

Supplementary Materials for
The interferon γ pathway enhances pluripotency and X-chromosome reactivation in iPSC reprogramming

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The PDF file includes:

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Other Supplementary Material for this manuscript includes the following:

Tables S1 to S6

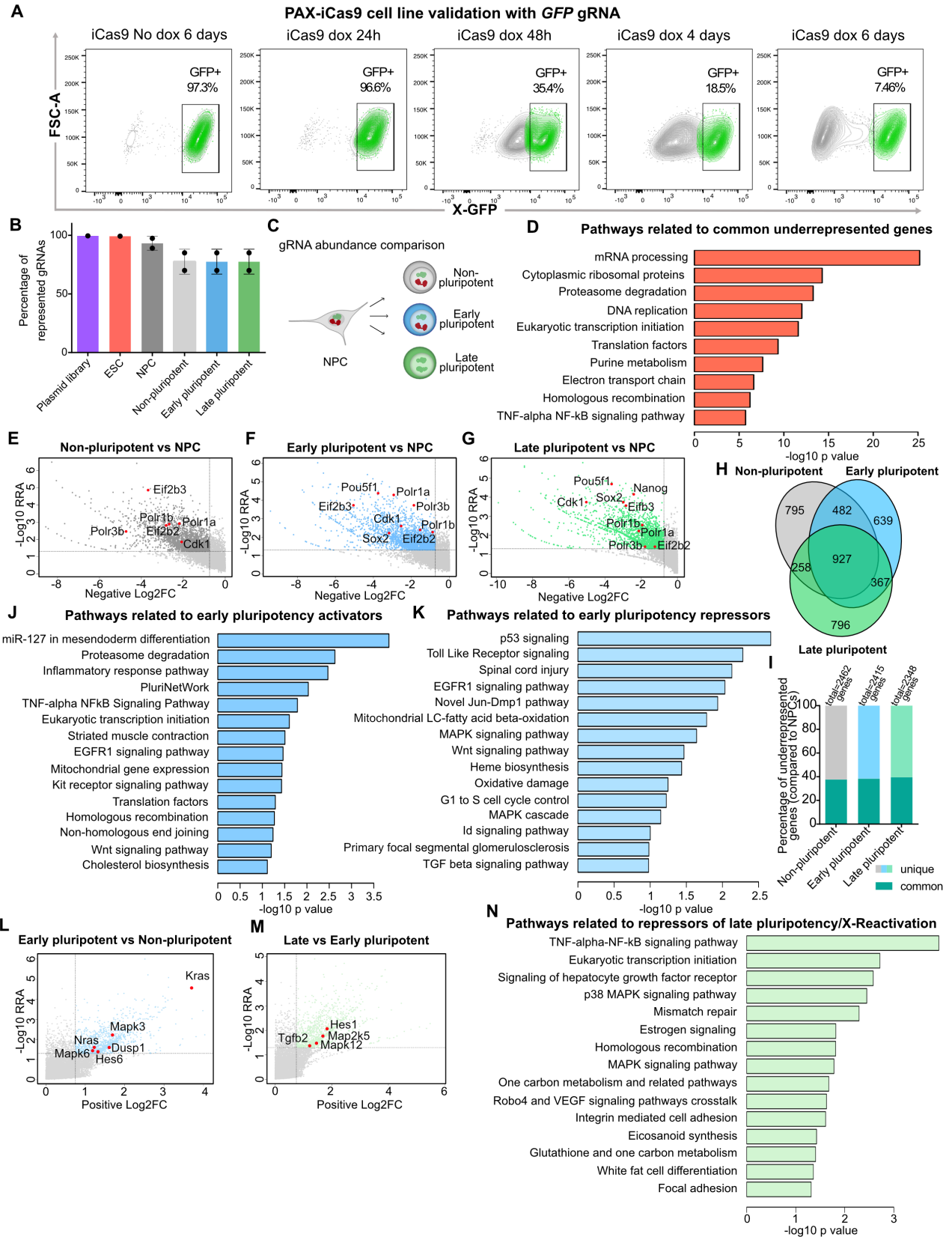


Fig. S1. Legend on the next page.

Fig. S1. CRISPR screen reveals molecular networks involved in reprogramming and X-chromosome reactivation. Related to **Fig. 1.** (A) Validation of knockout efficiency by flow cytometry. Flow cytometry analysis during 6 days of doxycycline treatment in the X-GFP *iCas9* ESC line was done to measure the X-GFP percentage decay in cells containing a gRNA targeting the *GFP* gene. Gating shows the X-GFP+ population. (B) Percentage of gRNA representation in the plasmid library, infected ESCs and the 4 populations analyzed in two independent screening rounds: NPCs and day 10 reprogramming populations (non-pluripotent, early pluripotent, late pluripotent). Error bars represent SD. (C) gRNA abundance comparisons (related to D-I): NPCs to non-pluripotent, early pluripotent and late pluripotent populations. (D) Pathways related to common underrepresented genes (n=927 genes) in the three reprogramming populations compared to NPCs (WikiPathways Mouse 2019). For all comparisons, an RRA score < 0.05 and Log2FC < -0.75 (underrepresented) filtering was applied. (E-G) Representation of genes with negative Log2FC (underrepresented) vs -log10 RRA in the non-pluripotent (E), early pluripotent (F) and late pluripotent (G) populations compared to NPCs (RRA cutoff = 0.05, Log2FC cutoff = -0.75). (H) Venn diagram (using Venny 2.1.0) representing overlap of underrepresented genes (compared to NPCs) in each of the sorted populations at day 10 of reprogramming. (I) Bar plot showing percentages of common and unique underrepresented genes (compared to NPCs) in each of the sorted populations at day 10 of reprogramming. (J) Pathways (WikiPathways Mouse 2019) related to underrepresented genes in the “early pluripotent vs non-pluripotent” comparison (activators of early pluripotency, n=1361 genes) (RRA score < 0.05 and Log2FC < -0.8 filtering was applied). (K) Pathways (WikiPathways Mouse 2019) related to overrepresented genes in the “early pluripotent vs non-pluripotent” comparison (repressors of early pluripotency, n=693 genes) (RRA score < 0.05 and Log2FC > 0.8 filtering was applied). (L) Representation of genes with positive Log2FC (overrepresented) vs -log10 RRA (RRA cutoff = 0.05, Log2FC cutoff = 0.75) in the “early pluripotent vs non-pluripotent” comparison (repressors of early pluripotency). (M) Representation of genes with positive Log2FC (overrepresented) vs -log10 RRA (RRA cutoff = 0.05, Log2FC cutoff = 0.75) in the “late pluripotent vs early pluripotent” comparison (repressors of late pluripotency, X-reactivation). (N) Pathways (WikiPathways Mouse 2019) related to overrepresented genes in the “late pluripotent vs early pluripotent” comparison (repressors of late pluripotency, X-reactivation, n=839 genes) (RRA score < 0.05 and Log2FC > 0.8 filtering was applied).

