# Epigenetic predisposition to reprogramming fates in somatic cells

(Supplementary Information)

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**SI text 1: Delay in dox induction does not affect reprogramming efficiency**

In **Figure 1B,** we show that the number of iPS colonies counted on day 14 is proportional to the number of cells on day of seeding. To eliminate the possibility that the delay in dox induction reduces reprogramming potential, we performed a reprogramming experiment in which we induced OKSM at a delay of 0 or 5 days. Cells were prepared as in all reprogramming experiments (**Methods**). After transduction with fluorescent proteins, cells were split onto 12-well plates at 20,000 cells per well. Two days later, cells from 4 wells (one row) were taken, pooled together and seeded on a new 12 wells plate, 10,000 cells per each well. Cells from 4 additional wells were taken after 5 more days, in the same procedure. In both cases, dox was added to the cells the day after re-seeding, as done in our standard reprogramming experiments, imaged for 14 days, and colonies scored by Nanog-GFP positive signal. To avoid counting satellite colonies, only large colonies that could be matched to a progenitor cell at the time of induction were counted. Additionally, Alkaline-Phosphatase (AP) staining was also performed (Methods), and only large AP-positive colonies were counted, resulting in a slightly lower count for this method, but overall providing highly similar results (**Figure S1**). These data confirm that reprogramming potential is not reduced due to pre-growth on the plate for several days. Thus, our Luria Delbruck assay is not impeded by prolonged proliferation over the durations used in this study.

**SI text 2: Estimation of reprogramming efficiency for each decision model**

We repeated the delayed reprogramming experiment in four 96-well plates (384 wells overall). In order to separate between the two decision models (described in text) we included only wells in which the number of cells had increased at least 2-fold during the 5 days prior to dox induction (71 wells). The 71 wells were divided into 6 groups, each containing 10-13 wells whose initial cell count falls within a certain range, though results were not affected by the resolution of binning. Each red cross mark in **Figure 1B** shows the mean and standard deviation of the initial count per bin. Each green cross mark shows the mean and standard deviation of the later count for the same group of wells. The variance in cell count numbers between the red and green cross marks reflects the natural variability in cell division number between

wells within a group. The failure rate parameter λ was estimated for each model separately using a maximal likelihood estimator, i.e. the parameter that maximizes the likelihood of the observed reprogramming result in the plate:

$$logP_{det}(W_1 \ldots W_N) = \sum_{i=1}^{N} logP(W_i)$$

Where *N* is the number of wells used and $w_i$ is the event corresponding to well *i*. For positive wells:

$$P(w_i = at\ least\ one\ cell\ reprogrammed) = 1 - \lambda^{n_i}$$

and for negative wells:

$$P(w_i = all\ the\ cells\ did\ not\ reprogram) = \lambda^{n_i}$$

Here $n_i$ is the number of cells in the well at the relevant time: initial count or count at day of dox. This calculation is distinct from the cross-mark graph presented (**Figure 1B**), as it takes into account the exact cell counting of each well and its reprogramming outcome (success or failure) without using any binning or averaging. To get more a robust estimate of λ and the sampling error, we used a sampling bootstrap procedure [1]: we generated 400 sets of wells, each containing randomly picked 49 (70%) of the wells. The parameter was estimated for each of those sets, resulting in a distribution of λ values, for which we report the mean and standard deviation.

The blue data points in **Figure 1B** represent the results of a control reprogramming experiment where dox is added shortly after plating, using 13 plates and an average cell count per well for each plate. In these plates the 'initial count' and 'count at day of dox' are the same.

The solid curve for each color plots $1-\lambda^n$, which is the expected fraction of wells with GFP+ colonies according to the corresponding model as a function of *n*, the number of cells per well.

