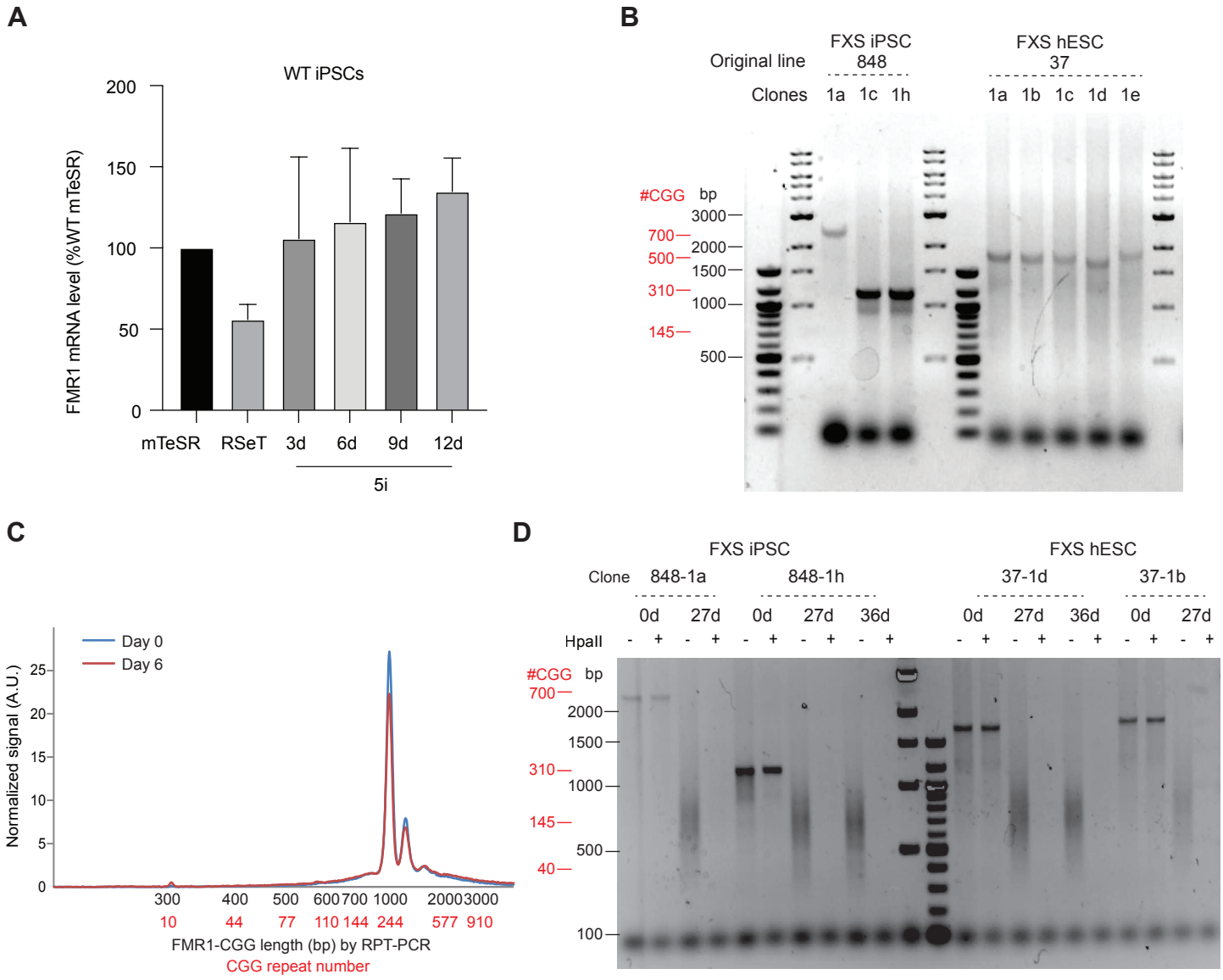
A
WT iPSCs
FMR1 mRNA level (%WT mTeSR)
200
150
100
50
0
mTeSR
RSeT
3d
6d
9d
12d
5i
B
Original line
FXS iPSC
848
FXS hESC
37
Clones
1a 1c 1h
1a 1b 1c 1d 1e
#CGG bp
3000
700
2000
500
1500
310
1000
145
500
C
Day 0
Day 6
Normalized signal (A.U.)
25
20
15
10
5
0
300 400 500 600 700 1000 2000 3000
10 44 77 110 144 244 577 910
FMR1-CGG length (bp) by RPT-PCR
CGG repeat number
D
FXS iPSC
FXS hESC
Clone
848-1a
848-1h
37-1d
37-1b
0d 27d 0d 27d 36d
0d 27d 36d 0d 27d
HpaII
- + - + - + - + - +
- + - + - + - + - +
#CGG bp
700
2000
1500
310
1000
145
500
40
100
E
Pre-mut iPSC clone
131-1a
media
RSeT
5i 27d
HpaII
- + - +
#CGG bp
310
1000
145
66
500
300
F
Wild-type iPSC
8330
media
mTeSR
RSeT
5i 12d
HpaII
- + - + - +
#CGG bp
310
1000
145
66
500
20
300