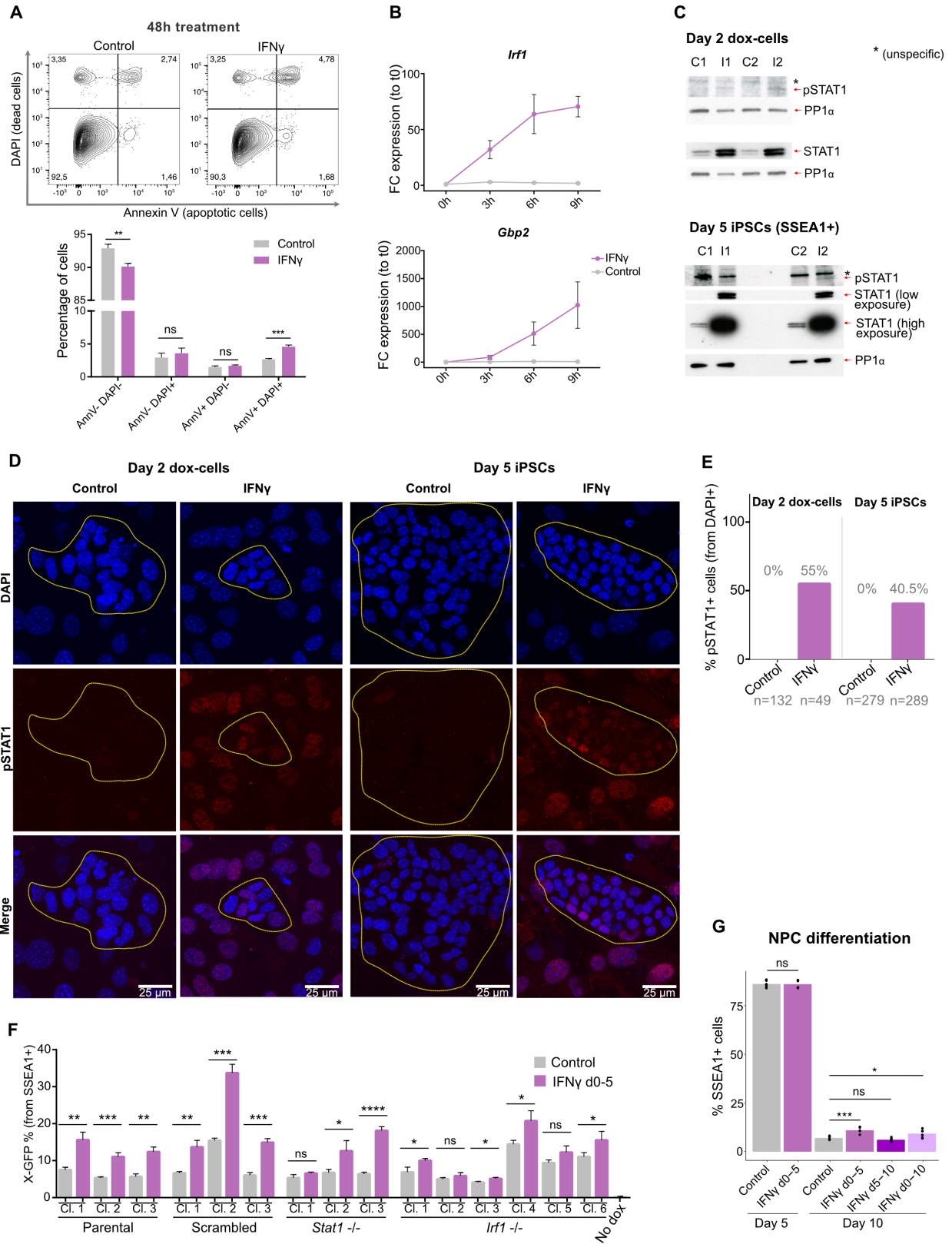


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Fig. S2. Interferon γ pathway activation during iPSC reprogramming. Related to **Fig. 2.** (A) Analysis of apoptosis by annexin V and DAPI staining with flow cytometry after 48h of reprogramming induction +/- IFN γ treatment (n=3 technical replicates). Statistics (unpaired t-tests): ns = non significant; ** = p<0.01; *** = p<0.001. Error bars represent SD. (B) RT-qPCR on mRNA for *Irf1* and *Gbp2* expression at 0h, 3h, 6h and 9h from reprogramming induction +/- IFN γ treatment (relative to t0). Error bars represent SD (n=3 technical replicates). (C) Western blotting of STAT1 and pSTAT1 (Tyr701) on day 2 and day 5 reprogramming cells +/- IFN γ treatment (loading control: PP1 α). (D) Immunofluorescence of pSTAT1 (Tyr701) on day 2 and day 5 reprogramming cells +/- IFN γ treatment. Scale bar = 25 μ m. Outlines highlight colonies of cells undergoing reprogramming, characterized by smaller nuclei and tight aggregation. (E) Percentage of pSTAT1-positive cells from immunofluorescence in (D). Numbers of counted cells are indicated on the bottom of the graph. (F) (Related to Fig. 2F-H). Flow cytometry quantification of total X-GFP percentages (from SSEA1+ cells) on day 7 of reprogramming for 3 clones from the parental cell line, 3 clones containing a scrambled gRNA, 3 *Stat1* -/- clones and 6 *Irf1* -/- clones, including three technical replicates for each clone, in IFN γ -treated cells and untreated controls. Statistics (unpaired t-tests): ns = non significant; * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001. Error bars represent SD. (G) (Related to Fig. 2I-K). Quantification of SSEA1 percentage on days 5 and 10 of NPC differentiation by flow cytometry in control and IFN γ treatment conditions (n=6 independent replicates). Statistics (paired t-tests): ns = non significant, * = p<0.05; *** = p<0.001.