**SI text 3: Paired lineages p-value calculation**

The p-values reported for the paired-lineages experiment use the following null model: the relevant decision (or potential) was acquired after the first cell division independently by both pair members. This model implies random pairing. Namely, the observed combinations of pairs will follow a random distribution. Below we denote by *NR*, *iPS* and *FD* the number of same-fate pairs of each type. We calculate the p-value using an analogy of *N* boxes (where *N=NR+FD+iPS)*. Each box contains two sub-lineages. Under the null model (which assumes random assignment to these boxes) there are $\frac{(2N)!}{2^N}$ possible assignments. Out of these, the number of assignments in which there are *NR*, *FD* and *iPS* pairs of same-fate lineages is:

$$\frac{(2NR)!}{2^{NR}} \cdot \frac{(2FD)!}{2^{FD}} \cdot \frac{(2iPS)!}{2^{iPS}} \cdot \frac{N!}{NR!\,FD!\,iPS!}$$

And so the probability of getting exactly *NR*, *FD* and *iPS* pairs of same-fate lineages is the fraction of perfect pairing orderings out of the total number of orderings:

$$\frac{\frac{(2NR)!}{2^{NR}} \cdot \frac{(2FD)!}{2^{FD}} \cdot \frac{(2iPS)!}{2^{iPS}} \cdot \frac{N!}{NR!FD!iPS!}}{\frac{(2N)!}{2^{N}}}$$

We can simplify this expression using:

$$\frac{(2N)!}{2^N} = \frac{1\cdot 2\cdot 3\cdot 4\cdot 5\cdot 6\cdot \ldots\cdot (2N-1)\cdot 2N}{2\ \cdot 2\ \cdot 2\ \cdot \ldots\cdot 2} = 1\cdot\frac{2}{2}\cdot 3\cdot\frac{4}{2}\cdot 5\cdot\ldots\cdot(2N-1)\cdot\frac{2N}{2} = (2N-1)!!\cdot N!$$

where $(2N-1)!! = 1\cdot 3\cdot 5\cdot\ldots\cdot(2N-1)$. Overall:

$$P(num\ of\ fd\ pairs = FD, num\ of\ nr\ pairs = NR, num\ of\ iPS\ pairs = iPS \mid 2FD, 2iPS, 2NR) = \frac{\frac{(2NR)!}{2^{NR}}\frac{(2FD)!}{2^{FD}}\frac{(2iPS)!}{2^{iPS}}\frac{N!}{NR!FD!iPS!}}{\frac{(2N)!}{2^N}} = \frac{(2NR-1)!!(2FD-1)!!(2iPS-1)!!}{(2N-1)!!}$$

Since in our case there are exactly N pairs of the same fate, the p-value is given by the expression above, since:

$$P(num\ of\ same\text{-}fate\ pairs >= N \mid 2FD, 2iPS, 2NR) =$$
$$P(num\ of\ same\text{-}fate\ pairs = N \mid 2FD, 2iPS, 2NR) =$$
$$P(num\ of\ fd\ pairs = FD, num\ of\ nr\ pairs = NR, num\ of\ iPS\ pairs = IPS \mid 2FD, 2iPS, 2NR)$$

## SI Materials and Methods

**Lentiviral preparation and transduction.** The lentiviral FUW plasmid containing either YFP, H2B-cerulean (CFP), Oct4, Sox2, Klf4 or c-Myc under control of the tetracycline operator and a minimal CMV promoter (7.5 µg) was packaged in HEK 293T cells cultured in DMEM with 10% FBS, L-glutamine and penicillin-streptomycin on 10cm dishes with the packaging plasmid Dr8.2 (4 µg) and VSV-G coat (3.4 µg) using *Trans*IT®-LT1 Transfection Reagent (Mirus). Viral supernatant was collected 48h after transfection, yielding a total of ~10 ml of supernatant. Viral supernatant was concentrated ~100-fold using PEG Virus Precipitation Kit (Abcam) according to manufacturer's instructions. Viral concentrates were stored at −80 °C. NGFP MEFs were transduced in 0.7 ml ES medium containing 8 µg per ml polybrene (Sigma) using 50 µl of the concentrated virus on a 12-well plate. ES cells medium was added ~16 h after infection and after another 24h fluorescent cells were observed by fluorescent microscope. The cells were expanded twice before seeding at

unique representations within control, uninfected inducible MEFs that were passaged in parallel.