Figure S1

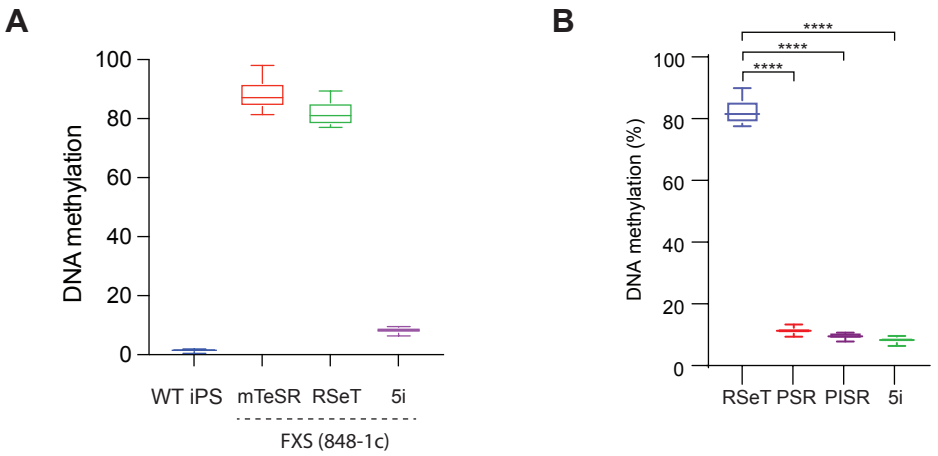
A
DNA methylation
100
80
60
40
20
0
WT iPS
mTeSR
RSeT
5i
FXS (848-1c)
B
DNA methylation (%)
****
****
****
RSeT
PSR
PISR
5i