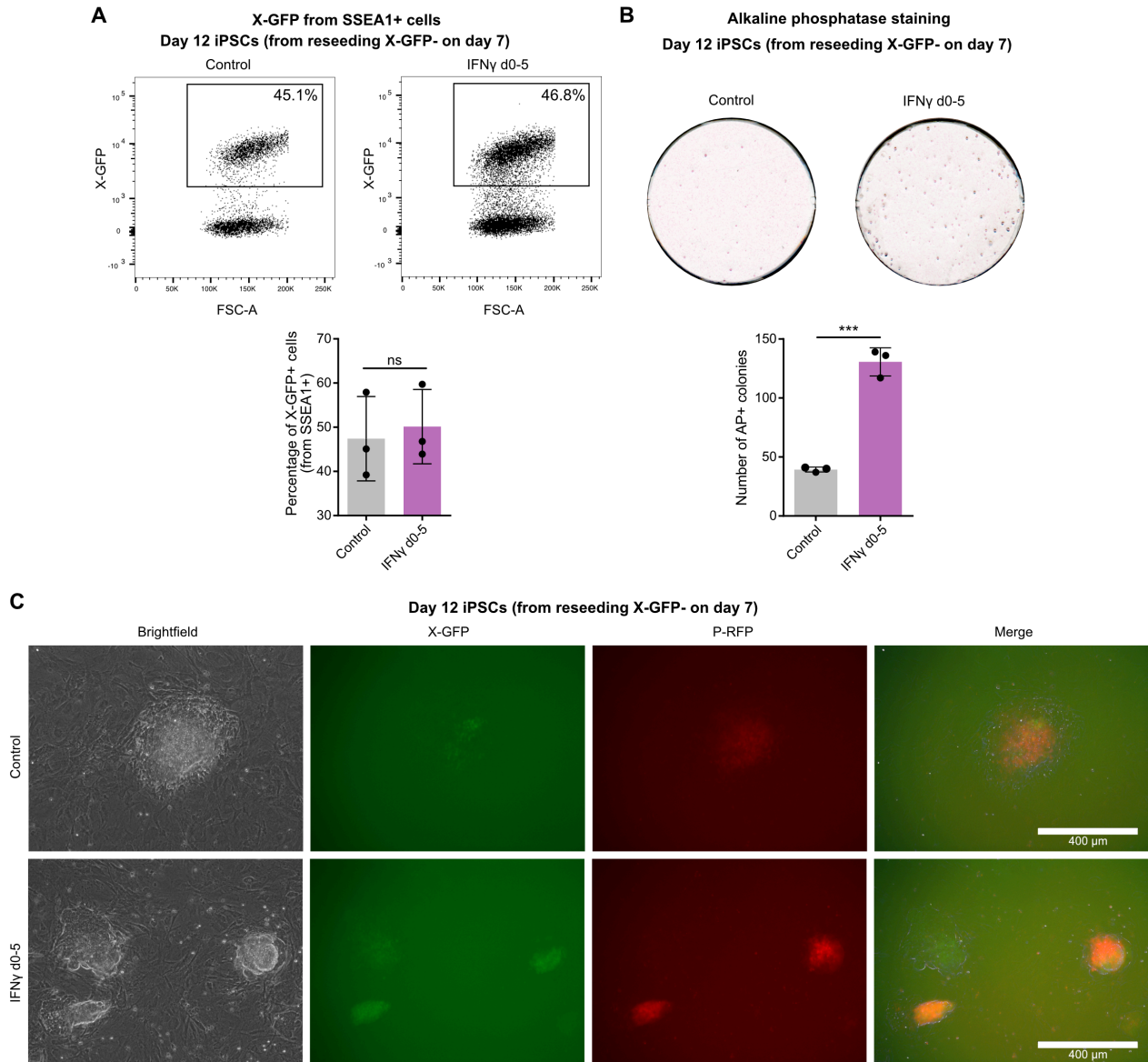


Fig. S3. Reseeding of IFN γ -treated day 7 X-GFP negative cells results in higher colony formation and equal X-GFP reactivation at day 12. Related to Fig. 2. (A) Flow cytometry plots of X-GFP expression (from SSEA1+ cells) in control and IFN γ treatment (day 0-5) on day 12 iPSCs after reseeding SSEA1+ X-GFP- cells on day 7 of reprogramming (gating shows the X-GFP+ population), and bar plot representation of X-GFP percentages (from SSEA1+ cells) (n=3 technical replicates). Statistics (unpaired t-tests): ns (non significant). Error bars represent SD. (B) Alkaline Phosphatase (AP) stainings on day 12 of reprogramming after reseeding SSEA1+ X-GFP- cells on day 7 of reprogramming, in control and IFN γ treatment (d0-5) and counting of AP+ colonies (n=3 technical replicates). Statistics (unpaired t-tests): *** = p<0.001. Error bars represent SD. (C) Brightfield and fluorescent images (X-GFP and P-RFP) of live cells at day 12 of reprogramming after reseeding SSEA1+ X-GFP- cells on day 7, in control and IFN γ treatment (d0-5). Scale bar = 400 μ m.

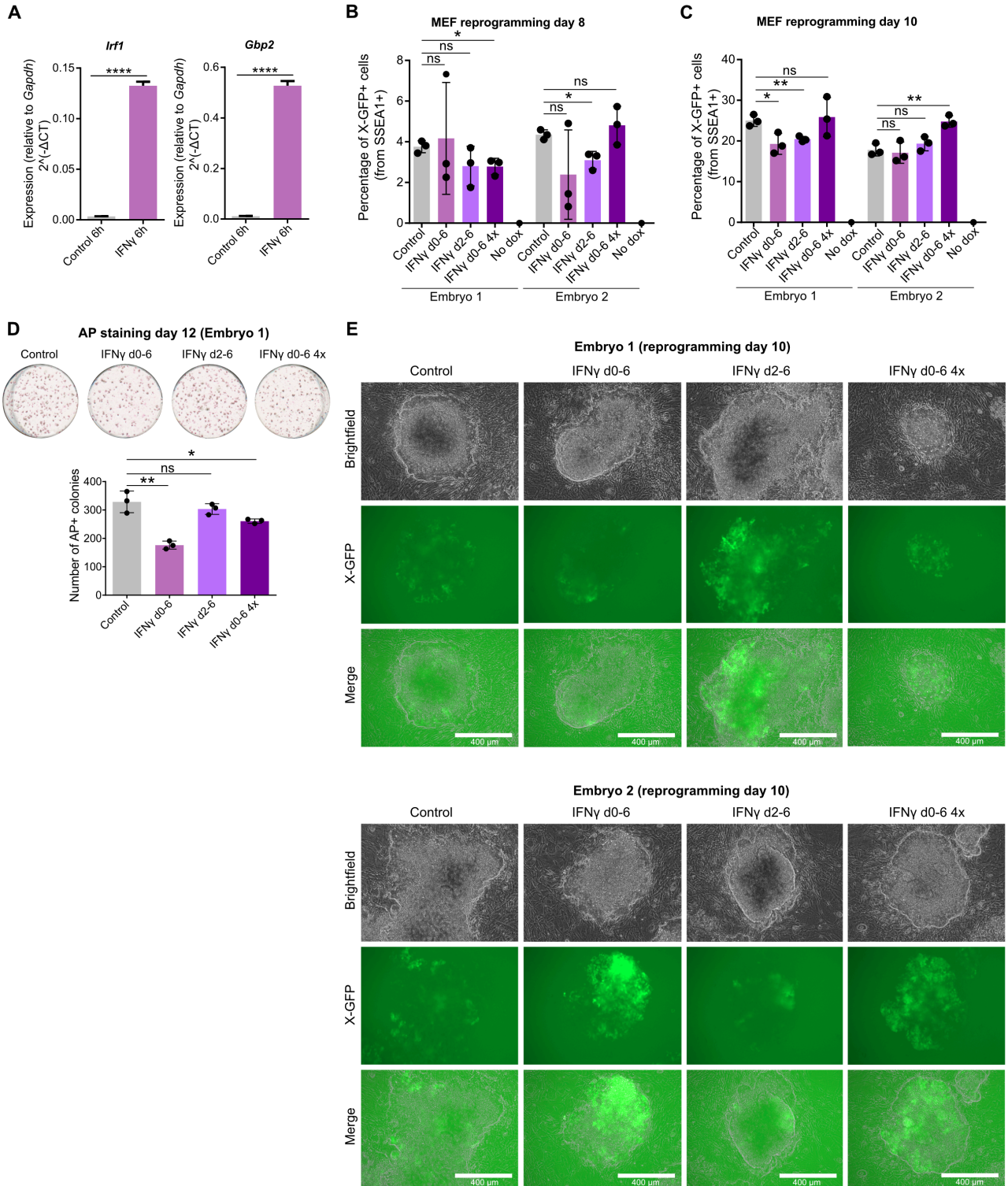


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Fig. S4. Early activation of the IFN γ pathway during MEF reprogramming reduces colony number and does not enhance X-GFP reactivation. (A) RT-qPCR on mRNA for *Irf1* and *Gbp2* expression in control and IFN γ -treated reprogrammable female MEFs after 6 hours since reprogramming induction. Expression levels are normalized to *Gapdh* ($2^{-\Delta CT}$) (n = 3 technical replicates). Statistics (unpaired t-tests): **** = p<0.0001. Error bars represent SD. (B,C) X-GFP percentages from SSEA1+ cells at days 8 (B) and 10 (C) of female MEF reprogramming, in control and different IFN γ treatment conditions (d0-6, d2-6 and d0-6 with 4 times more cells seeded) (n = 3 technical replicates). MEFs from two different embryos were used. Statistics (unpaired t-tests): ns = non-significant; * = p<0.05; ** = p<0.01. Error bars represent SD. (D) Alkaline Phosphatase (AP) stainings on day 12 of reprogramming of MEFs derived from female embryo 1 in control and IFN γ treatment conditions, and counting of AP+ colonies (n=3 technical replicates). Statistics (unpaired t-tests): ns = non-significant; * = p<0.05; ** = p<0.01. Error bars represent SD. (E) Brightfield and fluorescent images (X-GFP) of live cells at day 10 of reprogramming (embryos 1 and 2) in control and different IFN γ -treatment conditions. Scale bar = 400 μ m.