**Immunofluorescence and iPS cell colony scoring**. At the end of a given imaging experiment, plates were fixed in 4% paraformaldehyde and immunostained for Nanog (BD Pharmingen) and/or E-cadherin (Cell signaling) at 1:500 dilution and detected using Alexa488 or Alexa594 conjugated secondary antibodies diluted at 1:2000 (Invitrogen). Colonies that were stained both for Nanog and E-cadherin classified as positive iPSC colonies. For some experiments, alkaline phosphatase (AP) activity (Stemgent) was used for efficiency estimation. Positive AP colonies were counted as iPSC colonies. Immunostaining for measuring OSKM levels used Oct4, c-Myc, Sox2 and Klf4 antibodies (Santa Cruz) at 1:300 dilution. Cells were stained every two days (during reprogramming); each well was stained for two different factors. To analyze the experiment all images were automatically segmented using Cell Profiler [2]. To evaluate the Ezh2 inhibition effect, staining against a H3K27me3 (Millipore) at a dilution of 1:100, for 7 minutes in room temperature.

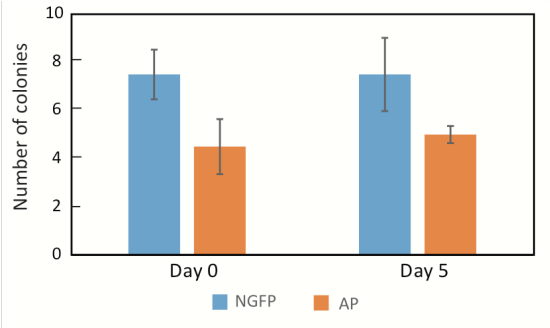
**Flow Cytometry and Live Immunostaining.** Live staining for Thy1 was done using an anti-mouse CD90.2 (Thy-1.2) antibody conjugated to APC (eBioscience). For lineage tracking (**Figure S11 a**) cells were initially plated in a 96-well plate containing feeders (30 cells per well). 16 hours after seeding, cells were incubated with Thy1 Ab at 1:50 dilution in mouse ES medium for 45 minutes at $4^0$C in the dark followed by an additional dilution of 1:4 in a fresh medium containing doxycycline to initiate reprogramming. For the Thy1+/- experiment (**Figure S11 b**) cells were incubated with Thy1 Ab as above, then sorted on a MoFlo XDP (Beckman-Coulter). Thy1+/Thy1-/unsorted cells were plated at a density of 25,000 cells per well on gelatin-coated 12-well plate. Reprogramming started 24h after plating. Cells were fixed on day 12, stained for AP and imaged.

**iPSC colonies counting.** To asses the efficiency of reprogramming in all experiments that were done in 12- or 6-well plates, counting was done by tracing colonies formation from day 6 until the end of experiment and Nanog appearance. This stringent method of counting estimates the true number of iPSC colony progenitors, rather than the total number of final colonies, which is dominated by “satelites” that fragment out of progenitor colonies around day 8, as previously shown [3]. In 96-well plate experiments, efficiency was estimated by counting the number of GFP+ wells, thus bypassing the problem of satelites. Cells were imaged every 24 hours. Colonies that formed around day 6, were traceable until day 14, and yielded large Nanog-GFP+ colonies, were scored as successful reprogramming events; satellite colonies that could not be traced to founding cells at the time of induction were excluded.

