis 1 or 3 months after transplantation

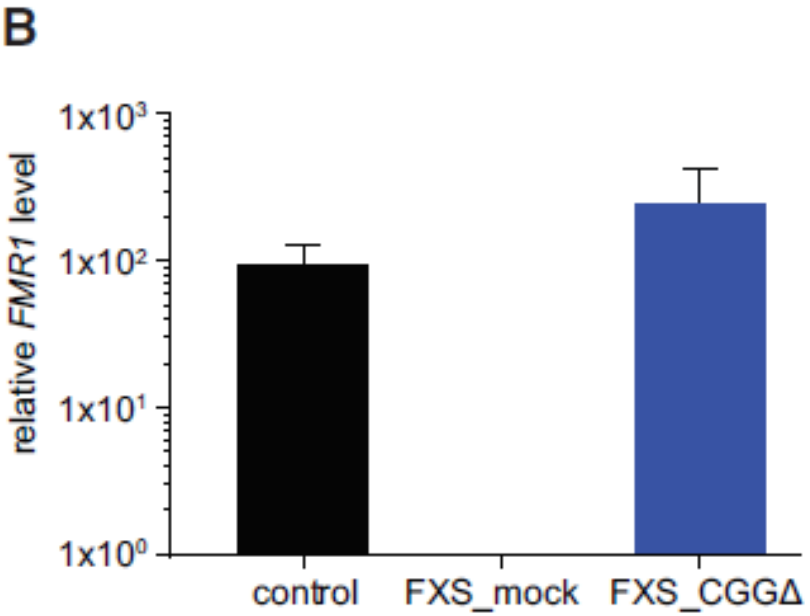


Edited FX52 neurons are positive for RFP neuron marker and FMRP expression -positive FX52 mock neurons (GFP-positive) were negative for FMRP expression (white arrow)