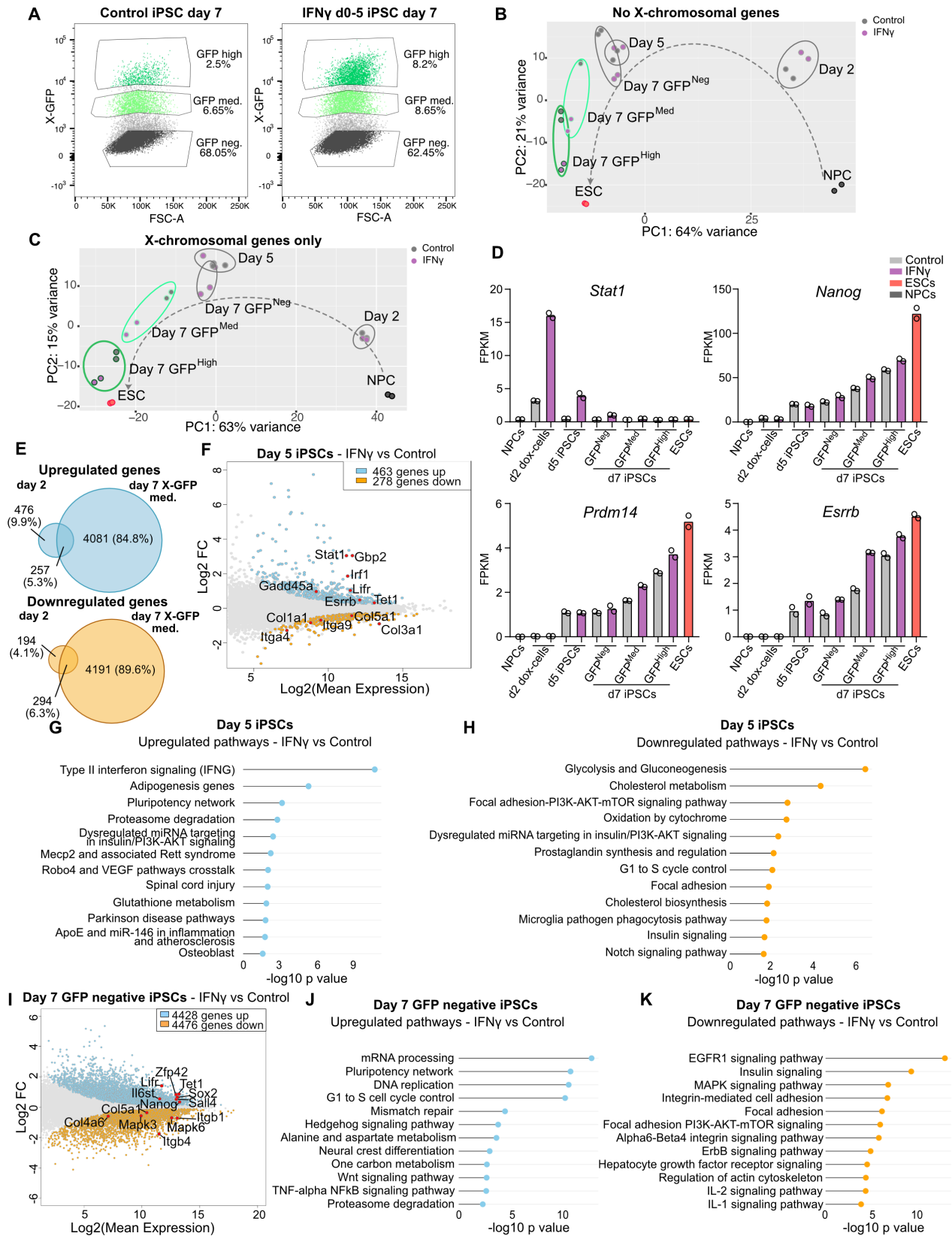


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Fig. S5. Transcriptomic analysis of interferon γ pathway activation during iPSC reprogramming. Related to **Fig. 3.** (A) Flow cytometry plots of X-GFP expression (from SSEA1+ cells) in control and IFN γ -treated day 7 iPSCs. Gating shows sorted populations for RNA-sequencing. Average percentages between two independent reprogramming inductions are indicated for each population. (B, C) Principal component analysis of RNA-sequencing of NPCs, day 2, day 5, day 7 reprogramming populations and ESCs, in control and IFN γ treatment (day 0-5), representing the top 500 most variable autosomal genes only (B) and X-chromosomal genes only (C). (D) Expression (FPKM) of selected genes (*Stat1*, *Nanog*, *Prdm14* and *Esrrb*) in NPCs, ESCs, day 2, day 5 and day 7 reprogramming populations +/- IFN γ treatment (two RNA-sequencing replicates shown). (E) Venn diagram (using Venny 2.1.0) representing overlapping of upregulated and downregulated genes upon IFN γ treatment between day 2 dox-treated cells and day 7 X-GFP medium cells. (F) MA plot displaying transcriptomic changes of IFN γ vs control day 5 iPSCs (adjusted p value = 0.1). Upregulated genes are highlighted in light blue, downregulated genes are highlighted in orange. Selected genes are shown with points in red. (G, H) Upregulated (G) and downregulated (H) pathways in IFN γ vs control day 5 iPSCs (WikiPathways Mouse 2019) (adjusted p value = 0.1). (I) MA plot displaying transcriptomic changes of IFN γ vs control day 7 X-GFP negative iPSCs (adjusted p value threshold = 0.1). Upregulated genes are highlighted in light blue, downregulated genes are highlighted in orange. Selected genes are shown with points in red. (J, K) Upregulated (J) and downregulated (K) pathways in IFN γ vs control day 7 X-GFP negative iPSCs (WikiPathways Mouse 2019) (adjusted p value = 0.1).

