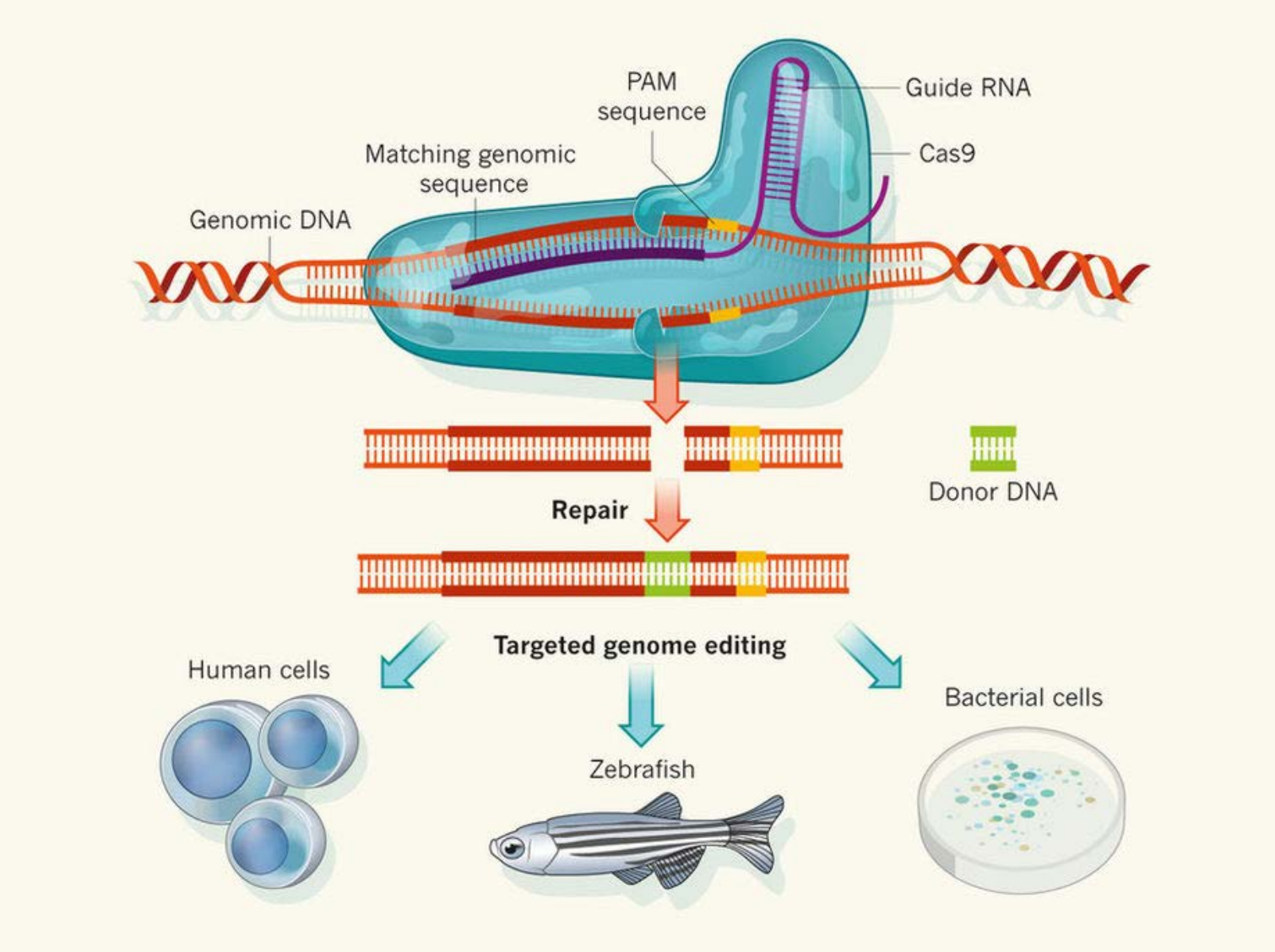
Gene activation and epigenetic modulation using CRISPR/ Cas9

Technical Journal Club

Juliane Bremer

17.4.2018

Crispr/ Cas9



Charpentier et al. Nature. 2013

Crispr/ Cas9

Benefits:

- Can be used to inactivate genes
- Can be used for targeted insertions/ modifications (homologous recombination)
  - For treatment of diseases
  - For basic research

Risks:

- Irreversible genetic alteration
- Off-target effects

Limitations:

- The original Crispr/ Cas9 cannot (directly) activate gene expression

Gene activation using modified CRISPR/ Cas9

1. Epigenetic modification for gene activation

Article

Cell

Cell 172, 979–992, February 22, 2018

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

X. Shawn Liu,[1] Hao Wu,[1,2] 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,*]

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

2. Target gene activation (with trans-epigenetic modulation)

Article

Cell

Cell 171, 1495–1507, December 14, 2017

In Vivo Target Gene Activation via CRISPR/Cas9-Mediated *Trans*-epigenetic Modulation

Hsin-Kai Liao,[1,7] Fumiyuki Hatanaka,[1,7] Toshikazu Araoka,[1,2,7] Pradeep Reddy,[1] Min-Zu Wu,[1,3] Yinghui Sui,[3] Takayoshi Yamauchi,[1,2] Masahiro Sakurai,[1] David D. O'Keefe,[1] Estrella Núñez-Delicado,[3] Pedro Guillen,[4] Josep M. Campistol,[5] Cheng-Jang Wu,[6] Li-Fan Lu,[6] Concepcion Rodriguez Esteban,[1] and Juan Carlos Izpisua Belmonte[1,8,*]

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Gene activation using modified Crispr/ Cas9

1. Epigenetic modification for gene activation

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Background I

- To avoid double-strand breaks: catalytically inactive Cas9
- Dnmt: DNA methyltransferase
- Tet: Ten-eleven translocation enzymes which demethylates DNA

➢ Fusion of catalytically inactive Cas9 with these DNA methylation modification enzymes Dnmt or Tet (dCas9-Dnmt/Tet)
(Choudhury 2016, Liu 2016, Vojta 2016, Xu 2016)

Background II

Fragile X syndrome (FXS)

- 1: 3,600 males
- Intellectual disabilities with spectrum of autistic phenotypes

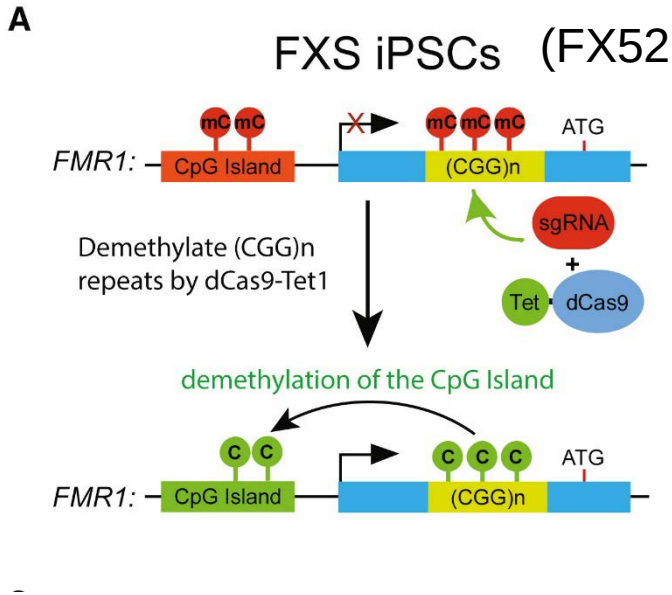
- Due to loss of fragile X mental retardation protein (FMRP) encoded by *FMR1* gene
- FMRP is an RNA binding protein, downregulates local protein synthesis at synapses
- FXS patient derived neurons show hyper-excitability

- CGG repeat expansion (>200) in 5’ UTR of FMR1
- Accompanied by DNA hypermethylation, heterochromatin formation and silencing of *FMR1*

- Currently no mouse model which recapitulates FXS (no effect of repeat expansion in mice)
- Mechanistic understanding limited
- Patient-derived embryonic stem cells (ESC) and induced pluripotent stem cells (iPSCs) model hypermethylation and *FMR1* silencing

Results

Demethylation of the CGG repeats to reactivate *FMR1* in FXS iPSCs

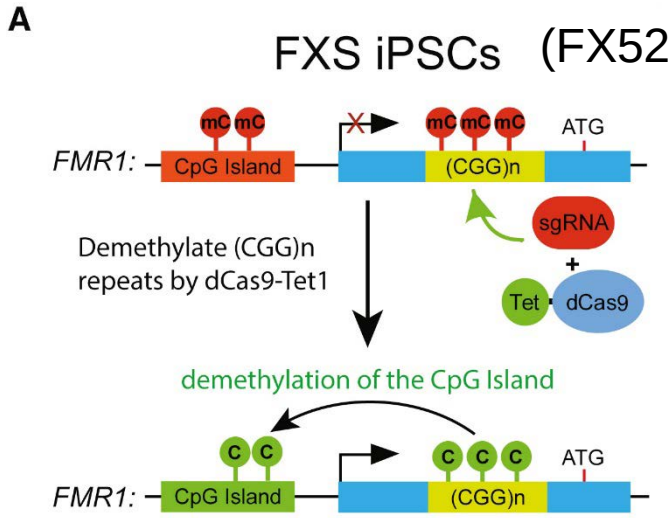


2 lentiviruses
- dCas9-Tet1 (dC-T)-p2a-TagBFP
- mCherry-expressing sgRNA (CGG)

FACS sorted (BFP, mCherry)

Results

Demethylation of the CGG repeats to reactivate *FMR1* in FXS iPSCs



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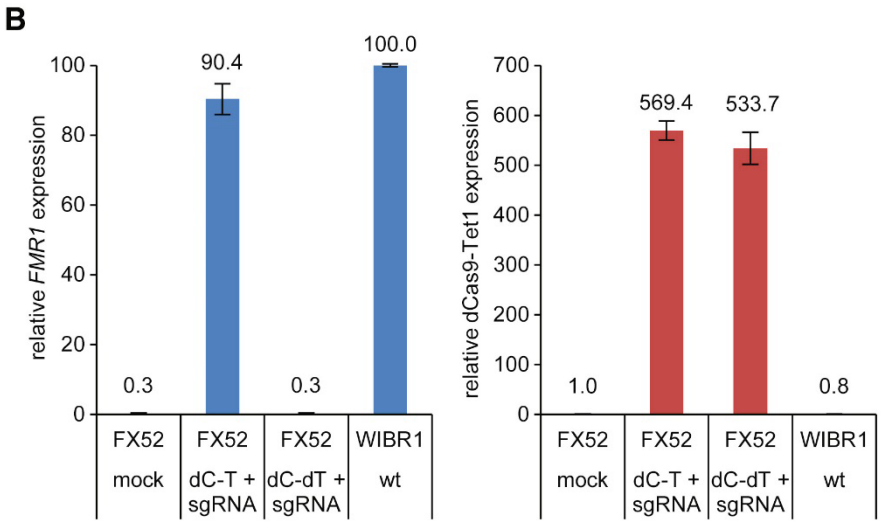
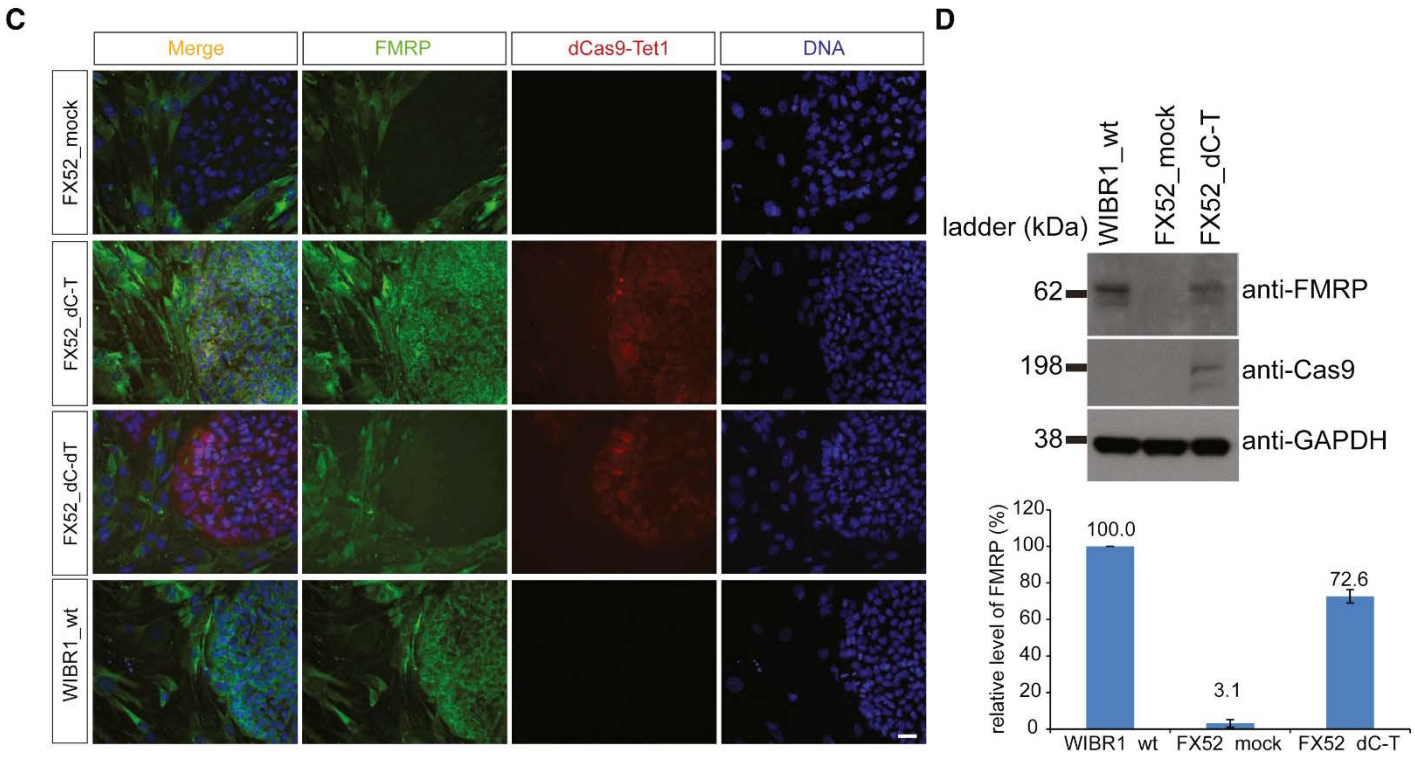