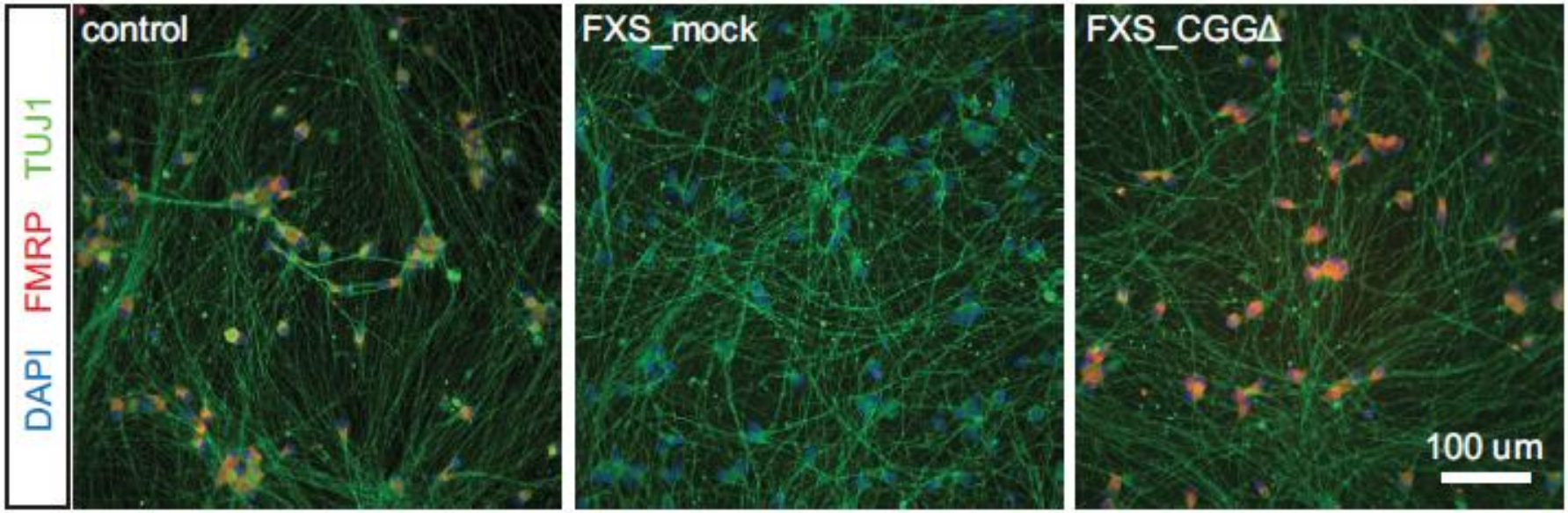


Deletion of *CGG* Repeats to Rescue FXS Phenotypes

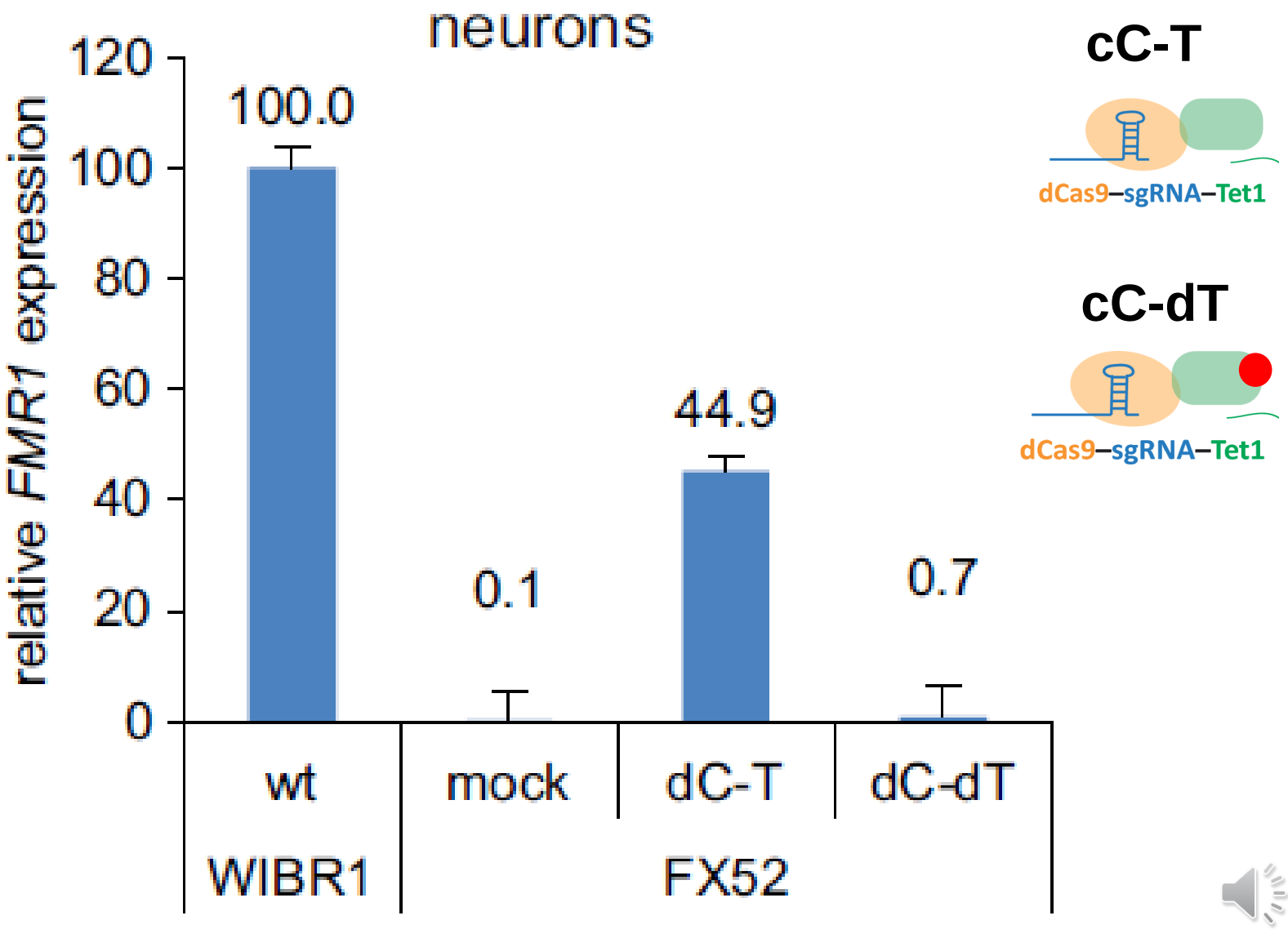