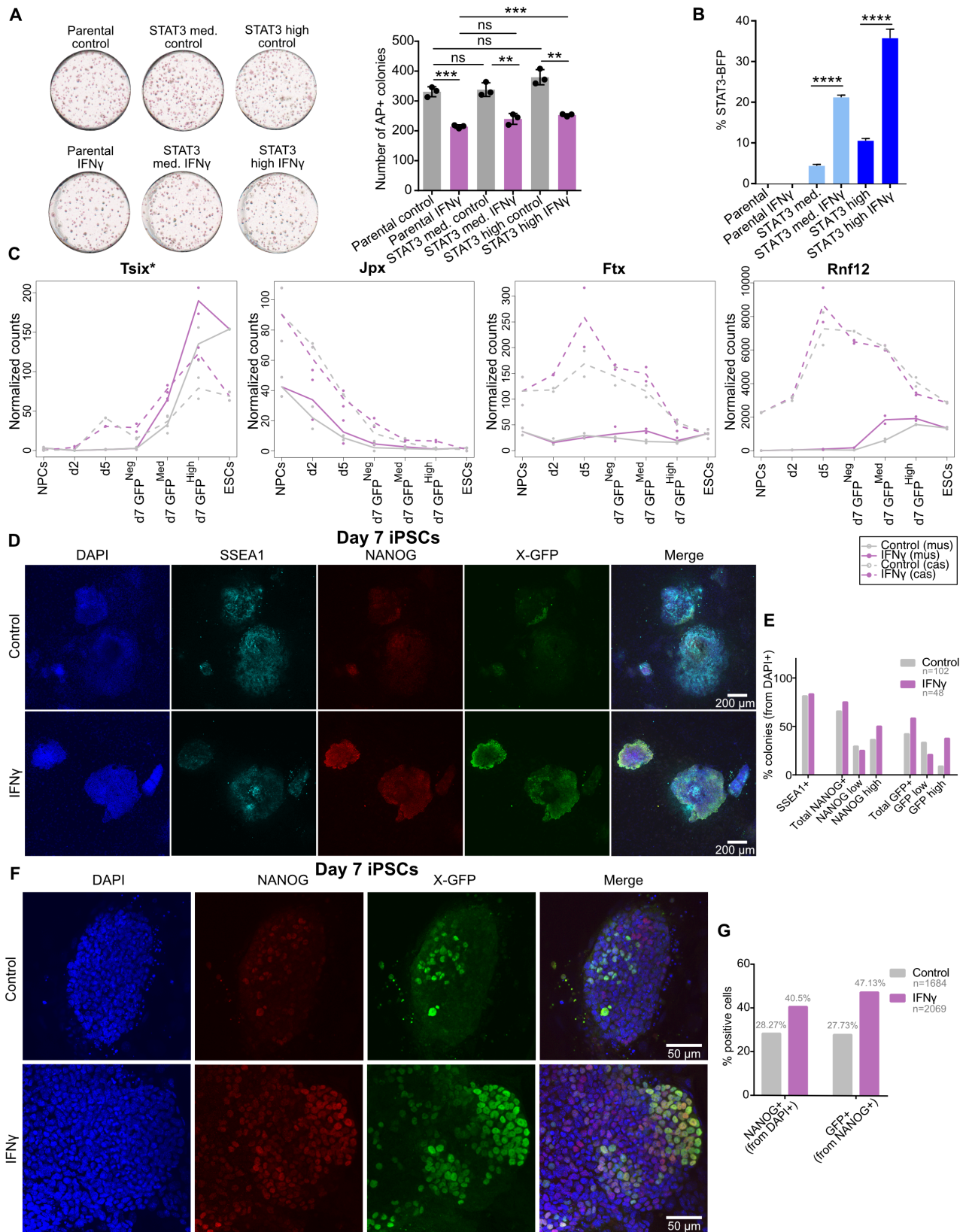


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Fig. S6. Increased expression of NANOG and X-GFP in iPSC colonies upon early interferon γ treatment. Related to **Fig. 4.** (A) Alkaline Phosphatase (AP) stainings on day 10 of reprogramming in parental, STAT3-BFP medium and STAT3-BFP high cells +/- IFN γ treatment (d0-5) and counting of AP+ colonies (n=3 technical replicates). Statistics (unpaired t-tests): ns = non-significant; ** = p<0.01; *** = p<0.001. Error bars represent SD. (B) Percentages of STAT3-BFP+ cells at day 7 of reprogramming in parental, STAT3-BFP medium and STAT3-BFP high cells +/- IFN γ treatment (d0-5) (n=3 technical replicates). Statistics (unpaired t-tests): **** = p<0.0001. Error bars represent SD. (C) Expression (normalized counts) of genes of the X-inactivation center (*Tsix*, *Jpx*, *Ftx*, *Rnf12*) from X mus and X cas on NPCs, ESCs, day 2, day 5 and day 7 reprogramming populations +/- IFN γ treatment (two RNA-sequencing replicates shown). The * at *Tsix* indicates that the gene contains a truncation on the X-mus (112) and therefore cannot regulate *Xist* expression *in cis*. (D) Immunofluorescence (low magnification, 4x) for SSEA1, NANOG and X-GFP (active X chromosome) of day 7 reprogramming colonies +/- IFN γ treatment. Scale bar = 200 μ m. (E) Percentages of SSEA1+, NANOG+ (low/high) and X-GFP+ (low/high) colonies from immunofluorescence in (D). The number (n) of counted colonies is indicated in the graph. NANOG+ or X-GFP+ colonies were scored as low or high if approximately less or more than half of the cells in the colony were positive for these markers, respectively. (F) Immunofluorescence (high magnification, 63x) for NANOG and X-GFP (active X chromosome) of day 7 reprogramming colonies +/- IFN γ treatment. Scale bar = 50 μ m. (G) Percentages of NANOG+ and X-GFP+ (from NANOG+) cells from immunofluorescence in (F). The number (n) of counted cells is indicated in the graph.