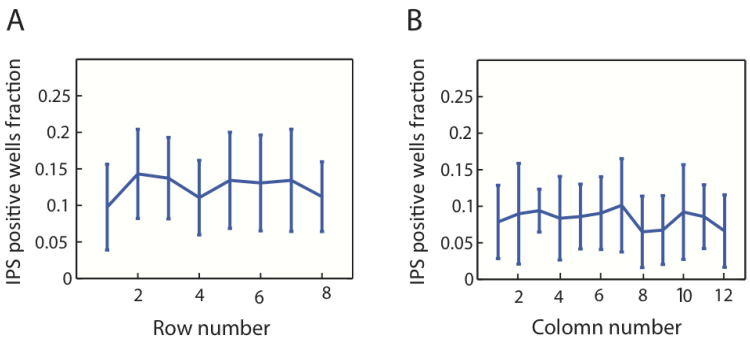
## References

1. Efron B, Tibshirani R (1993) *An introduction to the bootstrap*. Chapman & Hall, New York

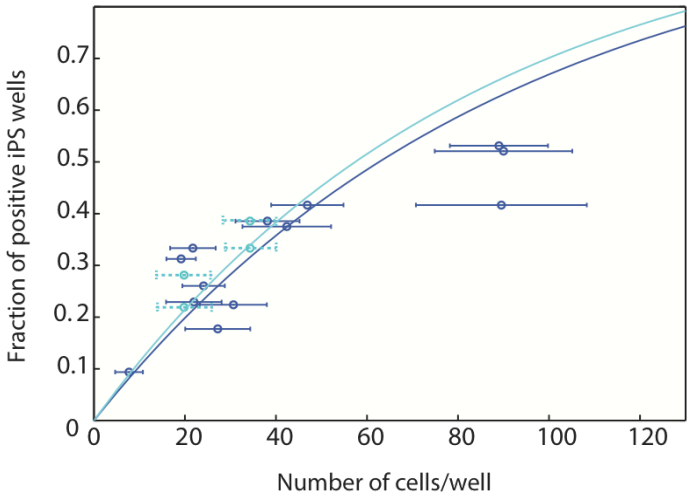
2. Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, Guertin DA, Chang JH, Lindquist RA, Moffat J *et al* (2006) CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol* **7:** R100
3. Smith ZD, Nachman I, Regev A, Meissner A (2010) Dynamic single-cell imaging of direct reprogramming reveals an early specifying event. *Nat Biotechnol* **28:** 521-526
4. Stadtfeld M, Maherali N, Borkent M, Hochedlinger K (2010) A reprogrammable mouse strain from gene-targeted embryonic stem cells. *Nat Methods* **7:** 53-55