Figure S2

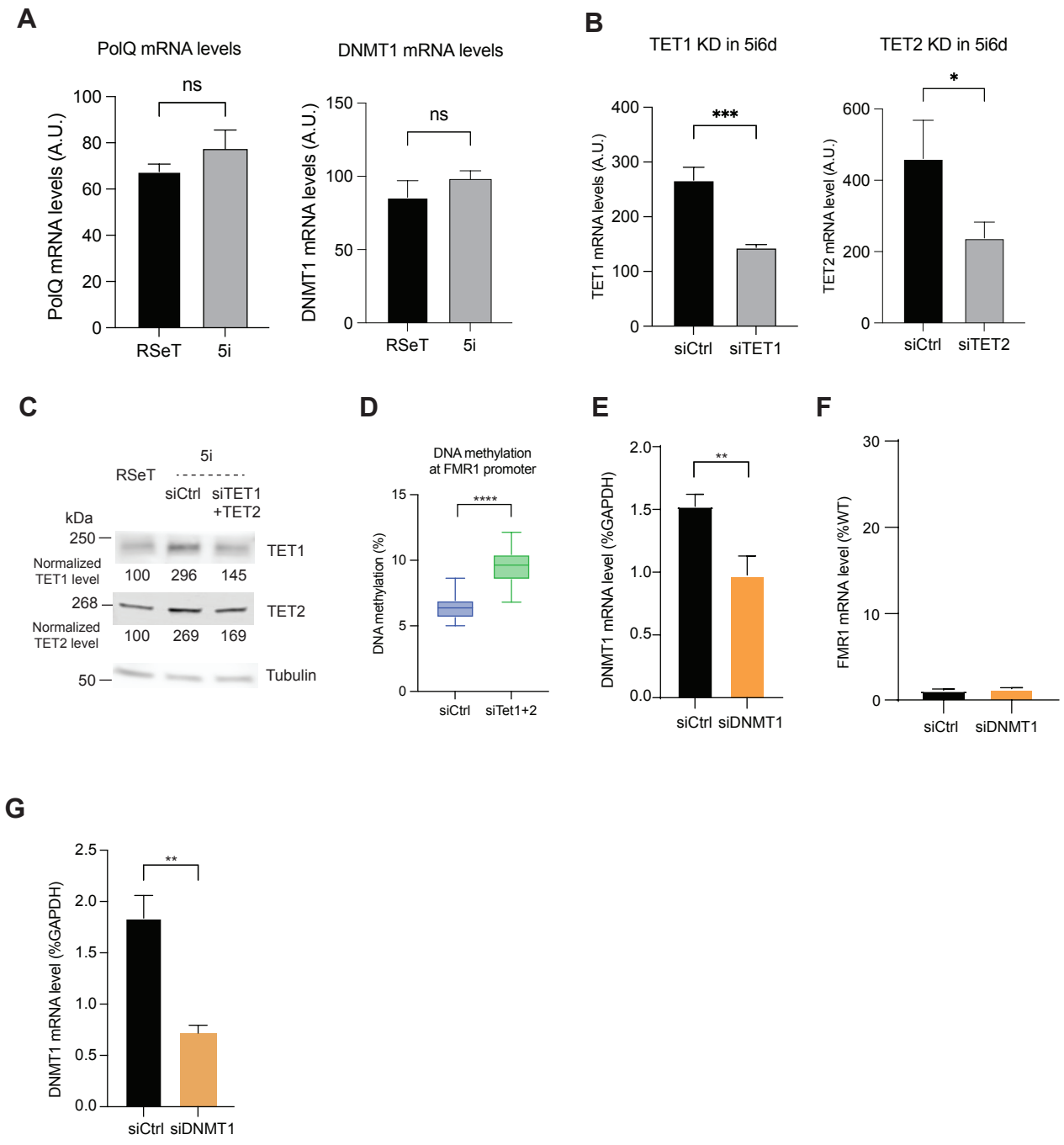
A
PolQ mRNA levels
ns
PolQ mRNA levels (A.U.)
100
80
60
40
20
0
RSeT
5i
DNMT1 mRNA levels
ns
DNMT1 mRNA levels (A.U.)
150
100
50
0
RSeT
5i
B
TET1 KD in 5i6d
***
TET1 mRNA levels (A.U.)
400
300
200
100
0
siCtrl
siTET1
TET2 KD in 5i6d
*
TET2 mRNA level (A.U.)
600
400
200
0
siCtrl
siTET2
C
5i
RSeT
siCtrl
siTET1 +TET2
kDa
250
TET1
Normalized TET1 level
100
296
145
268
TET2
Normalized TET2 level
100
269
169
50
Tubulin
D
DNA methylation at FMR1 promoter
****
DNA methylation (%)
15
10
5
0
siCtrl
siTet1+2
E
**
DNMT1 mRNA level (%GAPDH)
2.0
1.5
1.0
0.5
0.0
siCtrl
siDNMT1
F
FMR1 mRNA level (%WT)
30
20
10
0
siCtrl
siDNMT1
G
**
DNMT1 mRNA level (%GAPDH)
2.5
2.0
1.5
1.0
0.5
0.0
siCtrl
siDNMT1