To 90 % restored *FMR1* expression in dC-T (but not in dC-dT)

Results

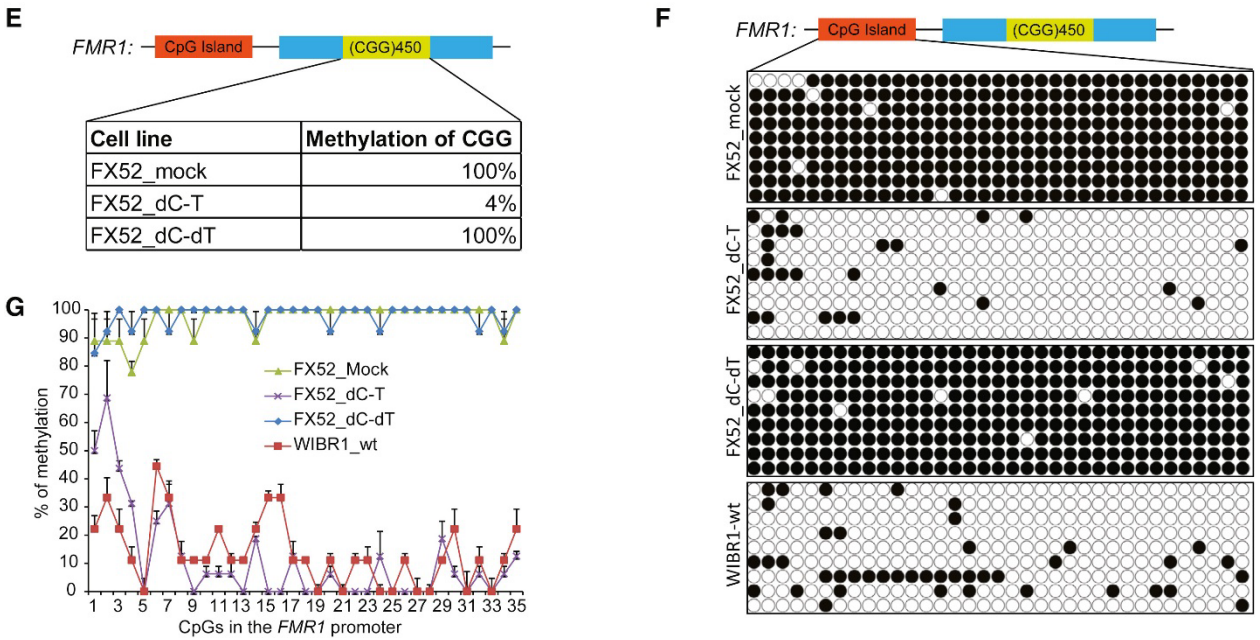
Demethylation of the CGG repeats to reactivate *FMR1* in FXS iPSCs

To 73 % restored FMRP expression in dC-T
(but not in dC-dT)



Results

Demethylation of the CGG repeats to reactivate *FMR1* in FXS iPSCs



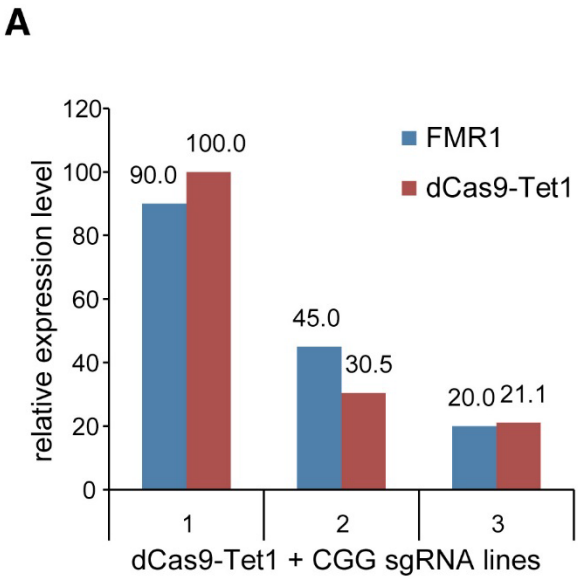
Bisulfide sequencing (BS-seq)

Methylation analysis of CGG repeats showed a significant reduction from 100% in mock FX52 iPSCs to 4% in dC-T/CGG

Robust demethylation of CpG islands in the *FMR1* promoter region

Results

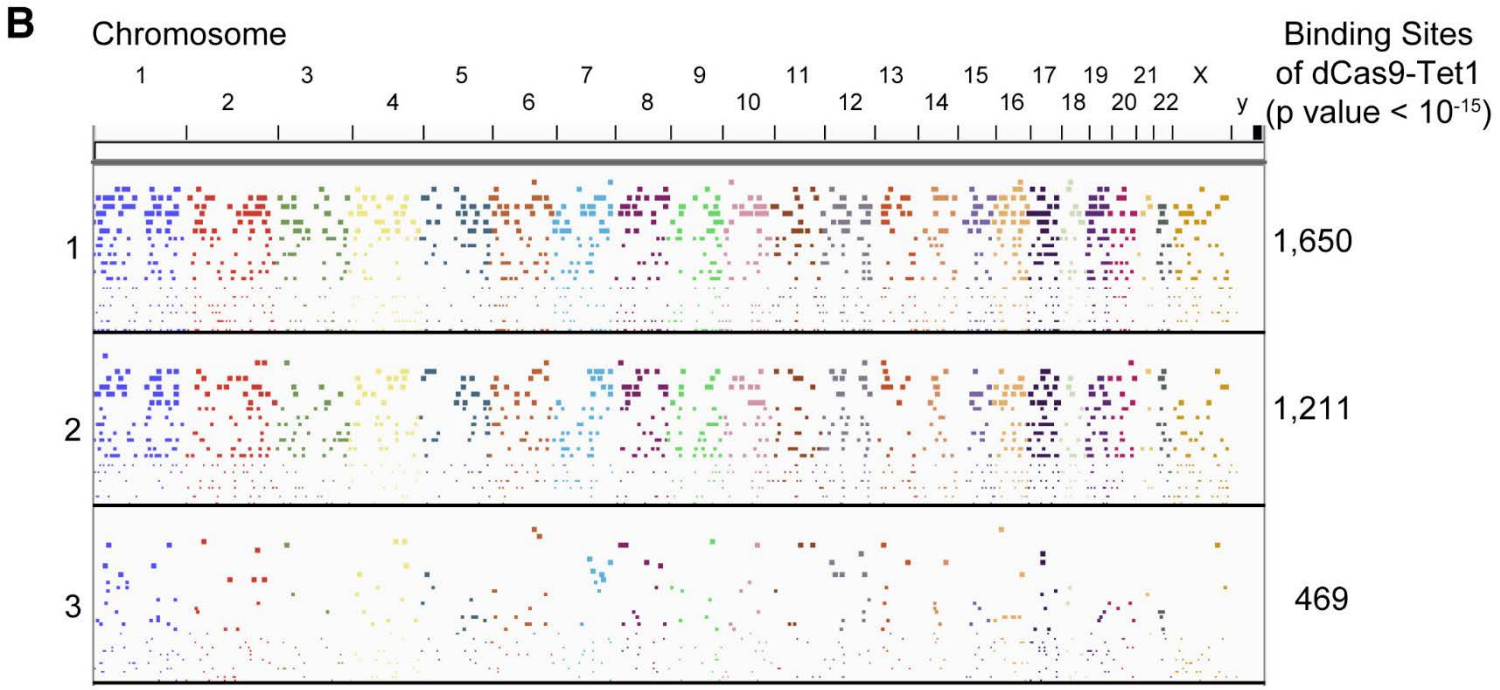
Demethylation of the CGG repeats to reactivate *FMR1* in FXS iPSCs



Restoration of *FMR1* in different FX52 iPSC lines with different levels of dCas9-Tet1
Restoration of *FMR1* decreased with lower expression of dCas9-Tet1

Results

Off-target effects of dCas9-TET1/CGG sgRNA



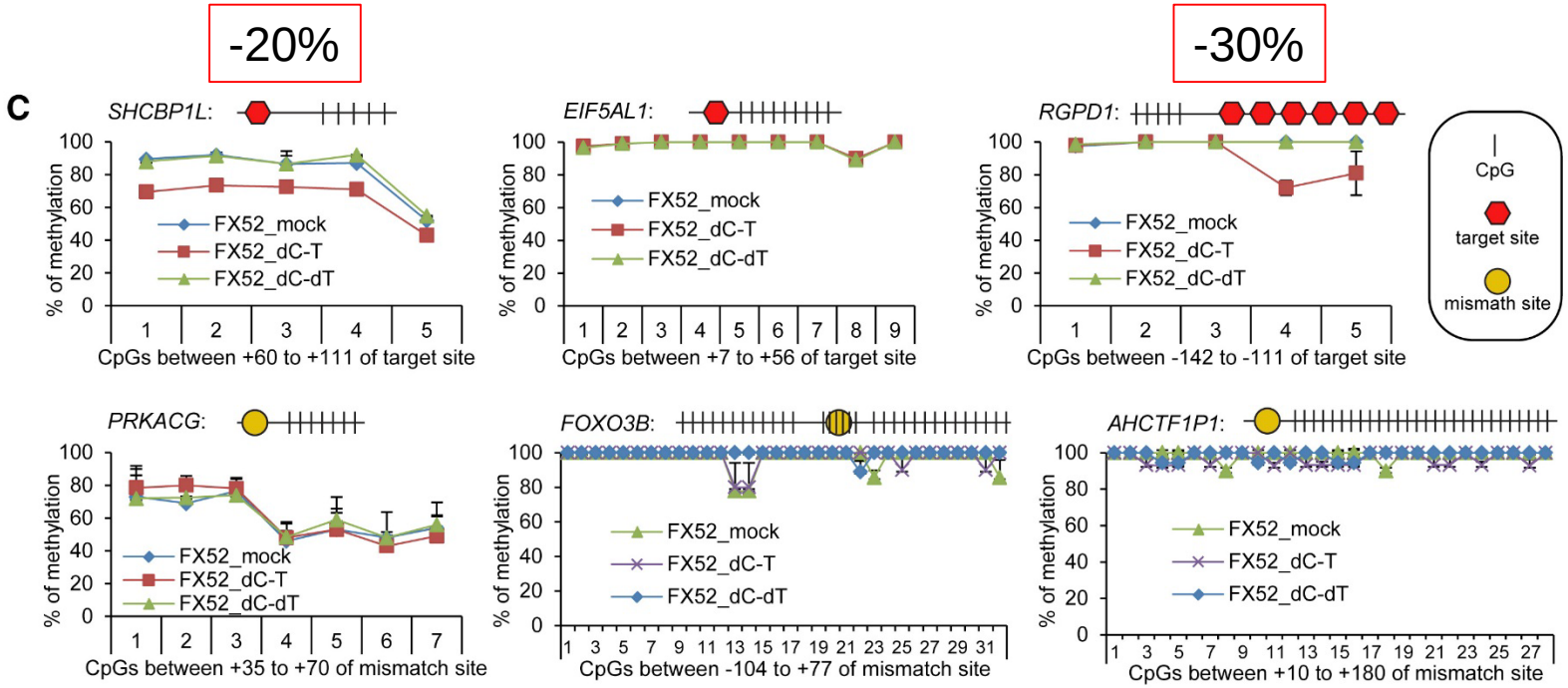
dCas9-Tet1 ChIP-seq

Genome-wide binding sites of dCas9-Tet1/CGG sgRNA in three cell lines

Number of binding sites decreased when expression level of dCas9-Tet1 was reduced