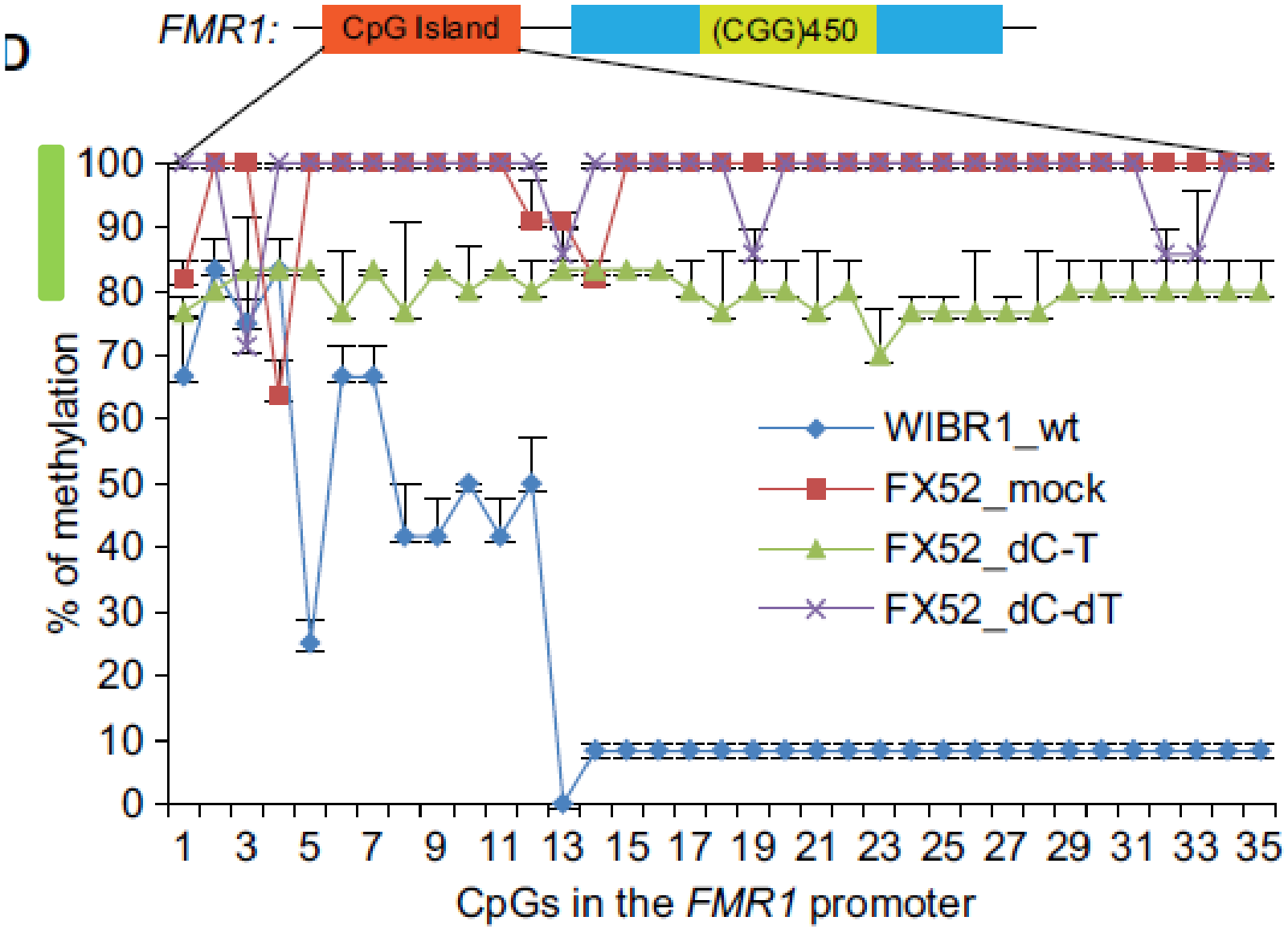
CGG deletion is associated with FMRP expression