Figure S3

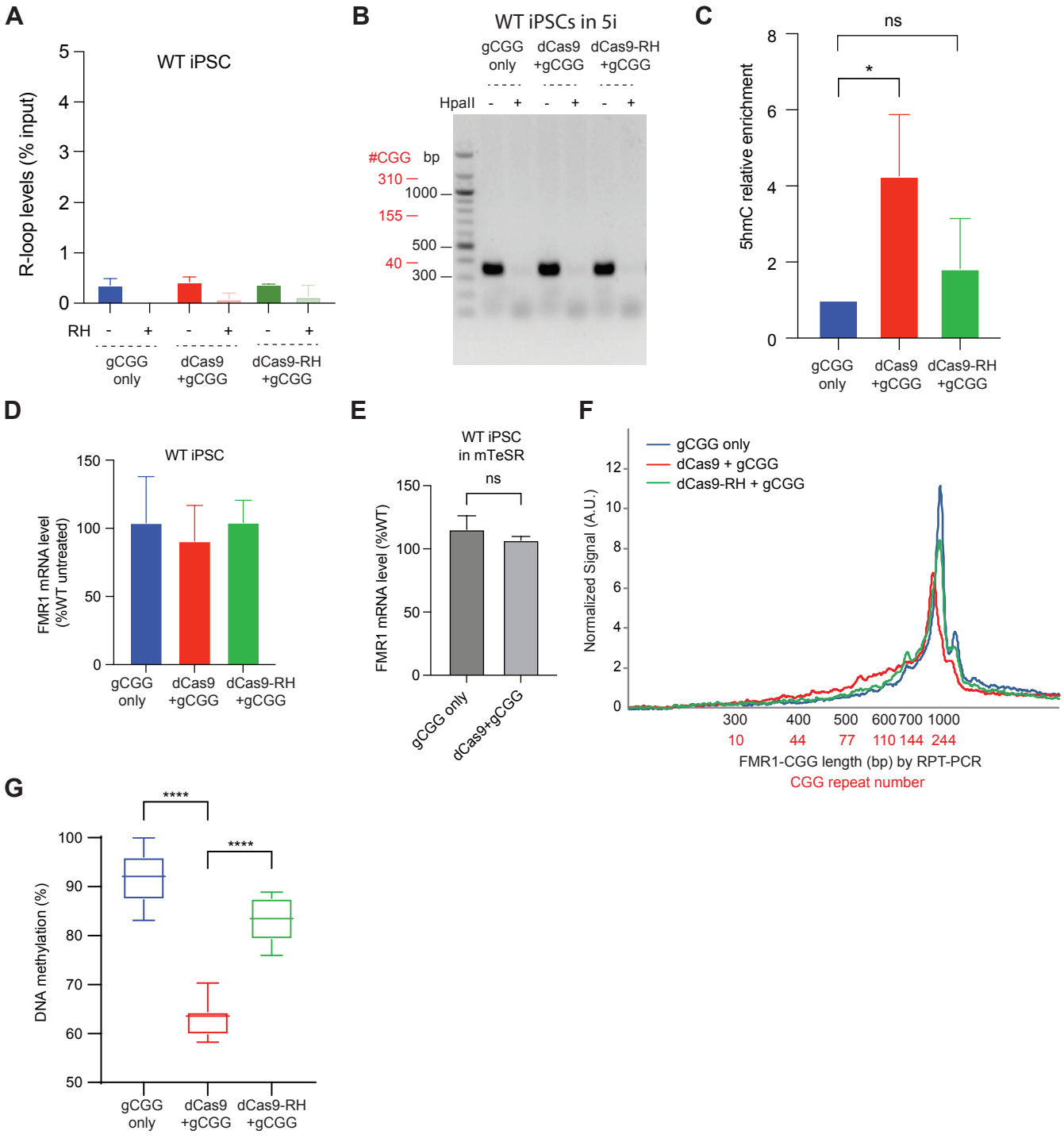
A
WT iPSC
R-loop levels (% input)
RH
gCGG only
dCas9 +gCGG
dCas9-RH +gCGG
B
WT iPSCs in 5i
gCGG only
dCas9 +gCGG
dCas9-RH +gCGG
HpaII
#CGG
bp
310
1000
155
500
40
300
C
ns
*
5hmC relative enrichment
D
WT iPSC
FMR1 mRNA level (%WT untreated)
E
WT iPSC in mTeSR
ns
FMR1 mRNA level (%WT)
gCGG only
dCas9+gCGG
F
gCGG only
dCas9 + gCGG
dCas9-RH + gCGG
Normalized Signal (A.U.)
300 400 500 600 700 1000
10 44 77 110 144 244
FMR1-CGG length (bp) by RPT-PCR
CGG repeat number
G
****
****
DNA methylation (%)