Results

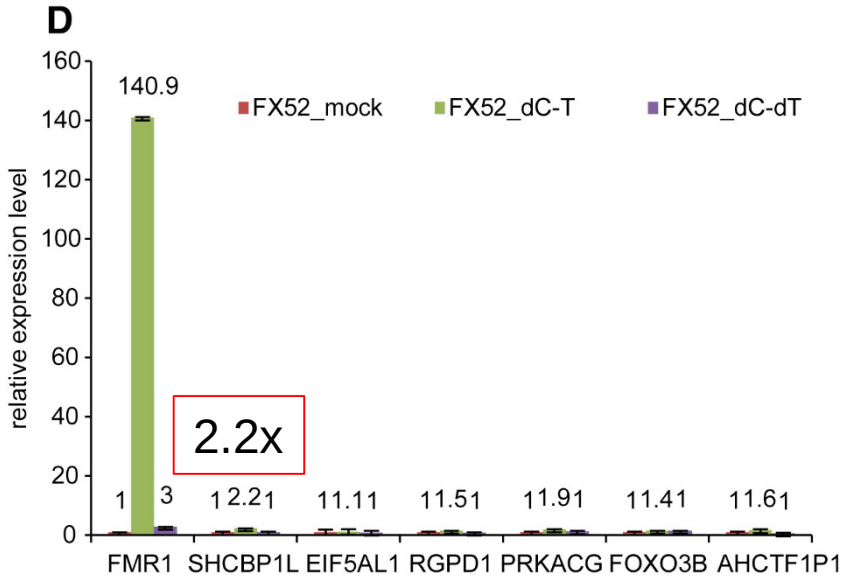
Off-target effects of dCas9-TET1/CGG sgRNA



BS-seq and pyrosequencing (Pyro-seq) of the 6 top off-target candidate gene loci that overlapped with methylated promoter regions according to a hESC/ iPSC methylome study previously reported (Lister et al. 2009) and showed the highest binding affinity of dCas9-Tet1

Results

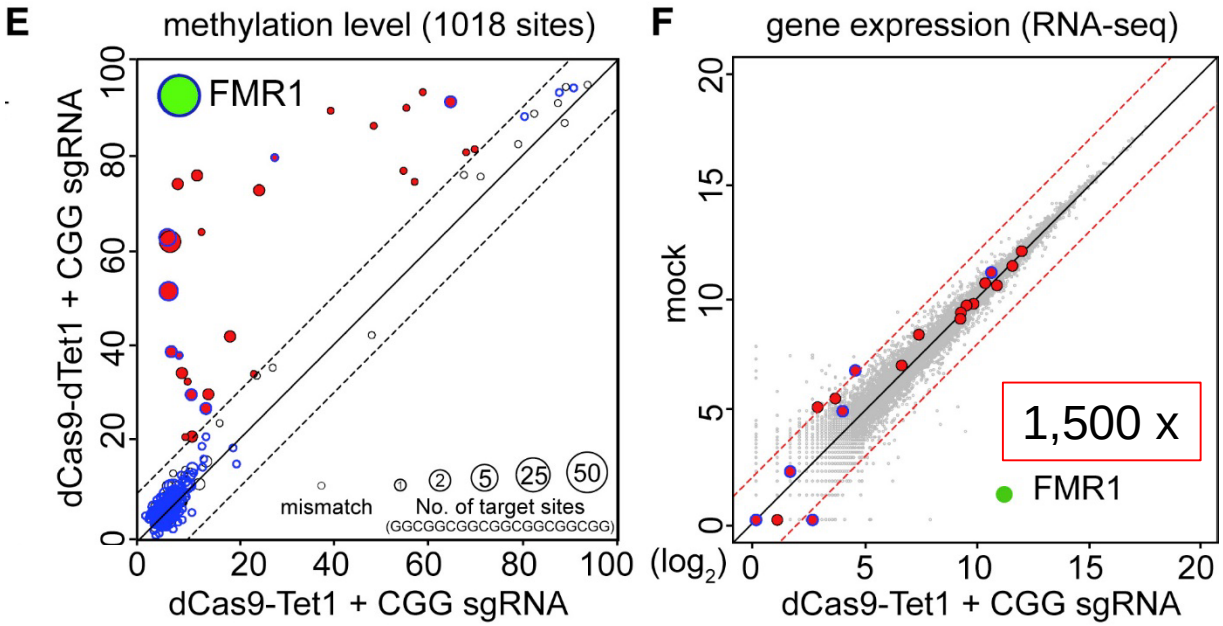
Off-target effects of dCas9-TET1/CGG sgRNA



Gene expression analyses of the 6 top off-target candidate genes by qPCR.

Results

Off-target effects of dCas9-TET1/CGG sgRNA – genome wide



Anti-Cas9 ChIP-BS-seq of FX52 iPSCs expressing dCas9-Tet1/CGG sgRNA or dCas9-**d**Tet1/CGG sgRNA

29 sites with a change of methylation larger than 10% (dashed lines) labeled in red

Blue lines of circles indicate binding sites overlapping with promoter region

RNA-seq of dCas9-Tet1/CGG sgRNA versus mock

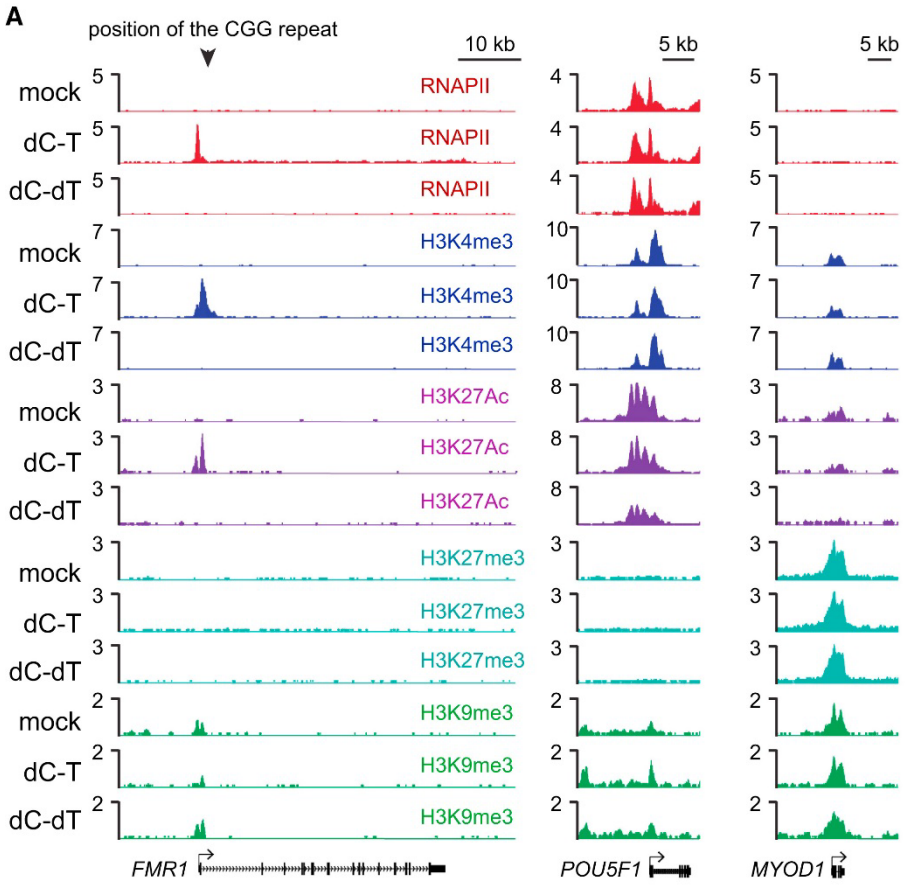
Red dots mark these 28 genes with change of methylation >10%. FMR1 in green.

Red line: 4 fold change range.

- Off-target effects of dCas9-Tet1/CGG are minimal

Results

Activation of heterochromatic *FMR1* promoter



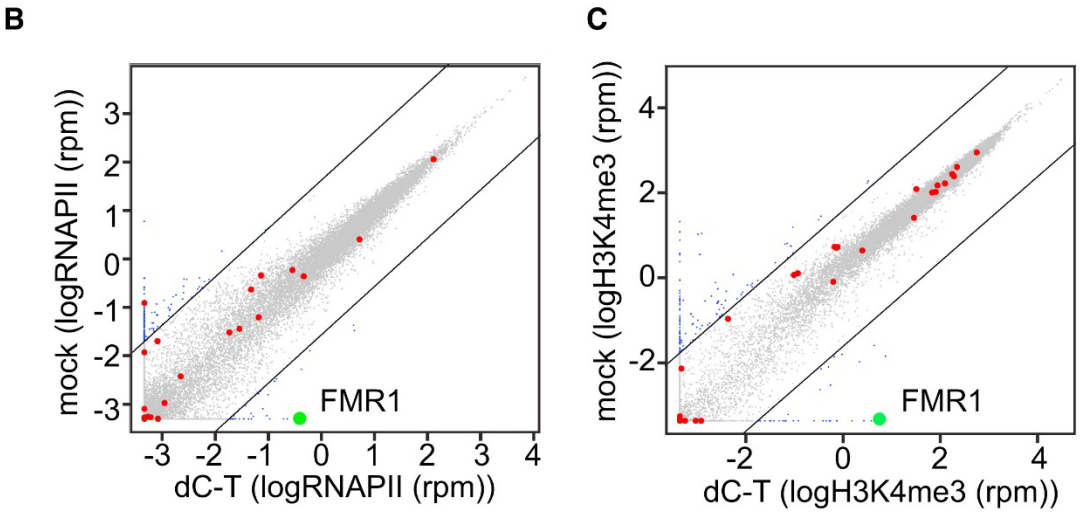
Epigenetic state of *FMR1* promoter:
ChIP-seq experiments with antibodies

- RNA pol II
- H3K4me3 (histone H3 lysine 4 trimethylation), active mark, decreased in *FMR1*
- H3K27Ac, active mark, decreased in FMR1
- H3K27me3, repressive mark
- H3K9me3, repressive mark, increased in *FMR1*

➢ Active *FMR1* chromatin conformation in methylation-edited cells
➢ No change in control genes

Results

Activation of heterochromatic *FMR1* promoter



Genome-wide analysis of Pol II occupancy:

FMR1 most upregulated gene

28 genes with >10% methylation change: no change or < 3 fold

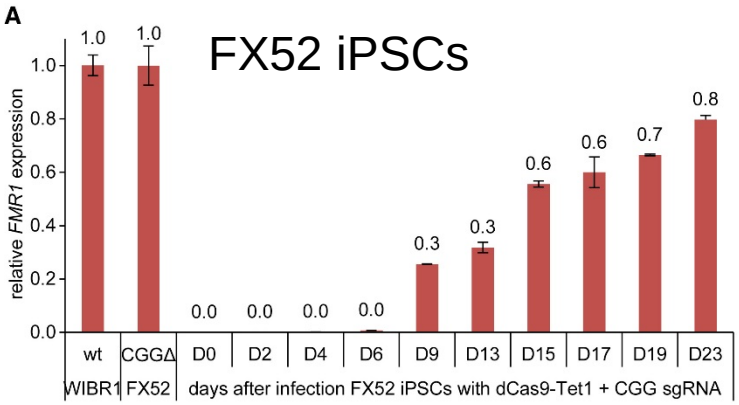
GSE1 5 fold (without expression change)

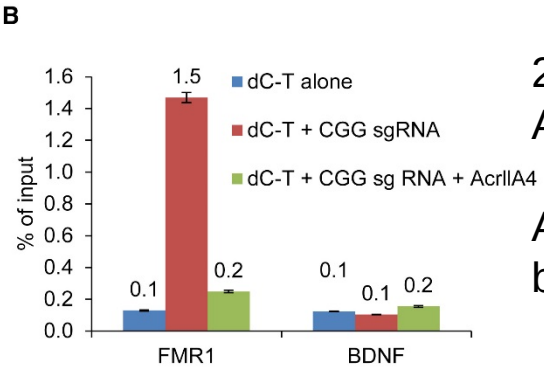
Genome-wide analysis of active histone H3K4me3 distribution:

effect on FMR1, but no change or < 3 fold for 28 genes

Results

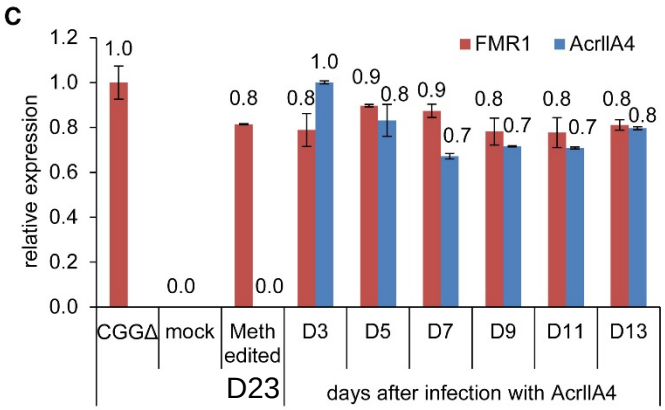
Kinetics and persistence of FMR1 reactivation