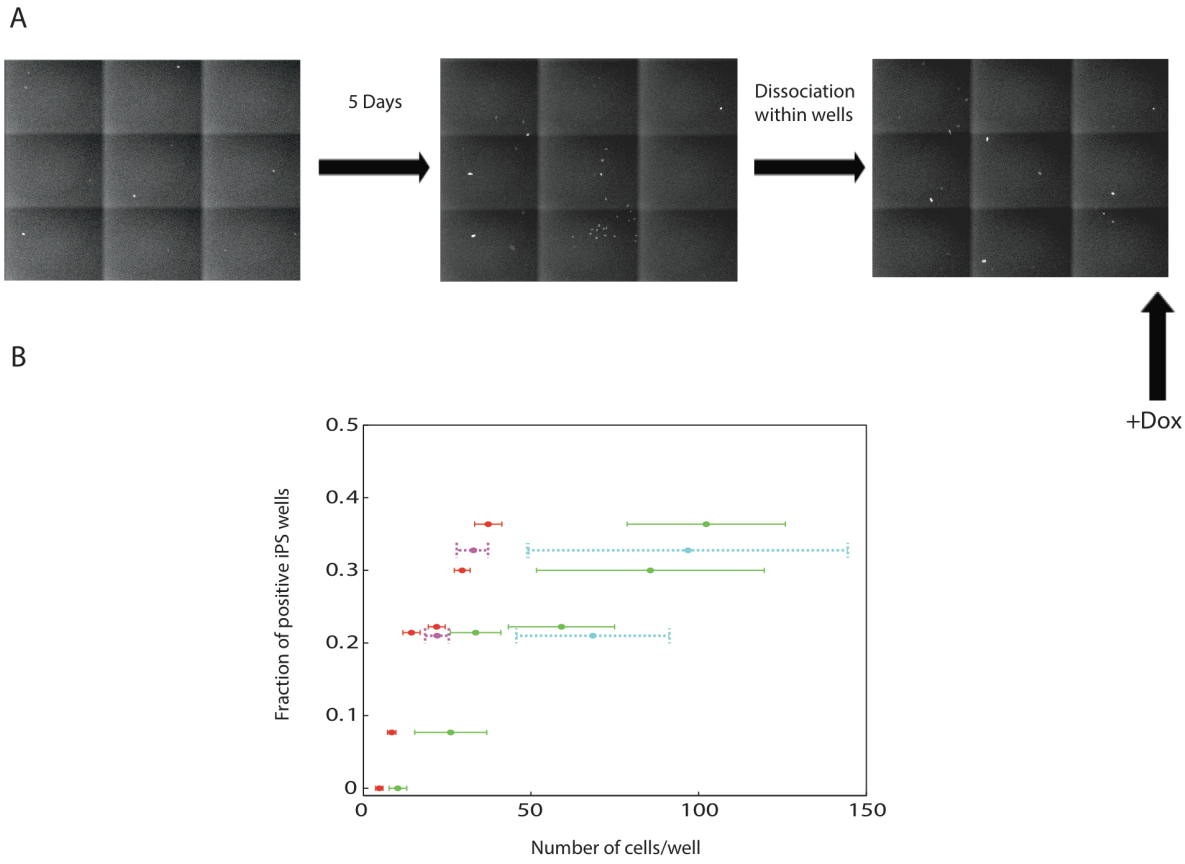

**SI Figure S1. Reprogramming efficiency does not decrease when delaying dox by up to 5 days.** Cells were induced by OKSM after being expanded for 5 days, followed by replating to control for cell number, or with no delay. Bars show average colony count after 14 days of reprogramming, according to Nanog and E-cadherin staining (blue) or according to alkaline phosphatase activity test (orange). Error bars represent standard deviation over 4 replicate wells. The expansion period causes no reduction in reprogramming efficiency.




**SI Figure S2. Well location on plate does not influence reprogramming outcome.** The number of positive (GFP+) wells in each row **(a)** or column **(b)** of a 96-well plate demonstrates no preferential reprogramming as an attribute of position within the 96 well plate format. Shown are mean and standard deviation over 13 plates.

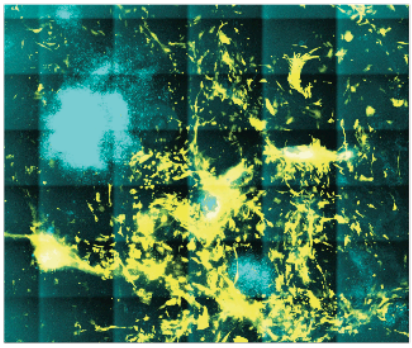



**SI Figure S3. Reprogramming efficiency is not reduced by low density in the Luria-Delbruck experiment.** The standard efficiency estimation (Figure 1C, blue marks) was repeated in increased cell density as follows: between 20-40 inducible CFP tagged cells were plated in each well together with 1000 inducible YFP tagged cells. Only CFP labeled Nanog-GFP+ wells were counted as positive wells. The reprogramming efficiency (as fraction of positive wells) for 4 such plates is shown (cyan marks) along with the 13 plates shown in **Figure 1B** (dark blue marks). Estimated "standard failure rate" parameter including these 4 additional plates is 0.988±0.003. Solid curves represent the reprogramming probability parameter, calculated as the maximum likelihood estimator according to the standard data, including (cyan) or excluding (blue) the high density plates. The results show similar efficiency estimates for the high and low density experiments.




**SI Figure S4. Local clump density does not affect reprogramming efficiency in the Luria-Delbruck experiment.** Efficiency estimation was repeated as in **Figure 1B** (red and green marks), but with local density and lineage relationships disrupted prior to dox induction. **(a)** Inducible MEFs were seeded as in **Figure 1**. After 5 days, cells were dissociated and allowed to resettle as single cells within each well, followed by induction with dox. **(b)** Reprogramming efficiency estimates. The magenta and cyan marks show 96 such wells (for which the cell count increased at least 2-fold within the 5 days) binned according to their initial cell number. Magenta mark - initial cell count; Cyan mark - cell count at day of dox induction. The red and green marks from the original experiment (**Figure 1B**) are shown for comparison. For these experiments, the estimated failure rate parameter is 0.991±0.001 according to starting cell count or and 0.9970±0.0003 according to count at time of dox induction.