Figure S4

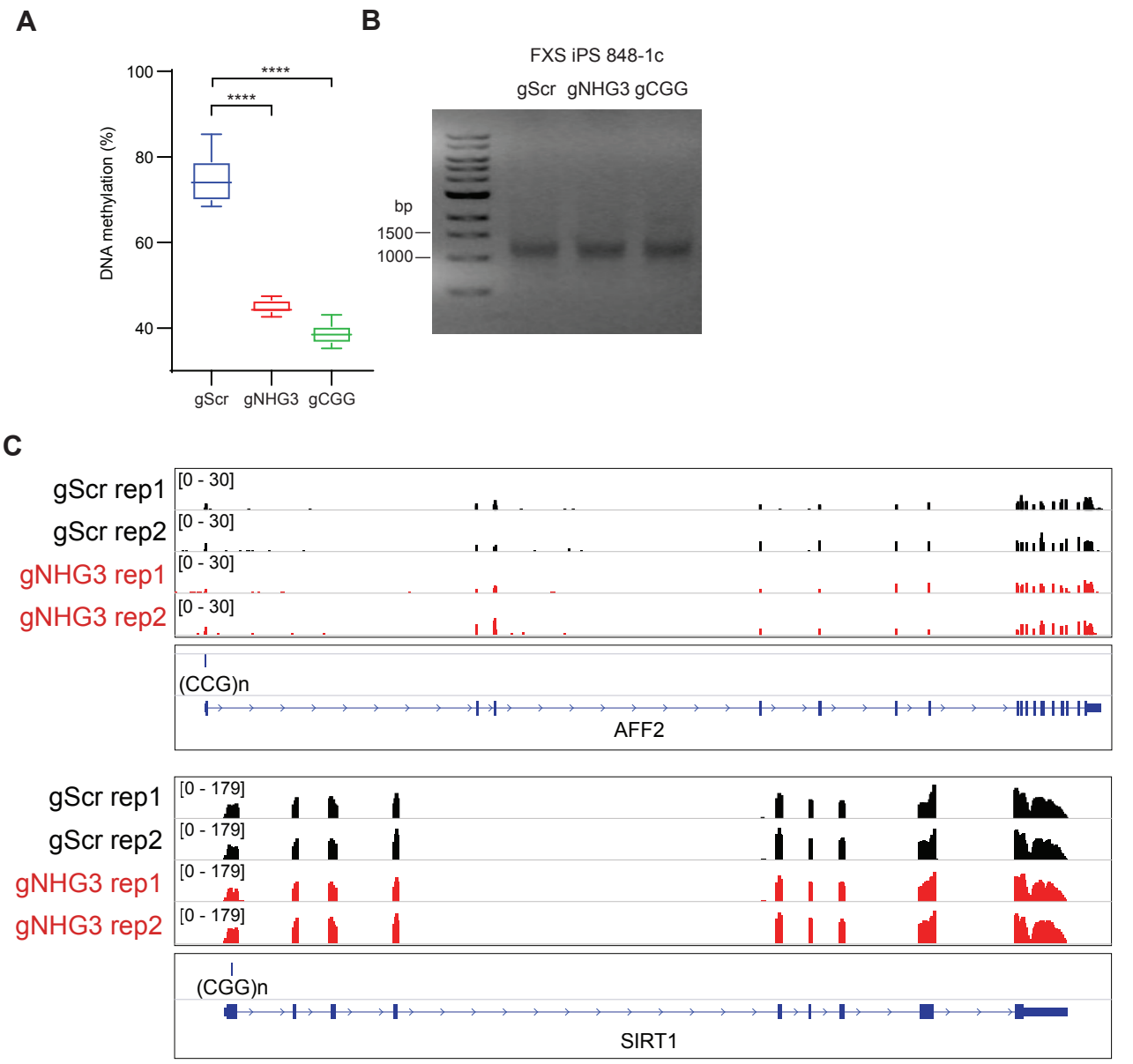
A
B
C
D
E
DNA methylation (%)
100
80
60
40
****
****
gScr
gNHG3
gCGG
FXS iPS 848-1c
gScr gNHG3 gCGG
bp
1500
1000
gScr rep1
gScr rep2
gNHG3 rep1
gNHG3 rep2
[0 - 30]
(CCG)n
AFF2
[0 - 179]
SIRT1
AFF2 CGG repeat
in 848-1c
5i
0d
25d
HpaII
#CGG
500
60
300
WT iPS