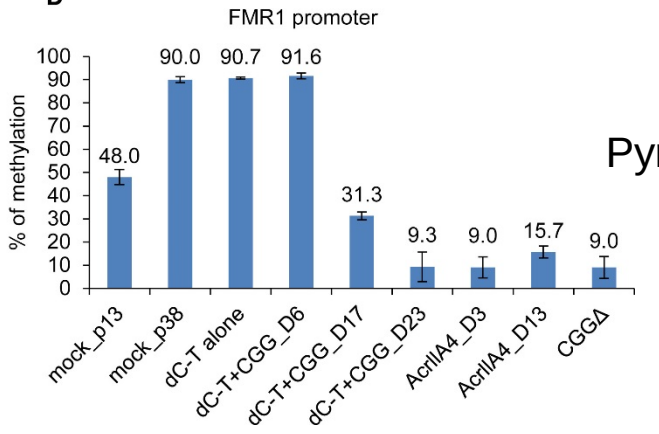




293T cells
Anti-Cas9 ChIP/ qPCR

AcrIIA4 inhibits
binding of Cas9 to FMR1



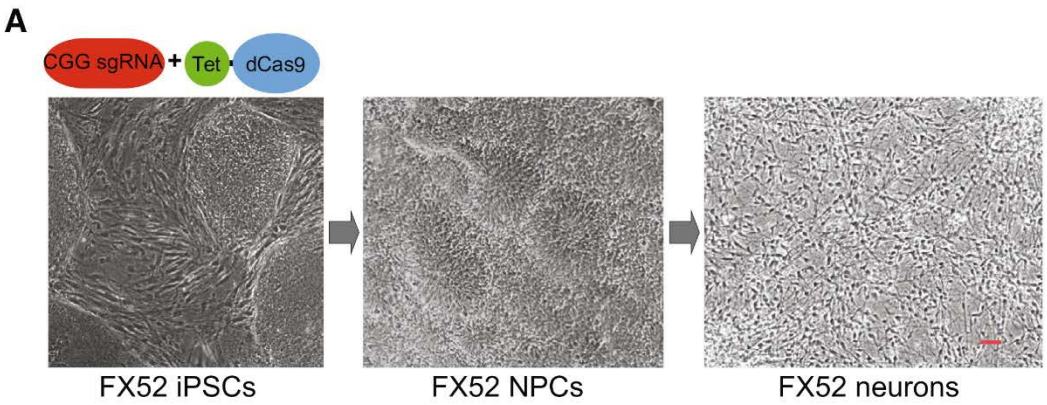


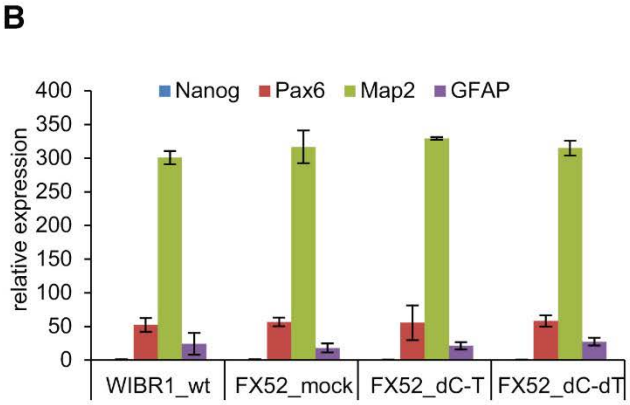
Pyro-seq

First *FMR1* detected 9 days after lentiviral infection (dCas9-Tet1/CGG sgRNA)
And peaked at 3 weeks, accompanied by demetylation of the *FMR1* promoter.
To test persistence, AcrIIA4 – a Cas9/dCas9 inhibitor was co-expressed
FMR1 expression & demethylation maintained for at least 2 weeks after Cas9 inhibition

Results

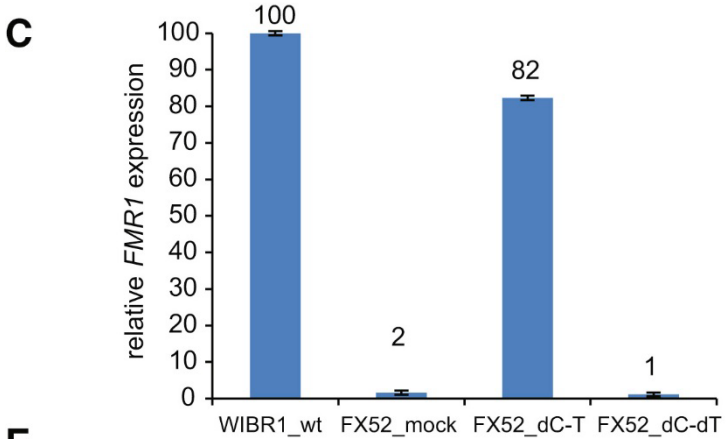
Rescue of FXS phenotype in edited neurons





Post-mitotic neurons derived from methylation-edited FX52 iPSCs

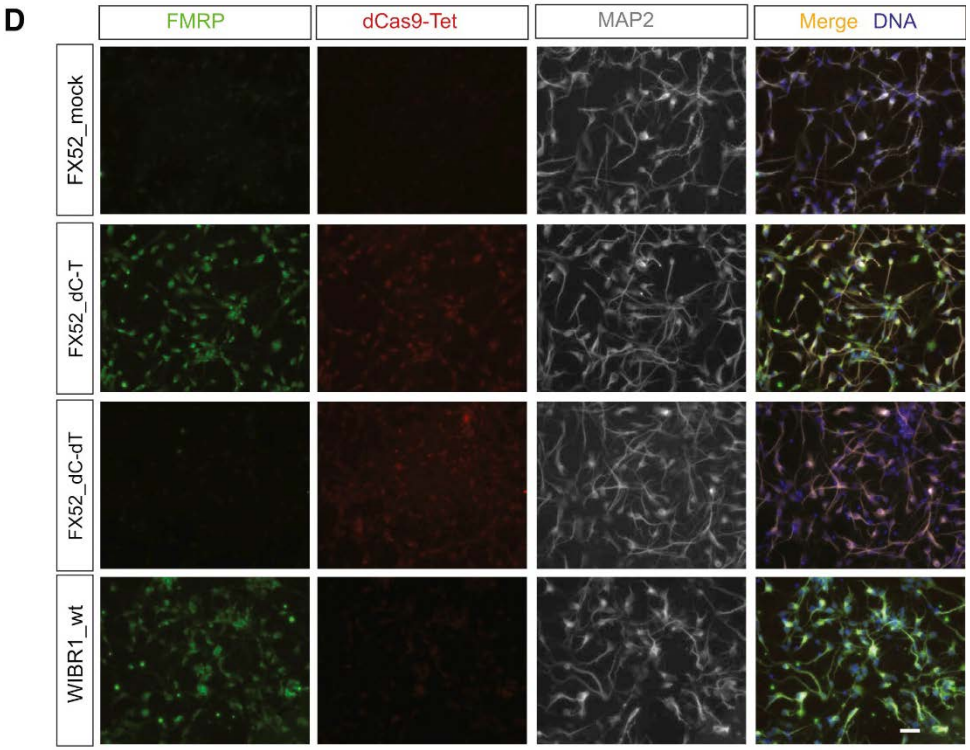
Gene expression analysis of lineage-specific markers suggest comparable differentiation states



Expression level of *FMR1* in neurons expressing dC-T/CGG restored to 82%

Results

Rescue of FXS phenotype in edited neurons

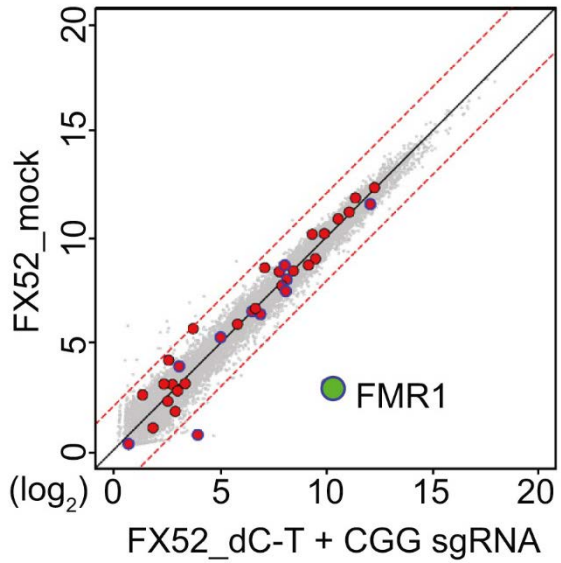


Post-mitotic neurons derived from methylation-edited FX52 iPSCs

FMRP in neurons expressing dC-T/CGG restored

Results

Rescue of FXS phenotype in edited neurons

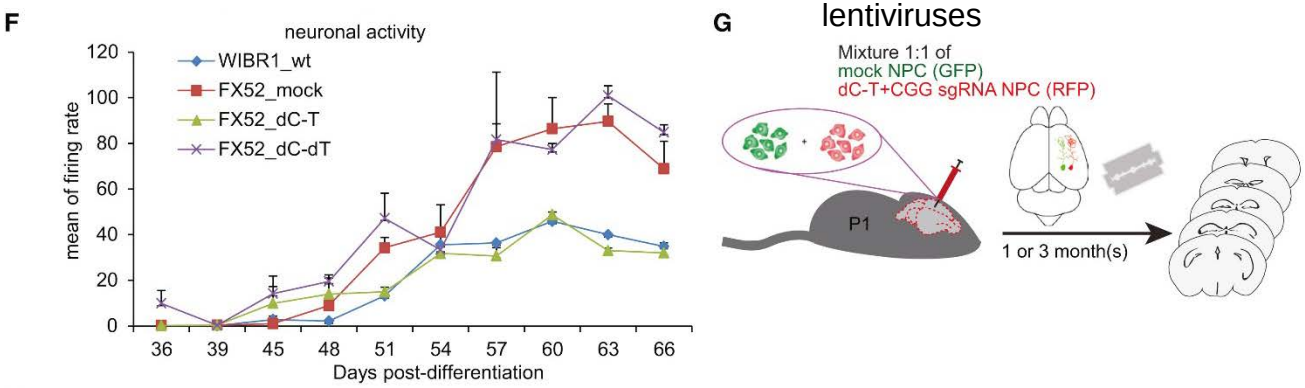


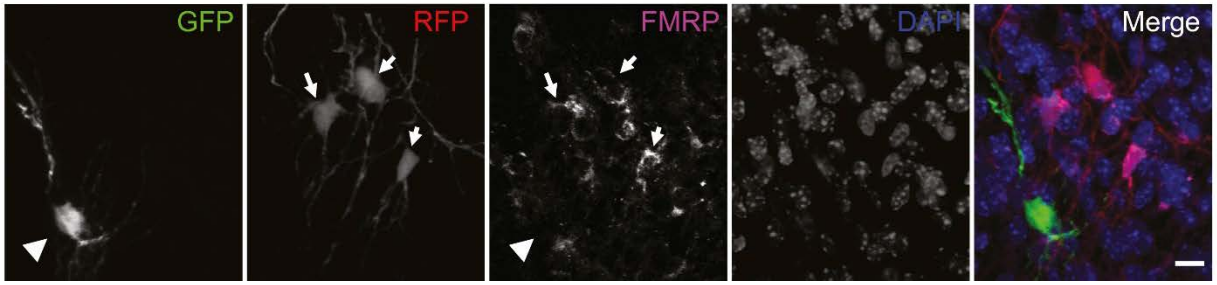
Transcriptome analysis of FX52 mock neurons and edited neurons:
FMR1 as the most upregulated gene (481 fold) and
no or < 4 fold change of expression for 41 genes with methylation changes <10% (ChIP-BS-seq)
9-fold change for RGPD1 gene