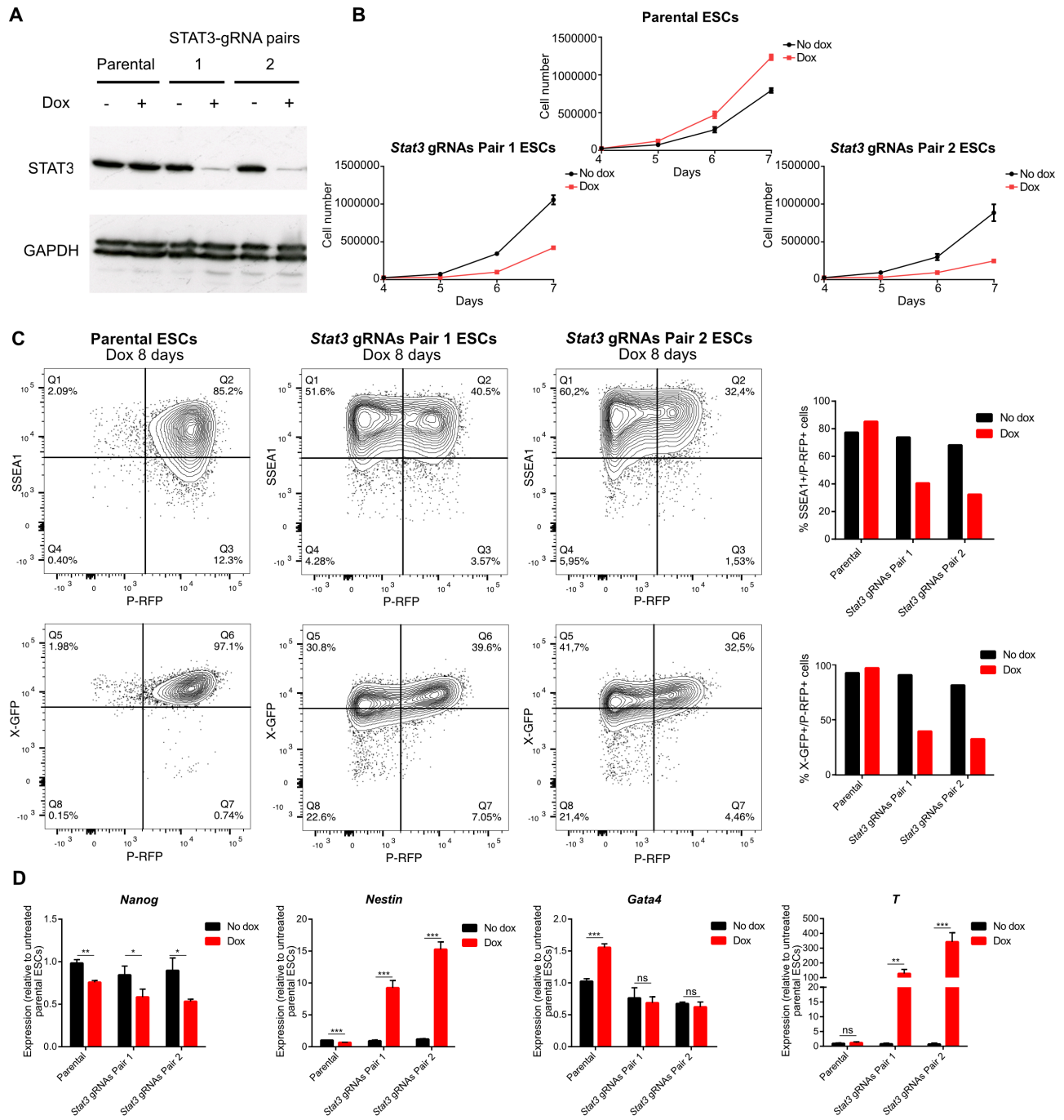


Fig. S7. Generation and characterization of *Stat3*^{-/-} knockout ESC pools. Related to **Fig. 4.** (A) Western blotting of STAT3 in parental ESCs and ESCs infected with *Stat3*-targeting gRNAs (Pairs 1 and 2) +/- doxycycline treatment for 7 days (loading control: GAPDH). (B) Cell number measurement for days 4-7 +/- doxycycline treatment in parental ESCs and ESCs infected with *Stat3*-targeting gRNAs (Pairs 1 and 2) (25,000 cells seeded on day 4). (C) Flow cytometry plots on parental ESCs and ESCs infected with *Stat3*-targeting gRNAs (Pairs 1 and 2) upon doxycycline treatment for 8 days, showing expression of SSEA1/P-RFP (top) and X-GFP/P-RFP (bottom) and bar plots showing percentages of these double-positive populations in +/- doxycycline treatment conditions. (D) RT-qPCR on mRNA for *Nanog*, *Nestin*, *Gata4* and *T* on parental ESCs and ESCs infected with *Stat3*-targeting gRNAs (Pairs 1 and 2) +/- doxycycline treatment for 7 days (n=3 technical replicates). Statistics (unpaired t-tests): ns = non significant; * = p<0.05; ** = p<0.01; *** = p<0.001. Error bars represent SD.

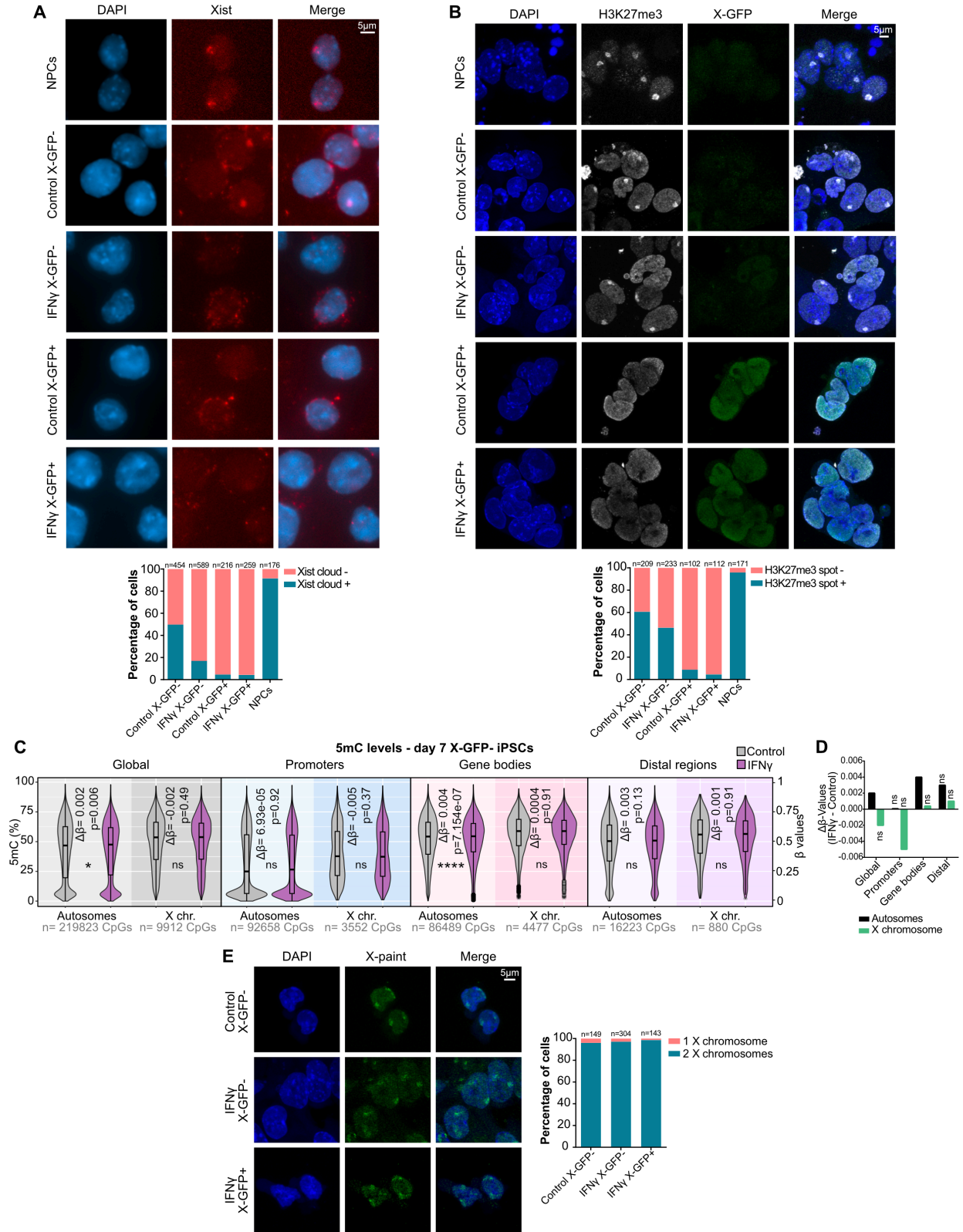


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Fig. S8. Day 7 X-GFP- reprogramming cells show a reduction in Xist clouds and in H3K27me3 spots, and equal X-chromosomal DNA methylation levels. Related to **Fig. 4.** (A) RNA FISH showing Xist clouds on the inactive X chromosome (Sx9 probe) in NPCs and day 7 SSEA1+ X-GFP-negative/positive control/IFN γ iPSCs (scale bar = 5 μ m) and quantification of Xist cloud-positive and -negative cells in all conditions. (B) H3K27me3 immunofluorescence in NPCs and day 7 SSEA1+ X-GFP negative/positive control/IFN γ iPSCs (scale bar = 5 μ m) and quantification of H3K27me3 spot-positive and -negative cells in all conditions. (C) Analysis of 5mC levels (β -values) of CpGs on autosomes and X chromosome in day 7 X-GFP-negative iPSCs for control and IFN γ conditions, globally and divided by genomic distribution: promoters (\leq 1kb from TSS), gene bodies and distal regions (number (n) of detected CpGs from each category is indicated on the bottom of the graphs). $\Delta\beta$ -values (mean β -value IFN γ - mean β -value control) and p values (comparison IFN γ vs control) are shown in the graphs. Statistics (unpaired t-tests): ns = non-significant; * = $p < 0.05$; **** = $p < 0.0001$. (D) $\Delta\beta$ -values for 5mC in day 7 X-GFP-negative iPSCs for each genomic region in autosomes and X chromosome (corresponding to analysis in (C)). Bars marked with “ns” correspond to non-significant changes from analysis in (C). (E) X-chromosome paint DNA FISH in control X-GFP-negative and IFN γ -treated (d0-5) X-GFP-negative and -positive day 7 iPSCs (scale bar = 5 μ m) and quantification of cells with 1 or 2 X chromosomes.