Figure S5

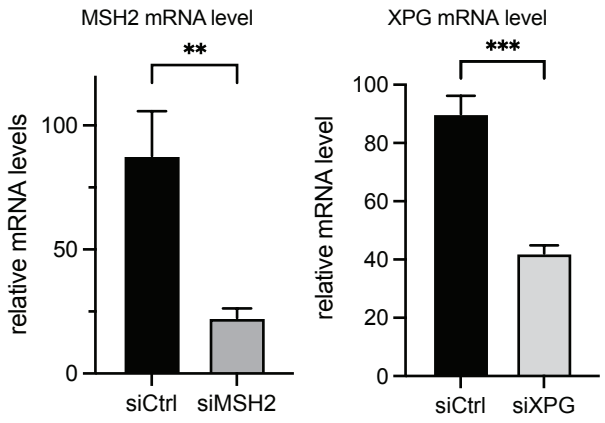
MSH2 mRNA level
**
relative mRNA levels
100
50
0
siCtrl
siMSH2
XPG mRNA level
***
relative mRNA level
100
80
60
40
20
0
siCtrl
siXPG

Figure S6

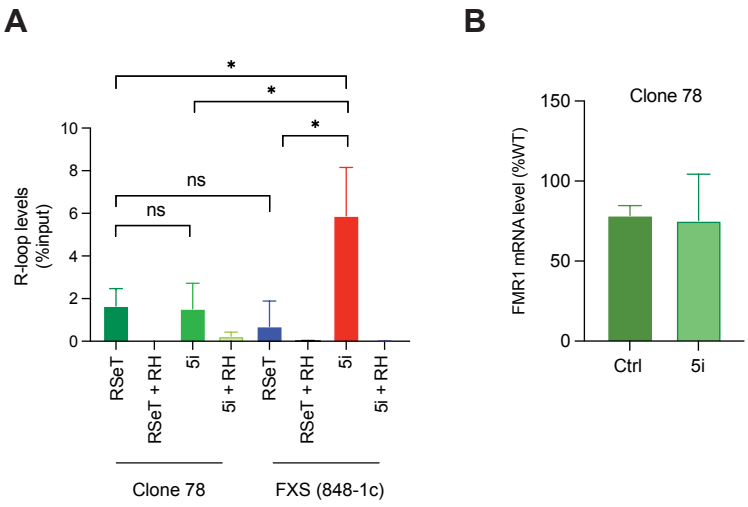
A
B
R-loop levels (%input)
10
8
6
4
2
0
*
*
*
ns
ns
RSeT
RSeT + RH
5i
5i + RH
RSeT
RSeT + RH
5i
5i + RH
Clone 78
FXS (848-1c)
Clone 78
FMR1 mRNA level (%WT)
150
100
50
0
Ctrl
5i