- (almost) specific reactivation of *FMR1*

Results

Rescue of FXS phenotype in edited neurons



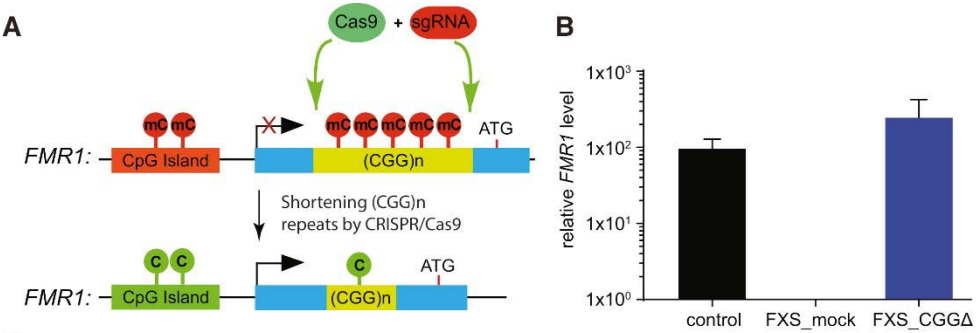


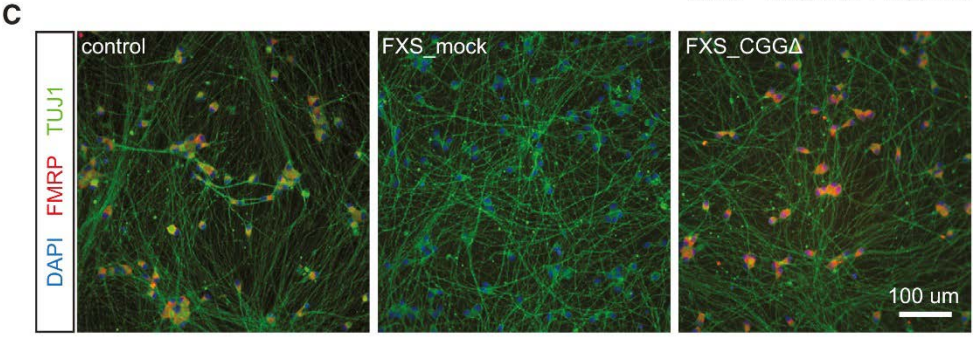
Multi-electrode array (MEA) showed that *FMR1* reactivation reversed the spontaneous hyperactive phenotype of FXS neurons.

FMR1 reactivation in edited FXS neurons is sustained after engrafting into mouse brains, 56% and 57% of edited neurons expressed FMRP after 1 and 3 months

Results

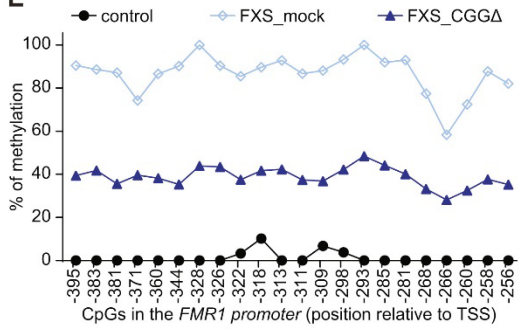
Deletion of CGG repeats (by CRISPR/Cas9) results in a similar phenotypic rescue as epigenetic editing

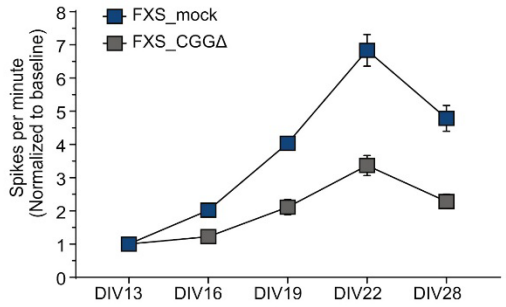




D

	CGG number	CGG methylation	promoter methylation
control	23	1%	1%
FXS_mock	>450	>90%	87%
FXS_CGGΔ	0	n/a	39%

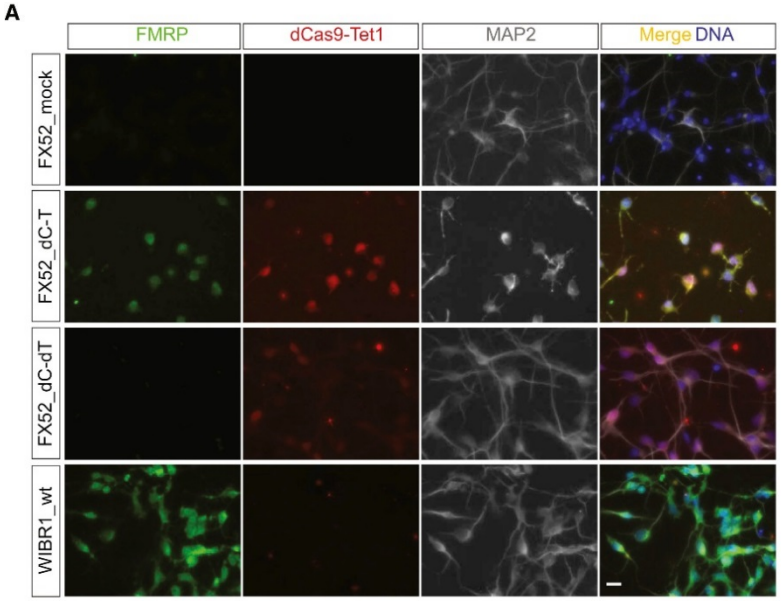


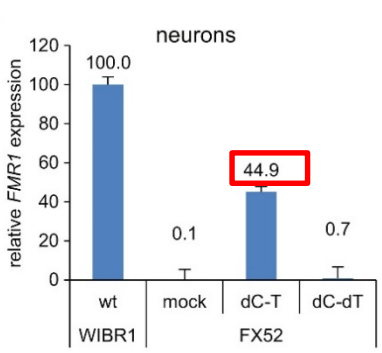


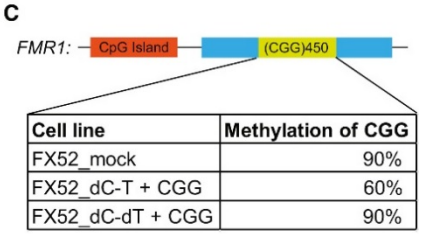
Reduced spontaneous hyperactivity

Results

Direct reactivation of *FMR1* in FXS neurons

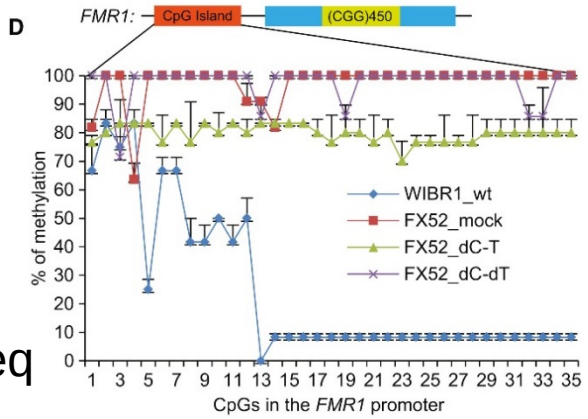


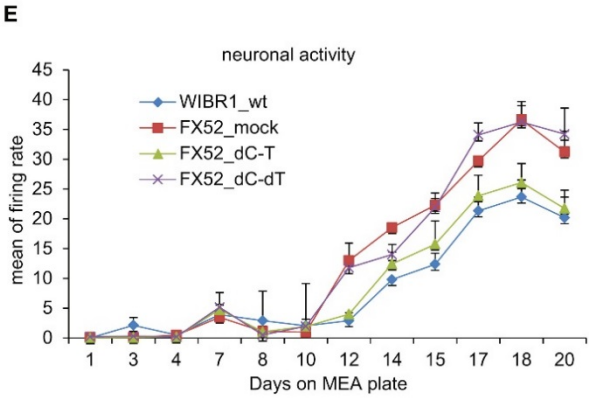




Cell line	Methylation of CGG
FX52_mock	90%
FX52_dC-T + CGG	60%
FX52_dC-dT + CGG	90%

BS-seq





dC-T/CGG lentivirus in post-mitotic neurons derived from FXS iPSCs

Less activation of FMR1 (inability to isolate double vector-infected neurons by FACS)

Discussion

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

Great experimental system to investigate the toxicity of CGG repeats in neurons.

Restoration of FMRP protein and rescue of FXS cellular phenotypes after reactivation of *FMR1* argue that the cellular toxicity assumed for CGG repeat containing *FMR1* RNA is likely to be minimal in methylation edited FXS cells within a short period of culture time.

Whether FXS phenotype can be reversed postnatally is unknown, but the rescue of FMRP expression and neuronal hyperactivity suggest that functional deficits may be at least partially reversible.

Rescue of neuronal hyperactivity in edited neurons was normalized to wt levels, although FMR1 was only restored to 45%, full restoration may not be necessary for functional rescue.

Gene activation using modified Crispr/ Cas9

2. Target gene activation (with trans-epigenetic modulation)

Article

Cell

Cell 171, 1495–1507, December 14, 2017

In Vivo Target Gene Activation via CRISPR/Cas9-Mediated *Trans*-epigenetic Modulation

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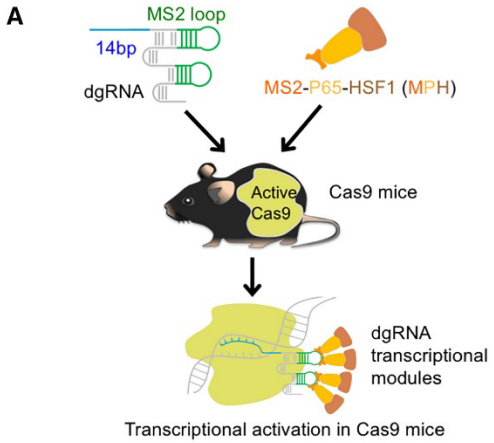
Background I

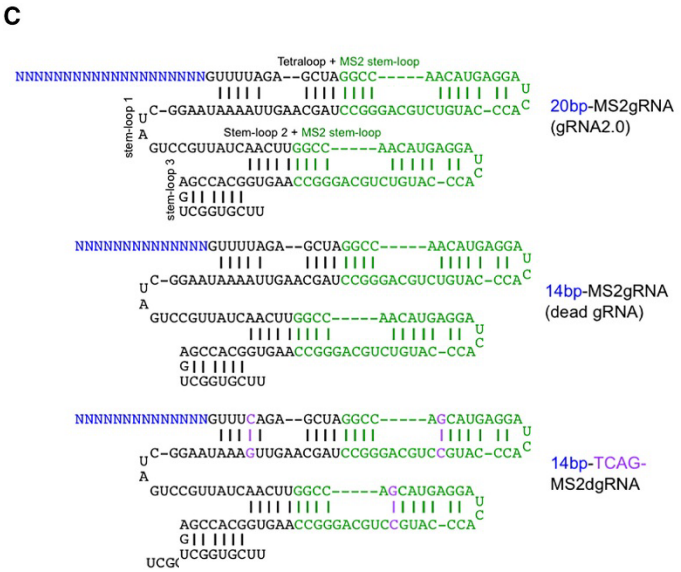
- To convert CRISPR/Cas9 into a transactivator by fusing a transcriptional activation domain (VP64) to catalytically inactive Cas9
  (Gilbert 2013, Perez-Pinera 2013)
- Original version required recruitment of multiple sgRNAs to the target gene for robust target gene activation (TGA), diminishing the utility
  (Gilbert 2013, Perez-Pinera 2013)
- To improve the efficiency, multiple transcriptional activation domains were fused or recruited to the dCas9/gRNA complex, e.g. dCas9-VPR, -SAM, -Suntag
  (Chavez 2015, Konermann 2015, Tanenbaum 2014)
- These Cas9-TGA systems proved effective for functional genetic studies *in vitro*, but insufficient transduction of the Cas9 fusion protein *in vivo*
  and low level of *in vivo* TGA
  sequences encoding the dCas9/gRNA and co-transcriptional activator complexes exceed the capacity of most viral vectors, e.g. AAV.