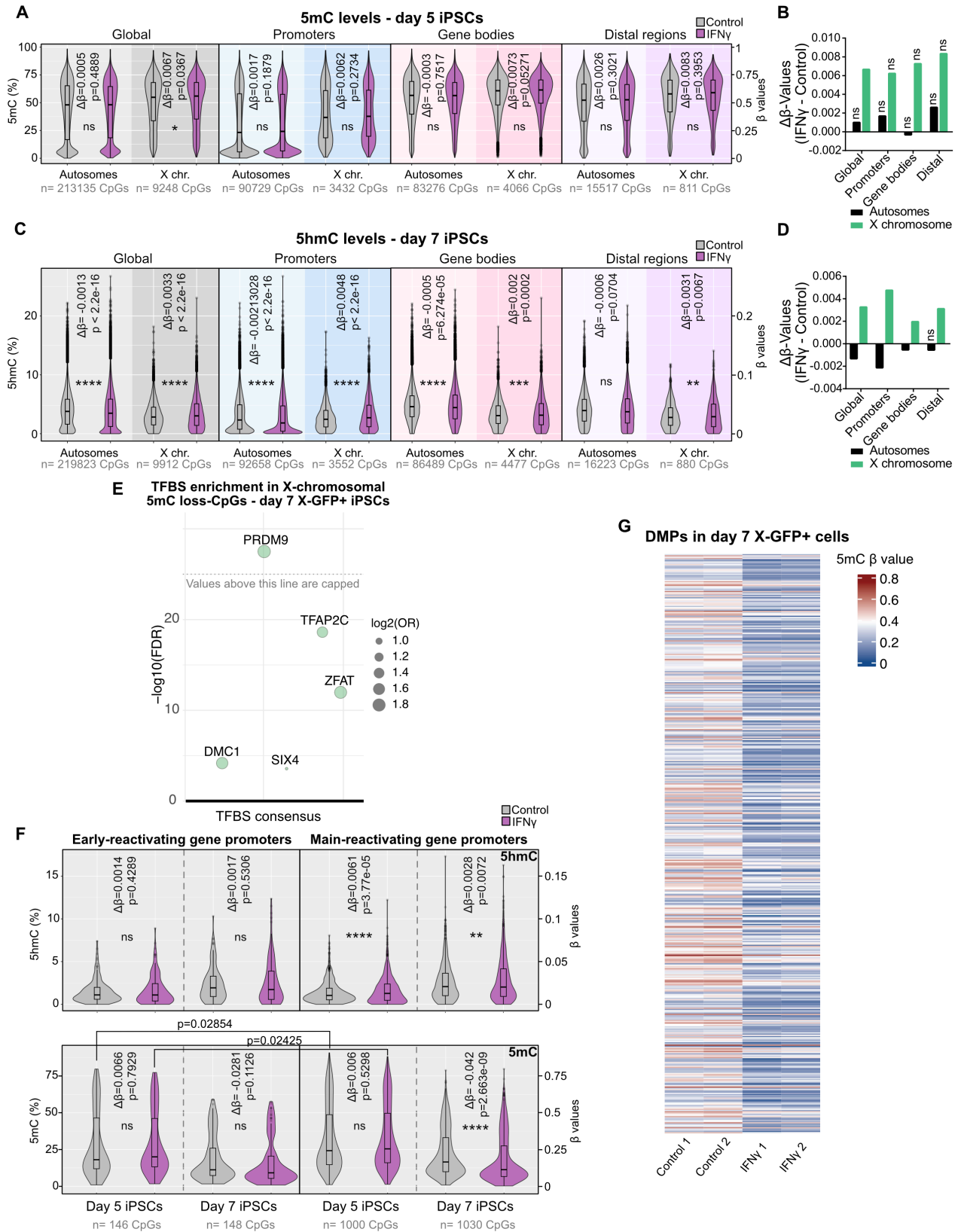


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Fig. S9. Interferon γ treatment promotes TET-mediated DNA demethylation in cells undergoing reprogramming. Related to **Fig. 5.** (A, C) Analysis of 5mC levels in day 5 (A) or 5hmC levels in day 7 X-GFP+ cells (C) (β -values) of CpGs in autosomes and X chromosome for control and IFN γ (d0-5) conditions, globally and divided by genomic distribution: promoters (\leq 1kb from TSS), gene bodies and distal regions (number (n) of detected CpGs from each category is indicated on the bottom of the graphs). $\Delta\beta$ -values (mean β -value IFN γ - mean β -value control) and p values (comparison IFN γ vs control) are shown in the graphs. Statistics: (unpaired t-tests): ns = non-significant; * = $p < 0.05$; ** = $p < 0.001$; **** = $p < 0.0001$. (B, D) $\Delta\beta$ -values for 5mC in day 5 (B, corresponding to analysis in A) or 5hmC in day 7 X-GFP+ iPSCs (D, corresponding to analysis in C) for each genomic region in autosomes and X chromosomes. Bars marked with “ns” correspond to non-significant changes from analysis in (A) or (C). (E) Transcription factor binding site (TFBS) enrichment analysis on differentially methylated X-chromosomal CpGs (DMPs, $\log_{FC} < (-0.1)$, $p < 0.01$, $n = 468$ CpGs) which lose methylation upon IFN γ treatment compared to control in day 7 X-GFP+ iPSCs. $-\log_{10}(\text{FDR})$ capped values are above 25. (F) Analysis of 5mC and 5hmC levels (β -values) of CpGs in early and main X-reactivating gene promoters at day 5 and day 7 X-GFP+ iPSCs for control and IFN γ conditions (gene lists were obtained from (24)). Number (n) of detected CpGs for each category and time point is indicated on the bottom of the graphs. $\Delta\beta$ -values and p values (comparison IFN γ vs control) are shown in the graphs. Statistics (unpaired t-tests): ns = non-significant; ** = $p < 0.001$; **** = $p < 0.0001$. (G) Heatmap showing 5mC levels (β -values) of all X-chromosomal differentially methylated CpGs ($n = 470$ DMPs, \log_{FC} cutoff = ± 0.1 , $p < 0.01$) sorted by chromosome position.