Figure S7

SUPPLEMENTAL DATA

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. FMRP restoration and single cell cloning of FXS hiPSC and hESC lines.

Related to Figure 1.

(A) FMR1 mRNA quantitation from the WT iPSC in mTeSR, RSeT, and 5i media for the indicated number of days. Expression levels normalized to WT iPSC levels in mTeSR media.

(B) Establishment of multiple single cell clones for FXS full mutation iPSC line 848 and hESC line WCMC37 ("37").

(C) Bioanalyzer profiles of RPT-PCR product length and estimated CGG repeat copy number for 848-1c iPSC on day 0 and day 6 of 5i treatment.

(D) RPT-PCR analysis of additional FXS iPSC and hESC clones after 0, 27 or 36 days of 5i treatment.

(E) Gel electrophoresis of CGG RPT-PCR products +/- *HpaII* digestion for pre-mutation iPSC clone 131-1a, grown in RSeT or treated for 27 days with 5i.

(F) Gel electrophoresis of CGG RPT-PCR products +/- *HpaII* digestion for WT iPSC line 8330, grown in mTeSR, RSeT, or treated for 12 days in 5i.

Figure S2. DNA de-methylation by MEKi and BRAFi

Related to Figure 2.

(A) Box plots for quantification of the DNA methylation levels at *FMR1* promoter CpG islands determined by pyrosequencing assay after treating 848-1c cells with mTeSR, RSeT, or 5i.

(B) Box plots for DNA methylation status at *FMR1* promoter CpG islands determined by pyrosequencing assay after treating 848-1c cells with RSeT, 3i (PSR), 4i (PISR), or 5i. P values determined by the Student *t*-test. ****, P<0.0001.

Figure S3. TET1/2 and DNMT1 knockdowns affect DNA methylation levels and repeat contraction in *FMR1*.

Related to Figure 3.

(A) TET1 and TET2 protein levels quantitated by Western blotting in FXS iPSC grown in RSeT media or 5i for 12 days with siRNA knockdown. Tubulin, loading control. TET1 and TET2 levels were quantified by imageJ (Schneider et al., 2012) and normalized to Tubulin.

(B) RT-qPCR for negative controls, PolQ and Dnmt1, for FXS iPSC grown in RSeT media or 5i for 6 days. Student *t*-test: ns, not significant.

(C) TET1 and TET2 mRNA quantitation by RT-qPCR from the FXS iPSC in RSeT media and 5i for 6 days after TET1 and TET2 knock-down as indicated. *t*-test: ***, P<0.001, *, P<0.05

(D) Box plots showing the DNA methylation levels at the *FMR1* promoter, as determined by pyrosequencing assay after treating 848-1c cells (grown in 5i) with siTET1 + siTET2 for 12 days. Student *t*-test: ****, P<0.0001.

(E) RT-qPCR of DNMT1 mRNA levels in the 848-1c cells treated with siCtrl or siDNMT1 for 6 days in RSeT media. *t*-test: **, P<0.01.

(F) RT-qPCR of FMR1 mRNA levels in 848-1c cells treated with siCtrl or siDNMT1 for 6 days in RSeT media.