Results

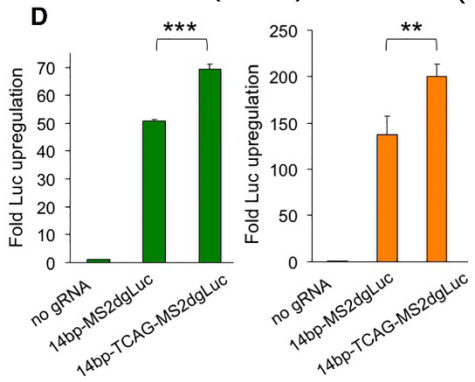
An optimized CRISPR/Cas9 system enables target gene activation

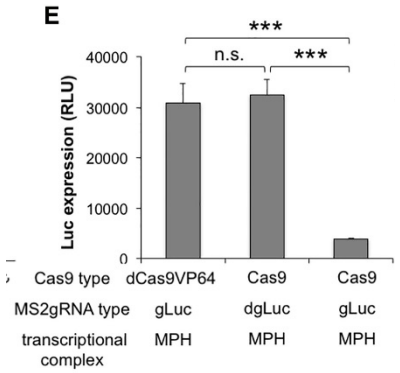
- Short sgRNA (14-15bp instead of 20bp) to guide wt Cas9, prevent active Cas9 from making double-strand breakes = dead sgRNA (dgRNA)
- dgRNA with two MS2 domains for recruiting MPH (MS2:P65:HSF1) transcriptional activator
  Optimization of MS2 dgRNA by mutagenesis, using Luciferase reporter





293T cells (Cas9) N2a cells (Cas9)





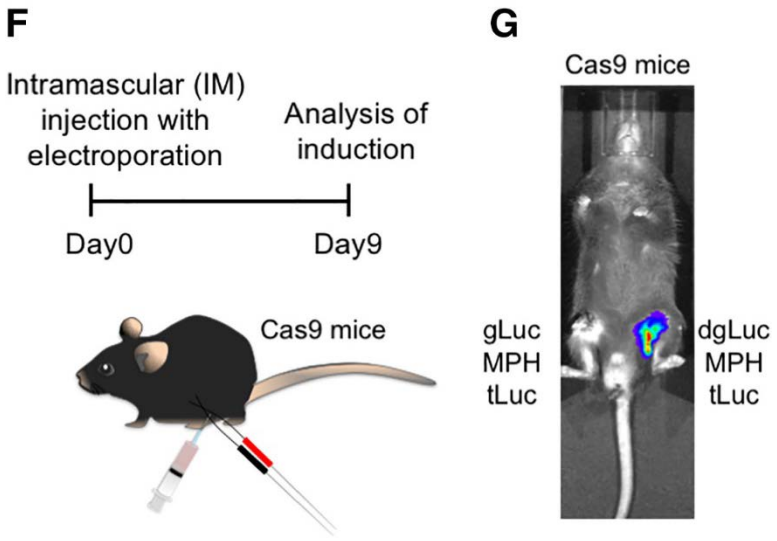
- High level of TGA in vitro

Results

An optimized CRISPR/Cas9 system enables target gene activation

- To test efficiency *in vivo*,
  1. plasmids containing luciferase reporter and
  2. plasmids containing optimized MS2dgLuc and MPH sequences were co-injected into hind limb muscles of adult Cas9-expressing mice electroporated into muscle cells
  luciferase was measured 9 days later.

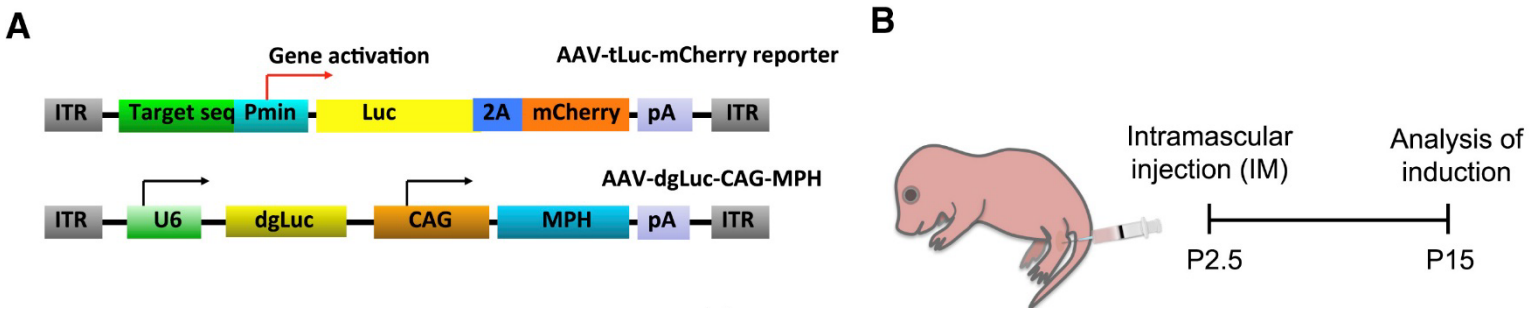
➢ TGA in vivo

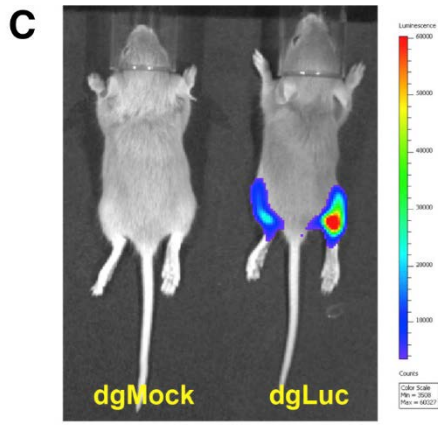


Results

AAV-mediated CRISPR/Cas9 TGA system activates reporters in different organs

- AAV-dgLuc-CAG-MPH
- AAV-tLuc-mCherry (reporter)
- Bilaterally co-injected these into hind-limb muscles of Cas9-expressing mice (P2.5) and assesses luciferase activity at P15

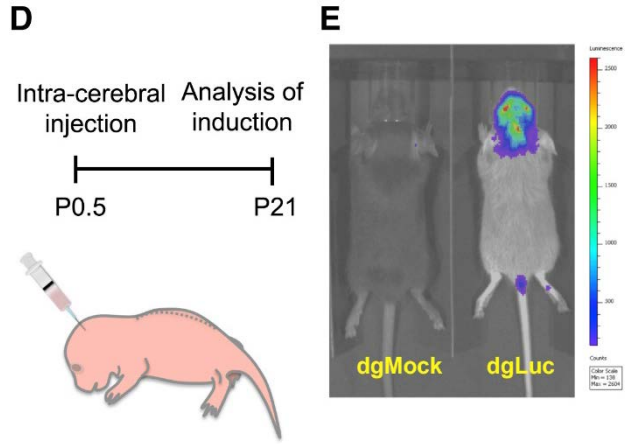


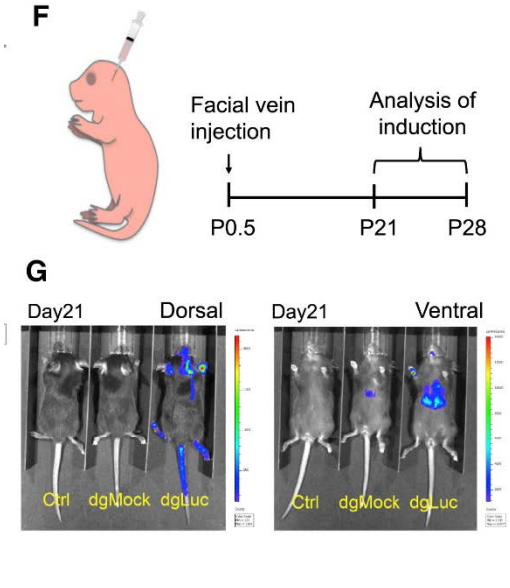


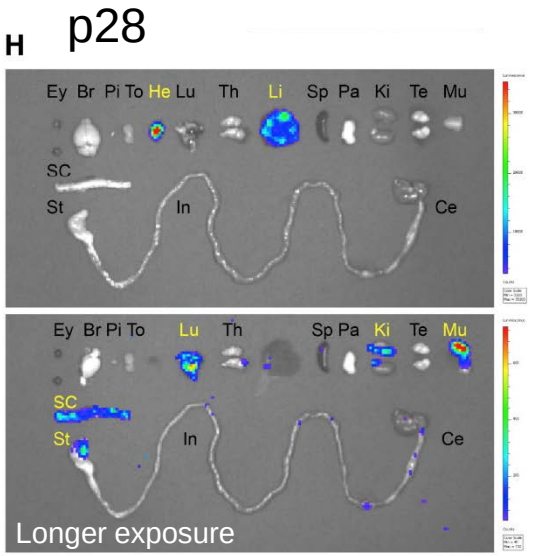
➢ *In vivo* expression in muscle

Results

AAV-mediated CRISPR/Cas9 TGA system activates reporters in different organs

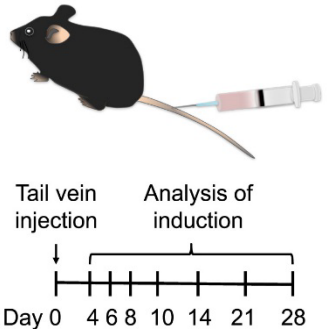


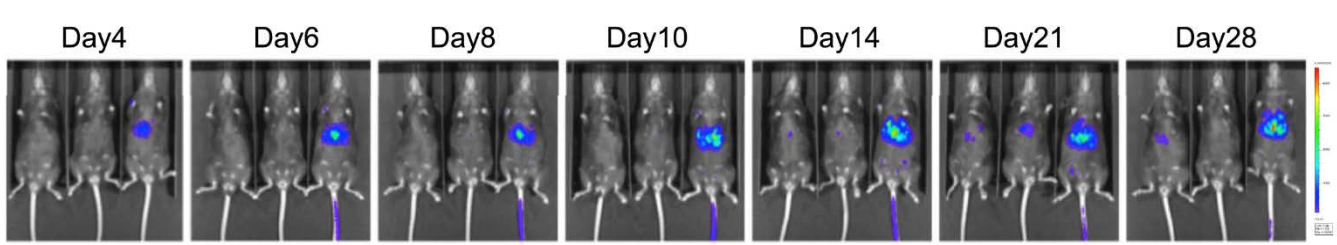




- In vivo expression in the brain

- Expression after systemic administration in neonatal mice



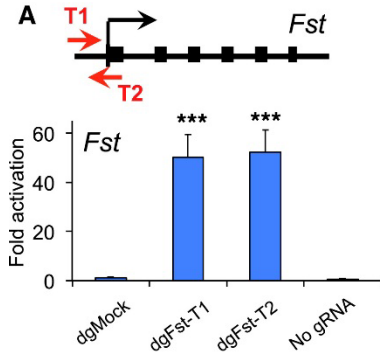


- Expression in liver after systemic administration in adult mice (11wks)

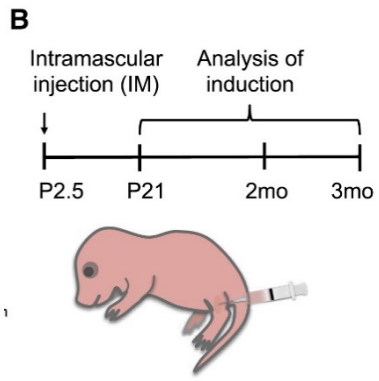
Results

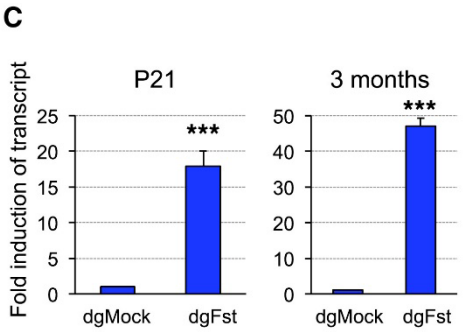
Phenotypic enhancement of muscle mass induced by *in vivo* CRIPR/Cas9

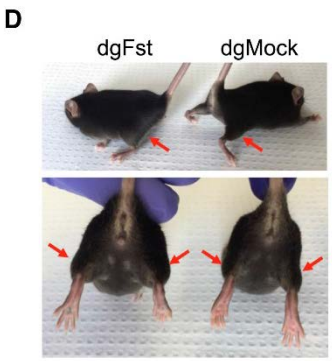
- Activate an endogenous gene (follistatin (*Fst*))
- N2a cells (Cas9) transfected with dgFst-target1-MPH or dgFst-target2-MPH plasmids

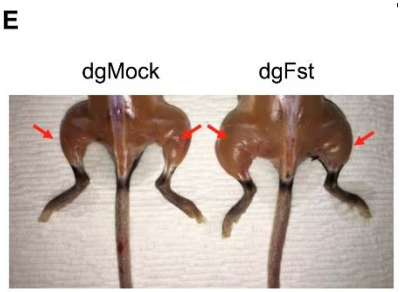


- AAV-dgFst-T2-MPH injected intramuscularly into the hind limb of Cas9-expressing mice (P2.5)