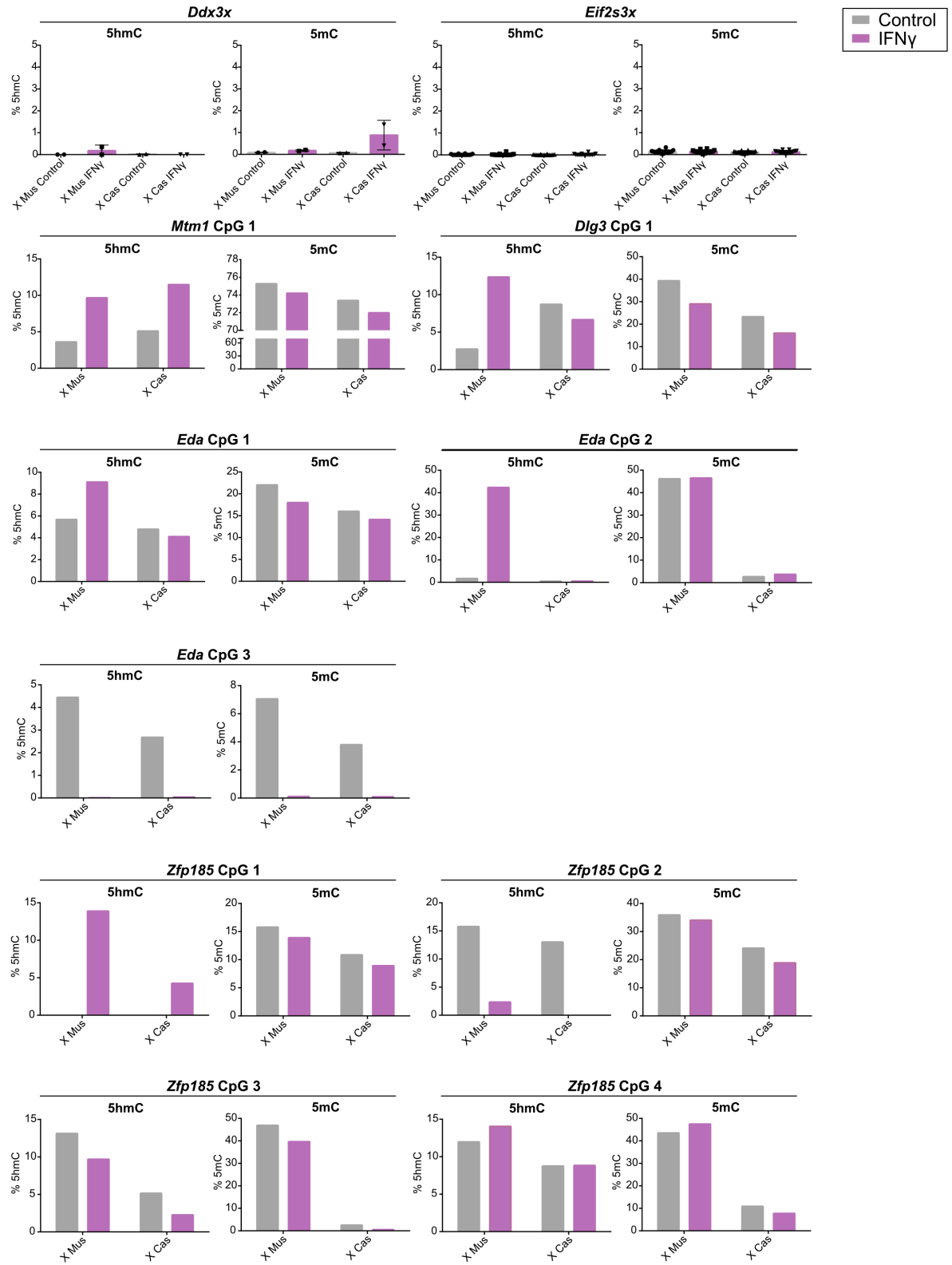


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Fig. S10. Allele-specific (hydroxy)methylation analysis by targeted amplicon oxidative BS-sequencing. Related to **Fig. 5**. Allele-specific analysis of 5mC and 5hmC percentages (relative to total C) at day 5 of reprogramming in control and IFN γ -treated samples, in specific promoter *loci* surrounding CpGs from X-reactivating genes that were found as differentially hydroxymethylated on day 5 or 7 in Fig. 5, including escapee gene controls. Analysed loci contained promoter regions of the escapee genes *Ddx3x* (2 CpGs) and *Eif2s3x* (16 CpGs) (plotted together because of low variability), and the X-reactivating genes *Mtm1* (1 CpG), *Dlg3* (1 CpG), *Eda* (3 CpGs) and *Zfp185* (4 CpGs) (plotted separately because of high variability).

Fig. S11. Absence of TET1 does not impede IFN γ -mediated enhanced X-GFP reactivation at day 7 of reprogramming. Related to **Fig. 5.** (A) PCR on genomic DNA of parental (WT) and *Tet1*^{-/-} clones showing an around 200 bp deletion in exon 3. The asterisk marks the clones used for further experiments. (B) Schematic representation of Sanger sequencing (and amino acid equivalence) of PCR products from genomic DNA in the parental clone (WT) and 5 *Tet1*^{-/-} clones used for the experiment, which showed a premature STOP codon (represented in black). (C) Experimental design for (D): *Tet1*^{-/-}, parental and scrambled gRNA control ESCs were differentiated into NPCs and then reprogrammed into iPSCs in the presence or absence of IFN γ (day 0-5). X-GFP percentages (from SSEA1⁺ cells) were measured by flow cytometry at day 7 of reprogramming. 3 clones from the parental cell line, 3 clones containing a scrambled gRNA and 5 *Tet1*^{-/-} clones were used, including three technical replicates for each clone. (D) Fold change of percentage of X-GFP⁺ cells (from SSEA1⁺ cells) in IFN γ -treated cells compared to untreated controls on day 7 of reprogramming, measured by flow cytometry. Bars represent the average X-GFP fold change (IFN γ vs control) for clones with the same genotype, listed in (C). Each dot represents the mean of three technical replicates for each clone. Statistics (unpaired t-tests): ns = non-significant. Error bars represent SD. (E) RT-qPCR on mRNA for *Tet1*, *Tet2* and *Tet3* expression in day 7 SSEA1⁺ cells (+/- IFN γ treatment day 0-5) from 3 scrambled and 3 *Tet1*^{-/-} clones. Expression levels are normalized to *Gapdh* ($2^{-\Delta CT}$). Statistics (paired t-tests between control and treatment within clones, unpaired t-tests for comparisons between different clones): ns = non-significant; * = p<0.05. Error bars represent SD.

Supplemental Tables Description

Supplemental Table S1. MAGeGK gene summary for CRISPR screen comparisons. Related to **Fig. 1** and **fig. S1**. Statistical comparisons for each gene in “non-pluripotent vs NPCs”, “early pluripotent vs NPCs”, “late pluripotent vs NPCs”, “early vs non-pluripotent” and “late vs early pluripotent”.

Supplemental Table S2. Lists of genes and pathways for CRISPR screen comparisons. Related to **Fig. 1** and **fig. S1**. Gene lists for each category and pathways (“WikiPathways mouse 2019”) corresponding to each of them: essentialome, repressors of colony formation, drivers and repressors of early pluripotency, drivers and repressors of late pluripotency and X-reactivation.

Supplemental Table S3. DESeq2 and pathway analysis from RNA-sequencing experiments. Related to **Fig. 3** and **fig. S5**. Differential gene expression analysis for each reprogramming timepoint (IFN γ vs control) and pathways (“WikiPathways mouse 2019”) associated to them (day 2, day 5, day 7 X-GFP negative, day 7 X-GFP medium, day 7 X-GFP high), and allelic ratio for X-linked genes in ESCs, NPCs and each reprogramming population.

Supplemental Table S4. DNA methylation: DMPs, TFBS enrichment, gene lists and pathway analysis. Related to **Fig. 5**, **fig. S8** and **fig. S9**. Differentially methylated CpGs (DMPs) for 5mC at days 5 and day 7 (X-GFP+) iPSCs (IFN γ vs control); overlap of upregulated genes in day 7 X-GFP+ cells by RNA-seq and genes with lower promoter 5mC levels in day 7 X-GFP+ cells and pathways (WikiPathways mouse 2019) associated to them; lists of X-reactivating, “early” and “main” X-reactivating, and escapee genes obtained from (24), SeSAmE TFBS enrichment (based on ChIP-seq data from Cistrome/ENCODE databases) at days 5 and 7 for CpGs losing 5mC globally, and for CpGs losing 5mC on the X chromosome at day 7.

Supplemental Table S5. Resources: oligonucleotides, antibodies, molecules for pathway validation, cell lines and softwares used in this study.

Supplemental Table S6. Source data for figure panels.

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