(G) RT-qPCR of DNMT1 mRNA levels in 848-1c cells treated with siCtrl or siDNMT1 for 6 days in 5i media. *t*-test: **, P<0.01.

Figure S4. CGG repeat contraction by 5i is R-loop-dependent and R-loop formation triggers active DNA demethylation.

Related to Figure 5.

(A) R-loop quantitation by DRIP assays at the *FMR1* TSS in WT control iPSC line, 8330, treated with dCas9 + gCGG, dCas9-RNaseH + gCGG, or gCGG alone in 5i media for 6 days.

(B) Gel electrophoresis of CGG RPT-PCR products +/- *HpaII* digestion for WT iPSC with dCas9 + gCGG, dCas9-RNaseH + gCGG, or gCGG alone, grown in 5i media for 12 days.

(C) 5hmC MeDIP assay at the FMR1 5'UTR in 848-1c iPSC treated with gCCG alone, dCas9+gCGG, or dCas9-RH+gCGG for 6 days. *t*-test: *, P<0.05. ns, not significant.

(D) RT-qPCR for FMR1 mRNA levels in WT iPSC line 8330 with dCas9 + gCGG, dCas9-RNaseH + gCGG, or gCGG alone in 5i media for 6 days.

(E) RT-qPCR for FMR1 mRNA levels in WT iPSC line 8330 with dCas9 + gCGG or gCGG alone in mTeSR media for 24 days.

(F) Bioanalyzer traces of repeat length/copy number distribution in 848-1c cells exposed to 24 days of dCas9 + gCGG, dCas9-RNaseH + gCGG, or gCGG alone in mTeSR media for 24 days.

(G) Box plot quantitation of pyrosequencing to examine DNA methylation levels at *FMR1* promoter-associated CpG islands for the profiles in (A). *t*-test: ****, P<0.0001.

Figure S5. R-loop formation by dCas9 with gCGG and gNHG3 triggers DNA demethylation in *FMR1*, with *FMR1*-specific transcription activation and repeat contraction.

Related to Figure 6.

(A) Box plot quantitation of pyrosequencing to examine DNA methylation status at *FMR1* promoter-associated CpG islands for 848-1c cells exposed to 36 days of dCas9 with gScr, gNHG3, or gCGG in mTeSR media. *t*-test: ****, P<0.0001.

(B) Gel electrophoresis of RPT-PCR products using RGPD1-specific primers to examine promoter-associated CGG repeat tracts. FXS iPS 848-1c cells exposed to dCas9 targeted by gScr, gNHG3, or gCGG for 36 days in mTeSR media.

(C) Genome browser (IGV) views for transcriptomic analysis in two biological replicates (rep1, rep2) treated with dCas9 + gScr versus dCas9 + gNHG3. Two representative negative control genes with CGG tracts at the 5' UTR are shown. Scale is indicated in brackets.

(D) Gel electrophoresis of RPT-PCR products +/- *HpaII* pre-digestion showing absence of off-target CGG contraction at *AFF2* following 25 days of 5i treatment in 848-1c FXS hiPSCs.

(E) Gel electrophoresis of FMR1 RPT-PCR products +/- *HpaII* pre-digestion for WT iPSC line 8330 exposed to dCas9 targeted by gScr, gNHG3, or gCGG for 36 days in mTeSR media.

Figure S6. Knockdown of MSH2 and XPG factors in DNA repair pathways.

Related to Figure 7.

RT-qPCR of MSH2 and XPG mRNA levels in the 848-1c cells treated with siCtrl or siMSH2/siXPG for 6 days in 5i media. *t*-test: **, P<0.01, ***, P<0.001.

Figure S7. CGG repeat threshold effect.

Related to the Discussion.

(A) R-loop levels quantitation by DRIP assays at the *FMR1* TSS region for clone 78 cells and FXS iPS cells (848-1c) in RSeT and 5i media for 6 days.

(B) RT-qPCR of FMR1 mRNA levels in clone #78 cells treated with 5i for 6 days.