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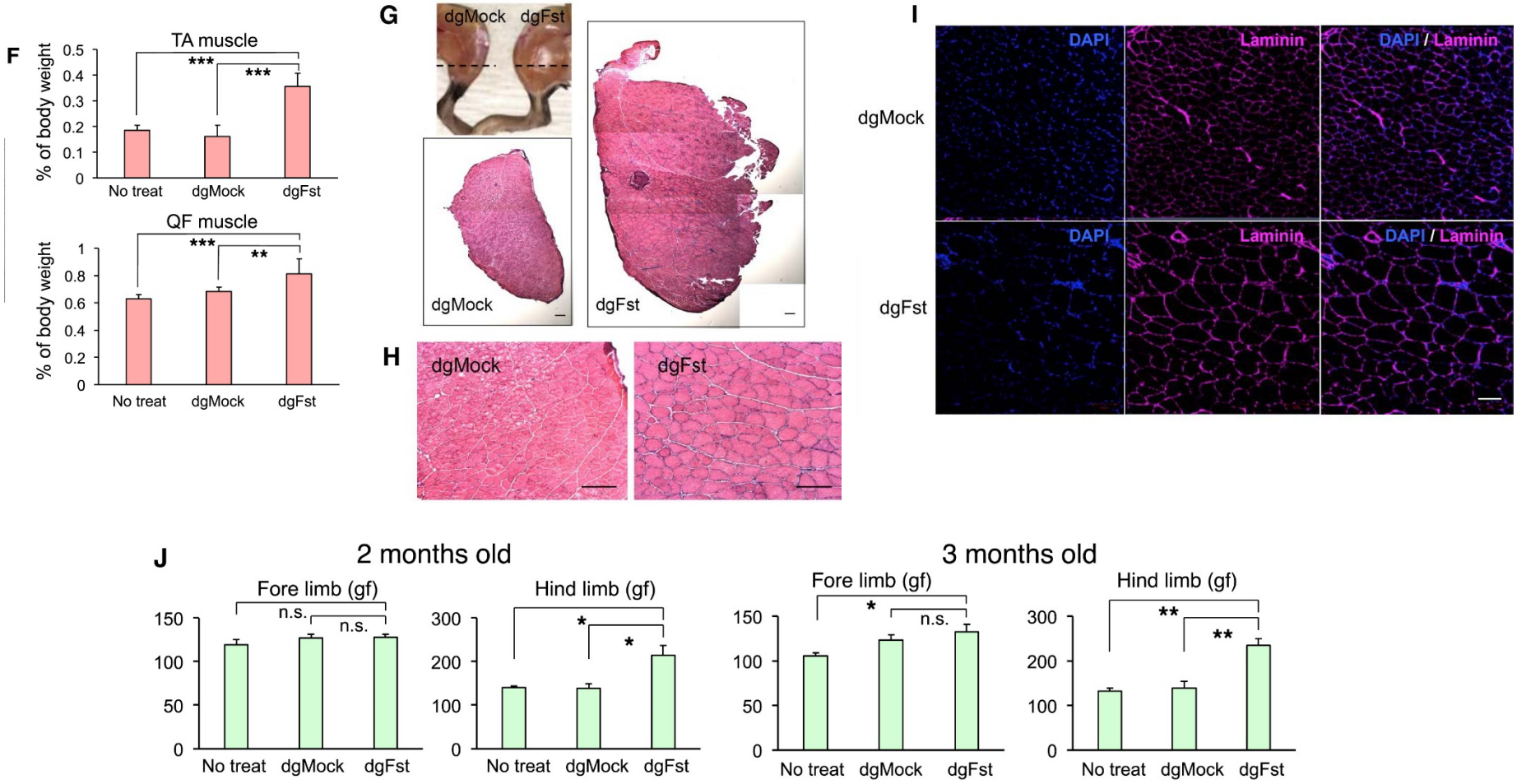
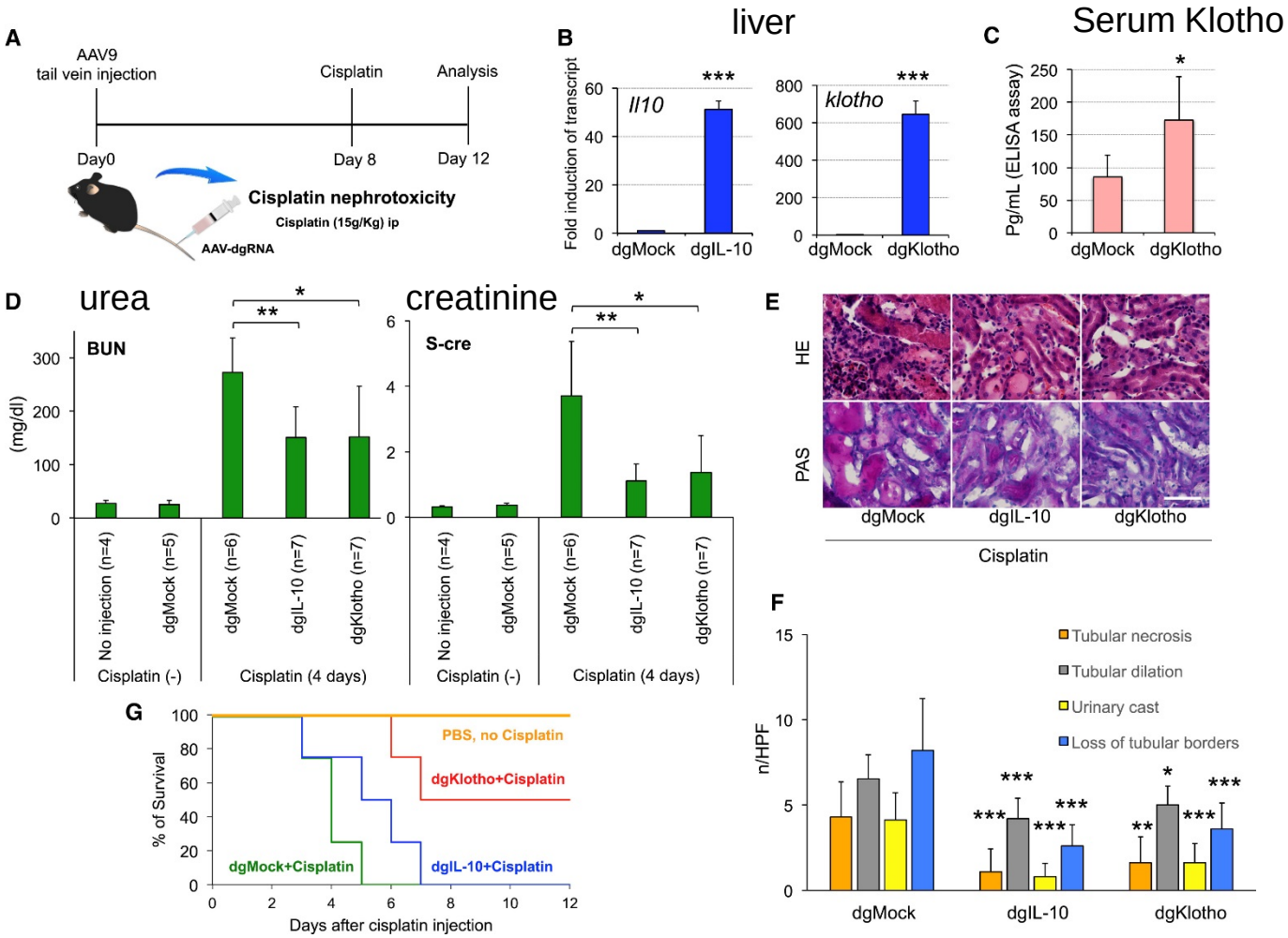


➢ Increased muscle mass and muscle fiber size after Fst activation

Results

CRISPR/Cas9 TGA of IL-10 or Klotho *in vivo* ameliorates acute kidney injury

- Klotho protects against renal damage and IL-10 is antiinflammatory/ ameliorates renal injury
- AAV-dgKlotho-MPH and AAV-dgIL-10-MPH injected into the tail vein of adult Cas9 mice

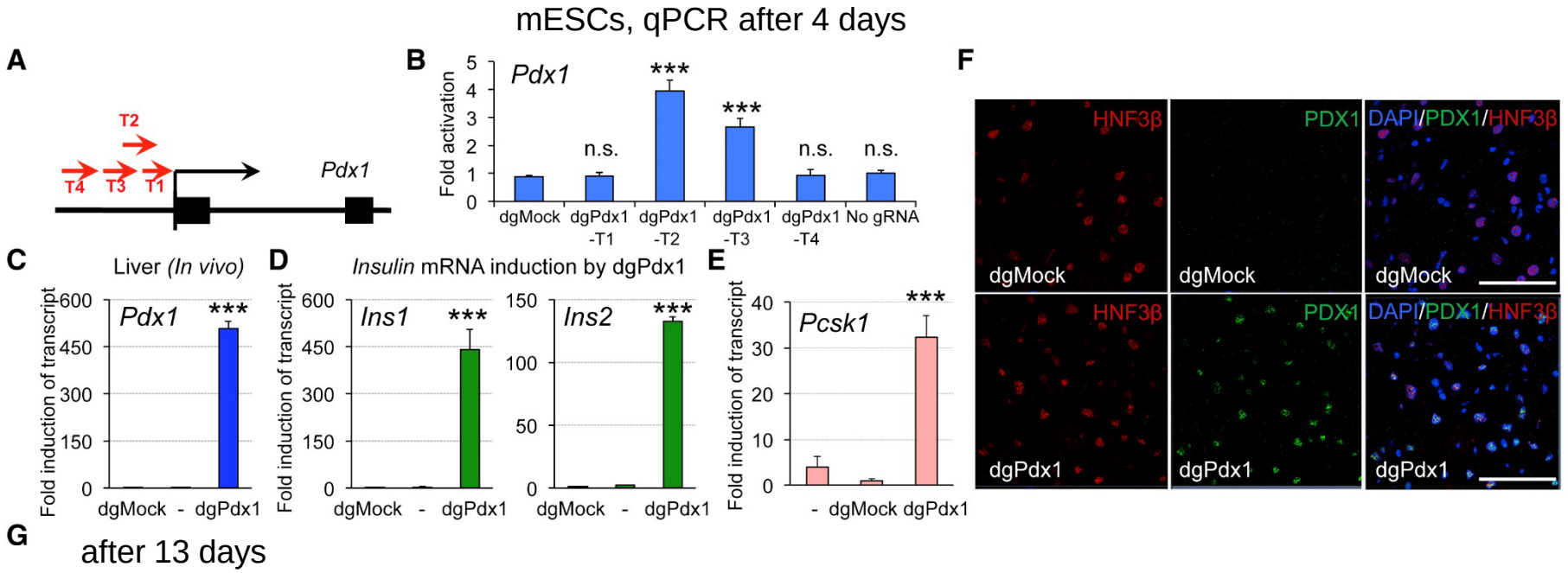


➢ Klotho and IL-10 activation by CRISPR/Cas9 ameliorates cisplatin-induced renal injury

Results

CRISPR/Cas9 TGA results in *in vivo* transdifferentiation of liver cells into insulin-producing cells via trans-epigenetic modulation

- Overexpression of pancreatic and duodenal homeobox gene 1 (*Pdx1*) in liver cells
- *Pdx1* can transdifferentiate hepatocytes into pancreatic beta-like insulin-producing cells
- AAV-dgPdx1-MPH (intravenous injection)
- Goal of generating insulin-secreting cells to treat diabetes type 1

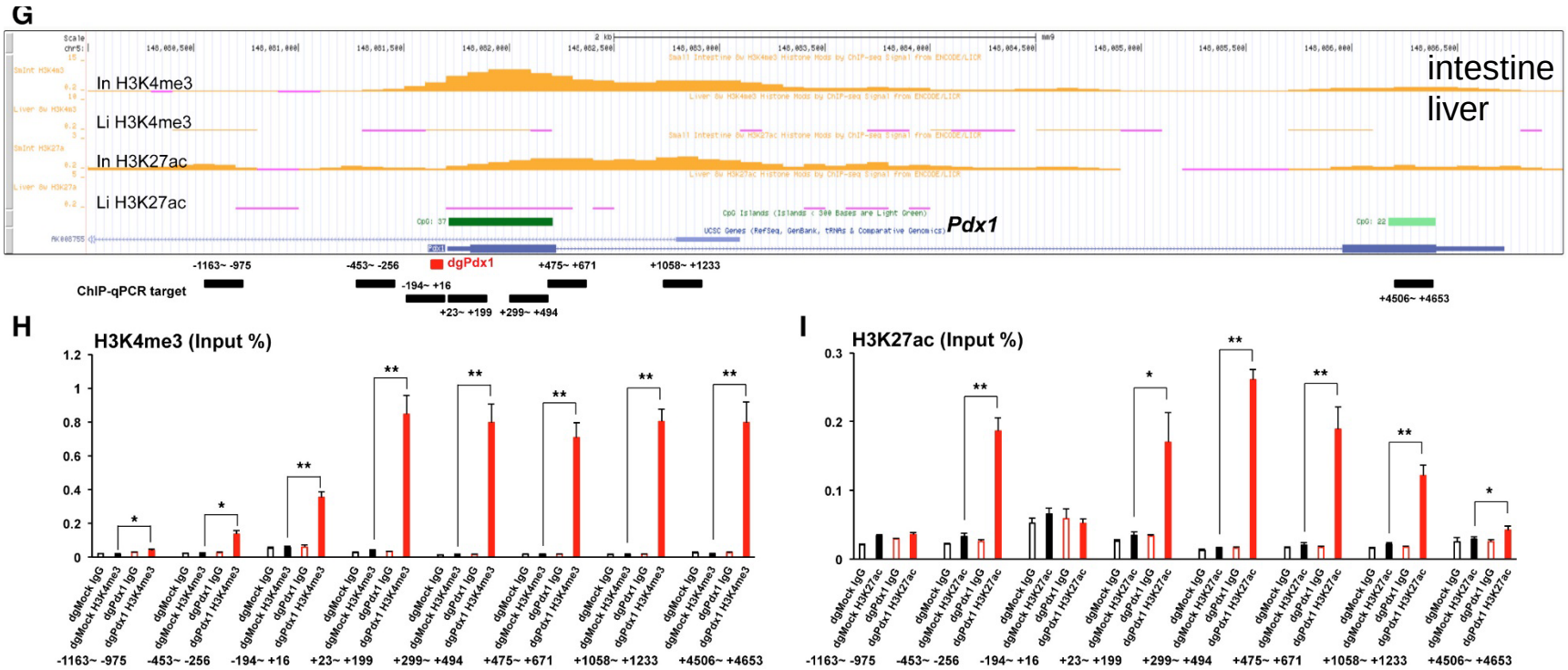


➢ *Pdx1* activation by CRISPR/Cas9 leads to insulin expression in liver cells

Results

CRISPR/Cas9 TGA results in *in vivo* transdifferentiation of liver cells into insulin-producing cells via trans-epigenetic modulation