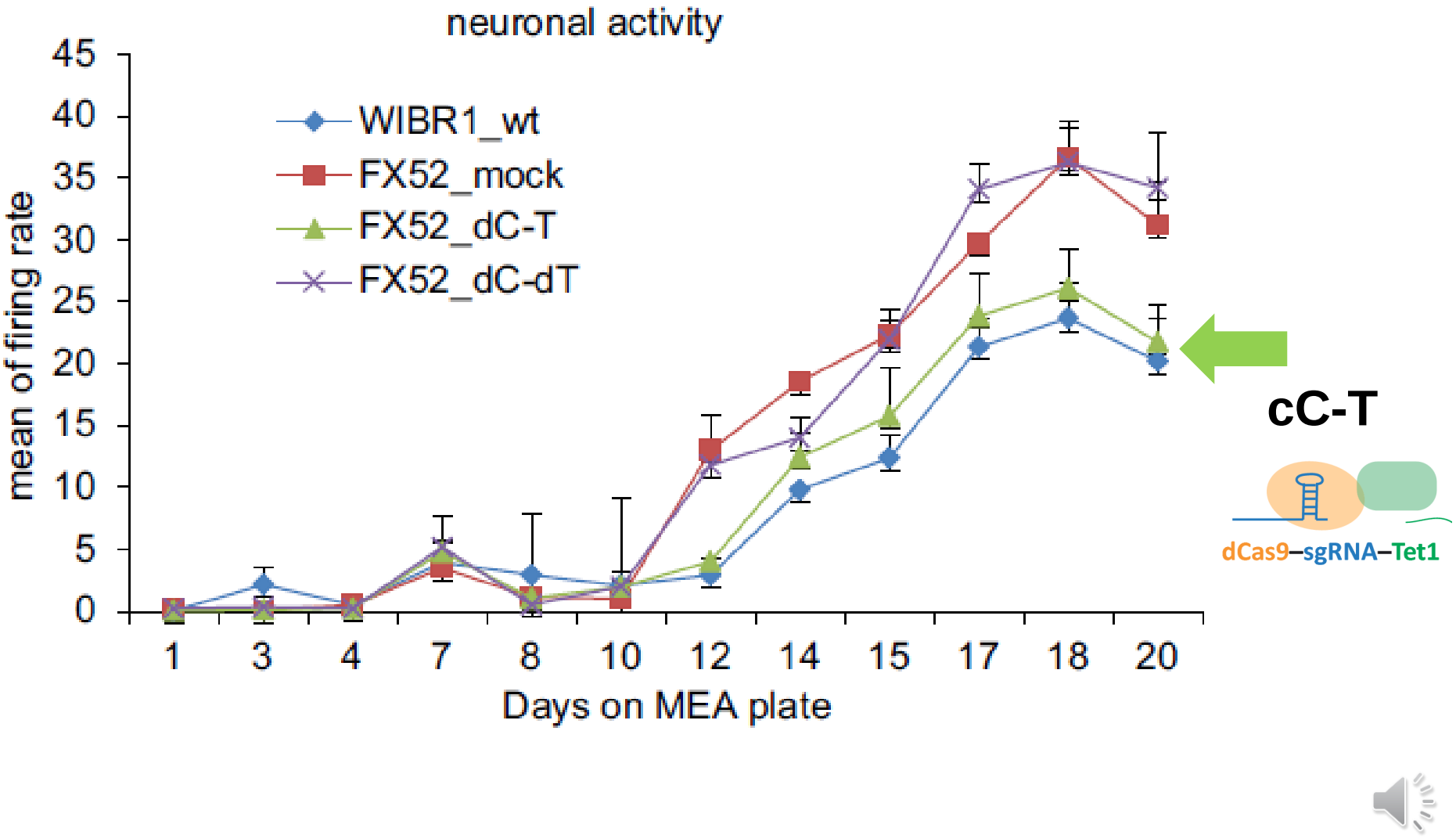
Reactivation of FMR1 expression with dCas9-Tet1/*CGG* sgRNA in post-mitotic neurons derived from FXS iPSCs



BS-seq of the FMR1 promoter in these neurons showed a 20% decrease of the methylation level in the edited FXS neurons compared with FXS mock neurons



Spontaneous hyperactivity associated with FXS neurons was reversed after reactivation of FMR1 in these neurons.



CONCLUSION

- Demethylation of the *CGG* repeats is sufficient to reactivate FMR1.
- Methylation editing is a valid strategy to reactivate FMR1 and to rescue the FXS-related cellular phenotypes

APPLICATION

Epigenome editing can be easily applied to examine the causality of disease-associated DNA methylation events and evaluate the consequences after targeted reversal of the DNA methylation status.