- ChIP-qPCR of liver samples of active histone marks
- Active histone marks enriched in livers of AAV-dgPdx1-MPH injected mice



- Pdx1 gene activated by <u>trans-epigenetic remodeling</u>
- CRISPR/Cas9 TGA for *in vivo* cell fate engineering

Results

CRISPR/Cas9 TGA of Klotho partially rescues dystrophin-deficient mice

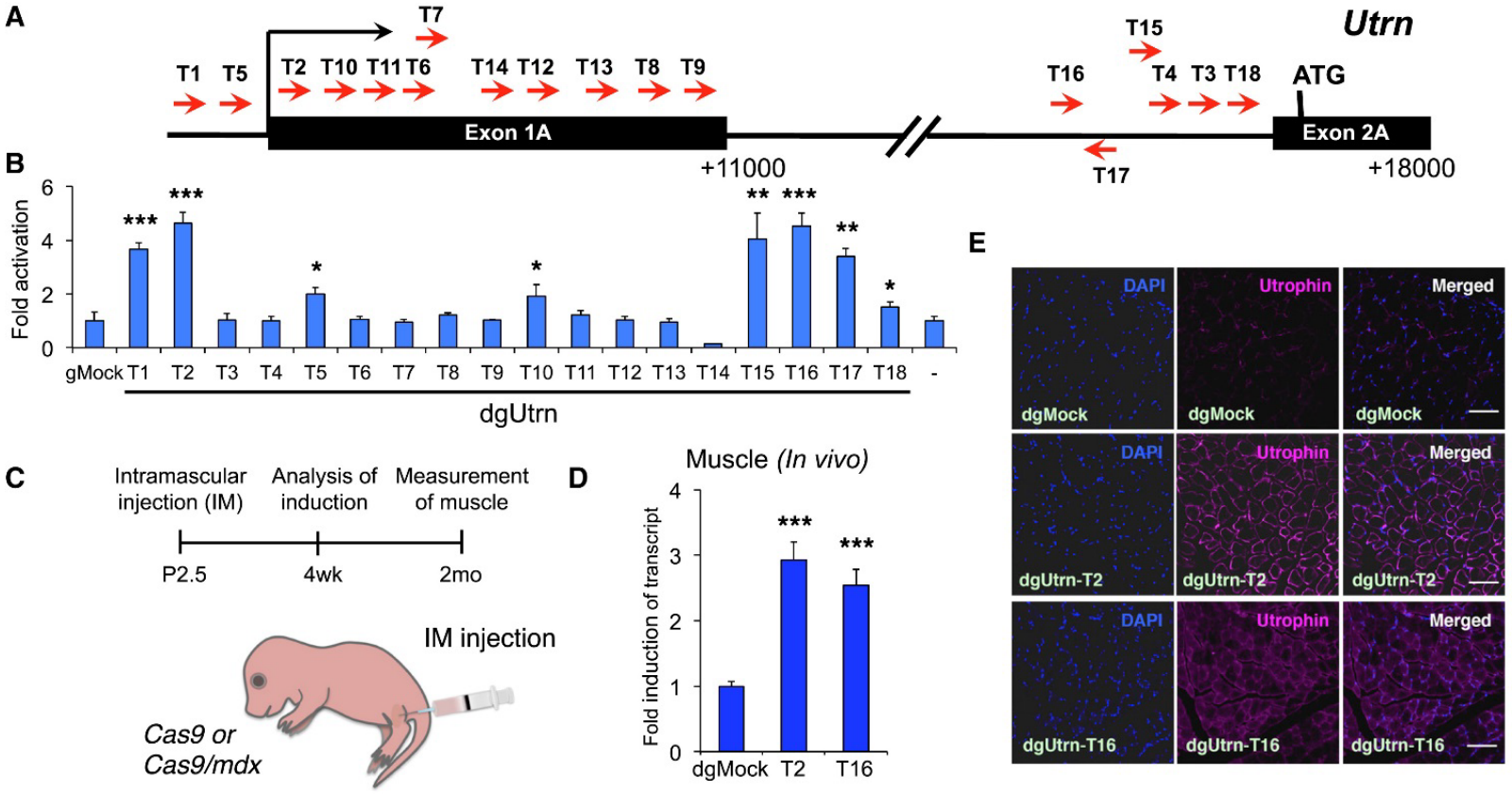
- *Mdx* mouse model of Duchenne muscular dystrophy (DMD), loss of function of dystrophin
- 14 kb protein, too large to be delivered in full length via virus
- Klotho is epigenetically silenced in DMD at disease onset/ OE ameliorates symptoms
- AAV-dgKlotho-MPH intravenously into neonatal mice

- Restored *klotho* expression
- Increased TA muscle mass
- Improved muscle strength after 2 months (wire hang and grip strength)

Results

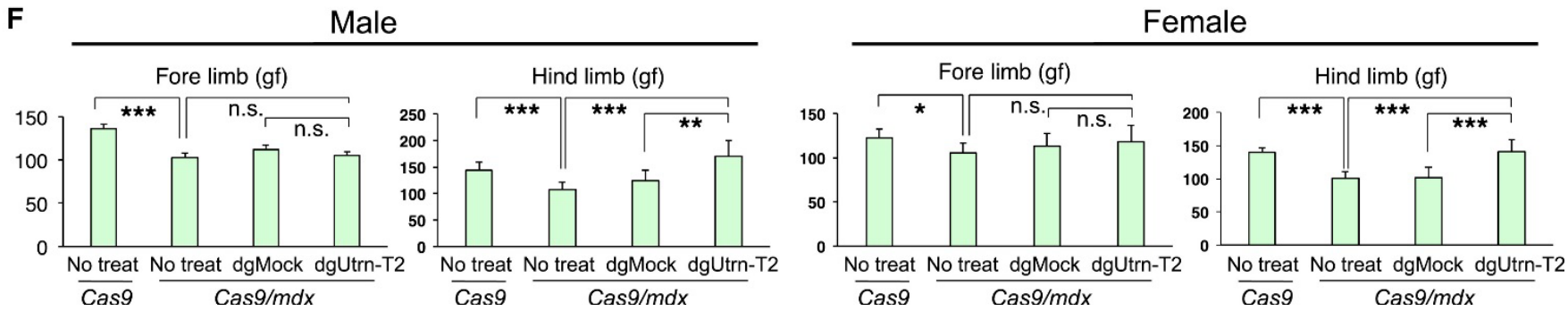
CRISPR/Cas9 TGA of utrophin partially rescues dystrophin-deficient mice

- Alternative to treat Duchenne muscular dystrophy (DMD) is to OE utrophin which is similar to dystrophin (89%)
- Too large to be delivered in full length via virus
- AAV-dgUtrnT2-MPH or AAV-dgUtrnT16-MPH intramuscularly into neonatal Cas9 mice



Results

CRISPR/Cas9 TGA of utrophin partially rescues dystrophin-deficient mice

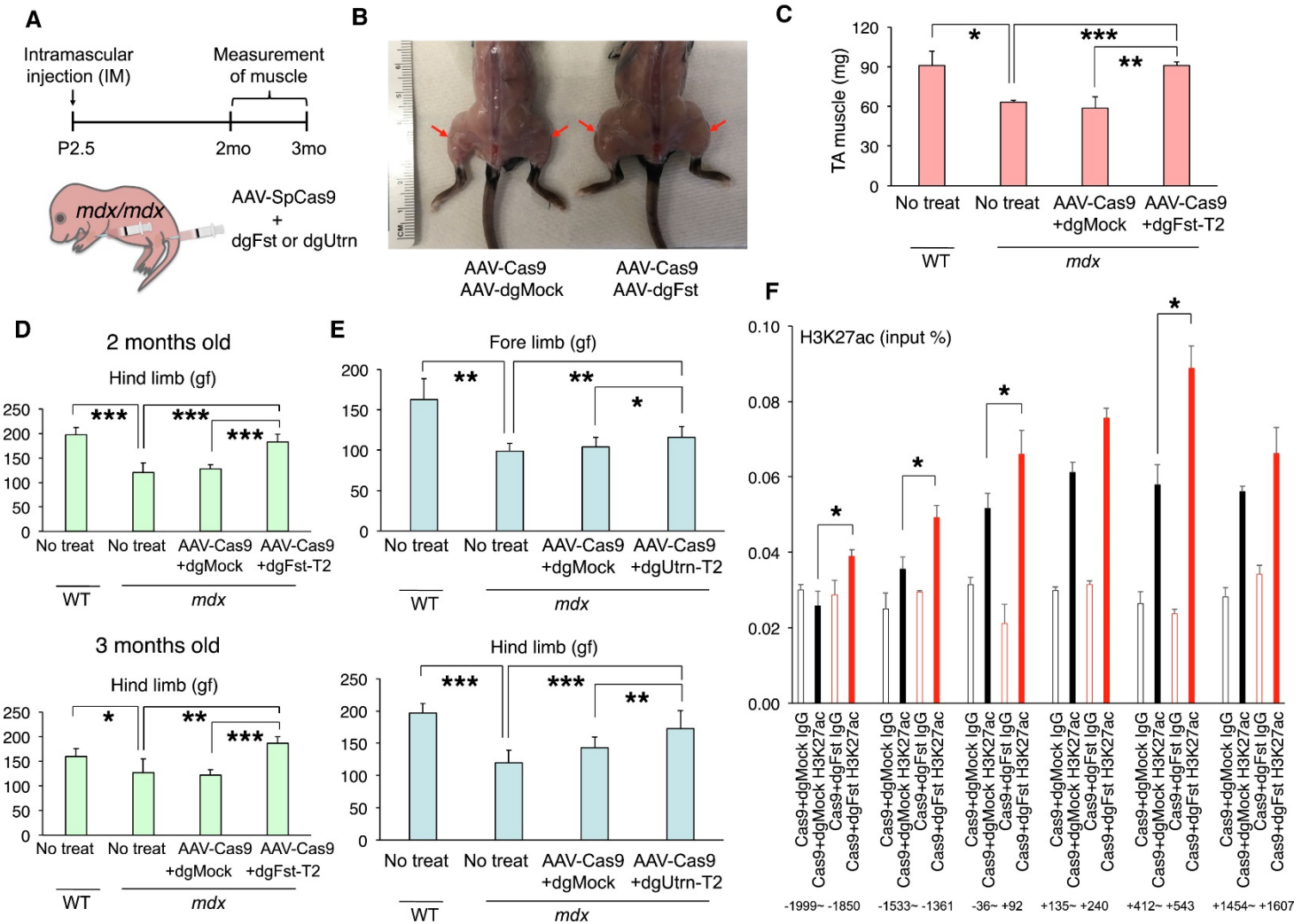


- Improved muscle strength after 2 months (hind limb grip strength)

Results

Amelioration of DMD phenotype by dual AAV-CRISPR/Cas9 TGA system that includes AAV-Cas9

- Co-injection of AAV-SpCas9 with AAV-dgFst-MPH or AAV-dgUtrn-MPH ameliorated disease symptoms of mdx mice
- Induced H3K4me3 and H3K27Ac activation marks within *Fst*



ChIP-qPCR

Discussion

CRISR/Cas9 TGA system can transcriptionally activate target genes *in vivo* by modulating histone marks rather than editing DNA sequences

Here, by recruiting transcriptional machinery, not by directly recruiting epigenetic modulators

Can be used especially for long genes, which cannot be packed into an AAV