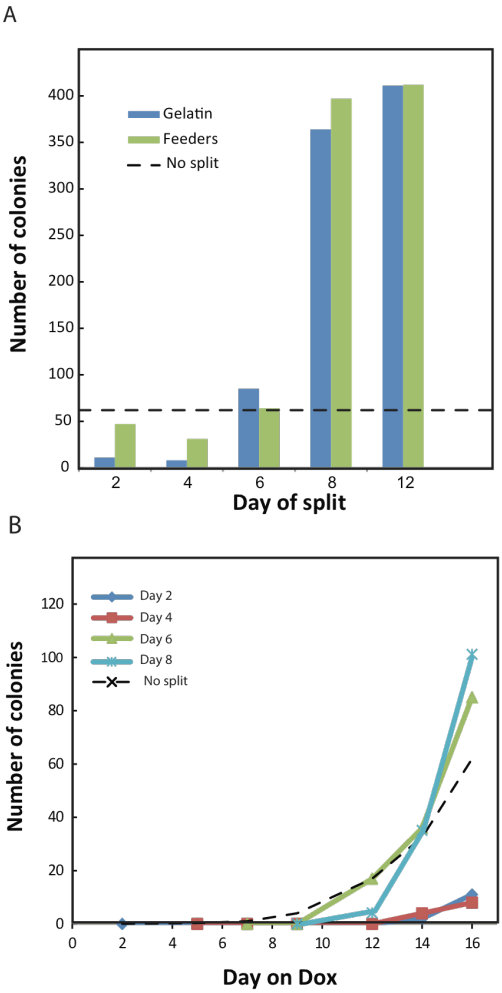
A



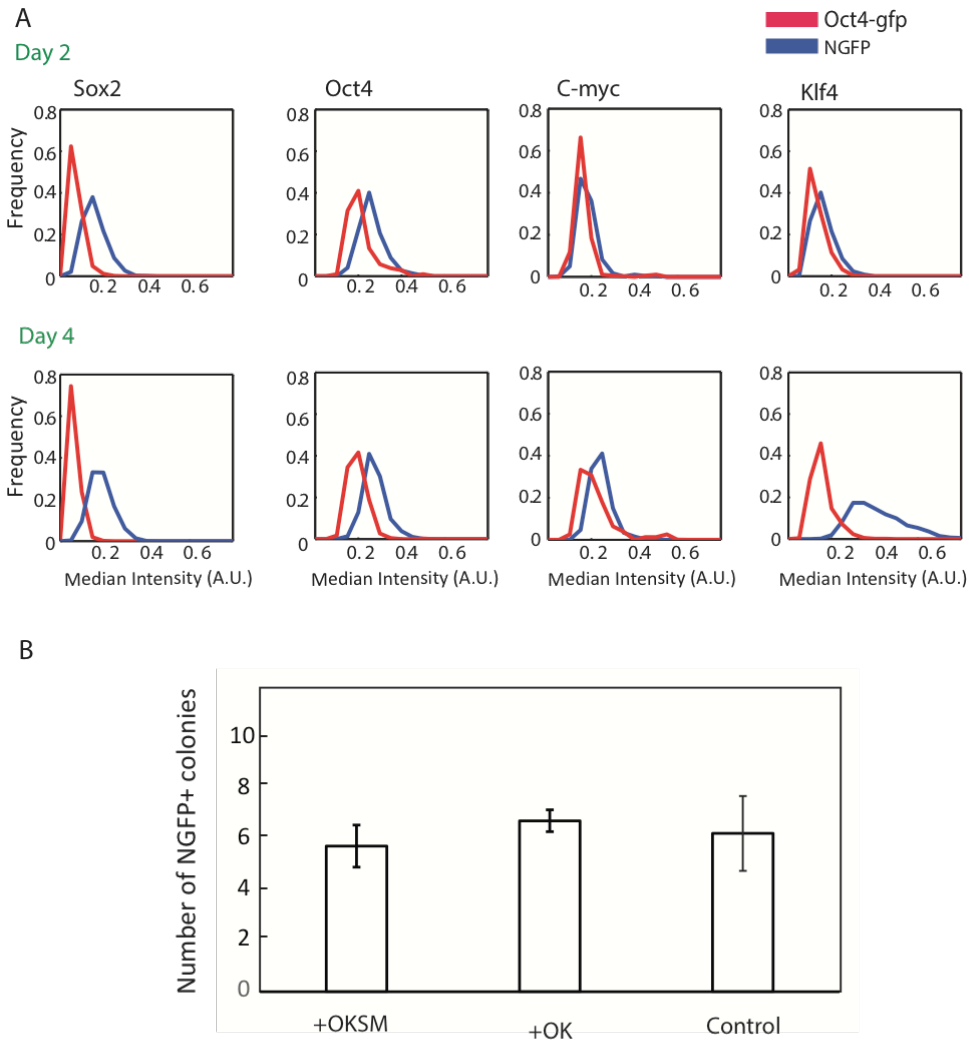
B

| Model | Number of pairs observed | | | | | | P-value |
|---|---|---|---|---|---|---|---|
| | FD-FD | iPS-iPS | FD-iPS | NR-NR | FD-NR | iPS-NR | |
| Proliferation and reprogramming | 56 | 9 | 0 | 77 | 0 | 0 | 2.63E-54 |
| Proliferation decision point | 65 | | | 77 | 0 | | 4.21E-43 |
| Reprogramming decision point | 56 | 9 | 0 | - | - | - | 6.25E-12 |

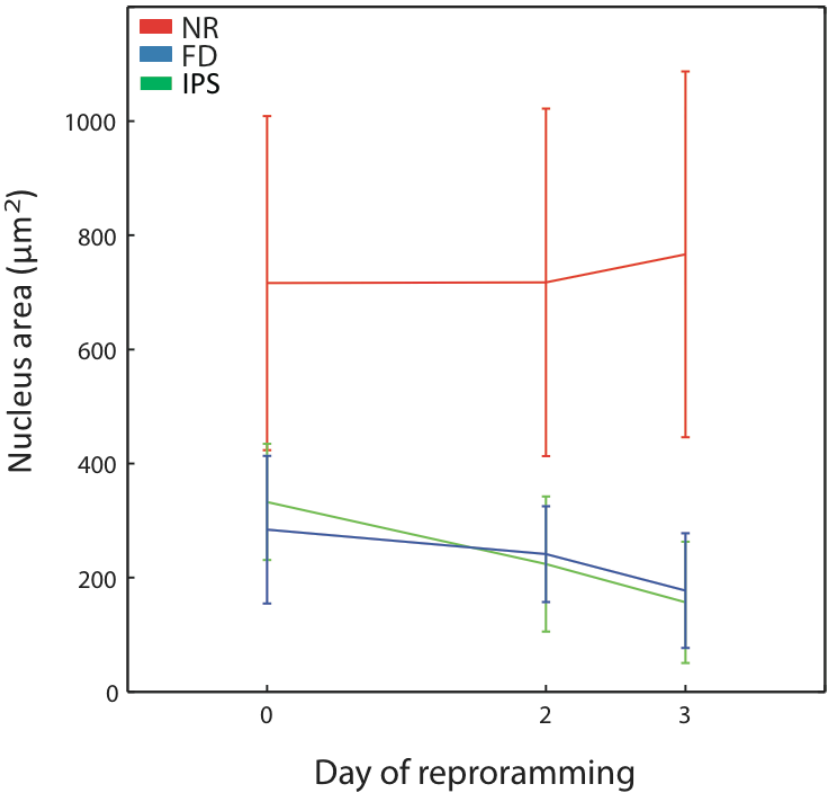
**SI Figure S5. Correlation between paired lineages is not affected by cell density.** The paired lineages experiment (**Figure 2**) was repeated at increased cell density as in **Figure S3**: In each well, 30 CFP inducible MEFs were plated together with 1000 inducible YFP MEFs. Lineage pairs were tracked only in the CFP population. **(a)** A sample well imaged at day 14. **(b)** Counts of lineage pairs with each corresponding fate combination (as in **Figure 2D**). Again, no mixed-fate pairs (e.g. iPS-FD) were observed.



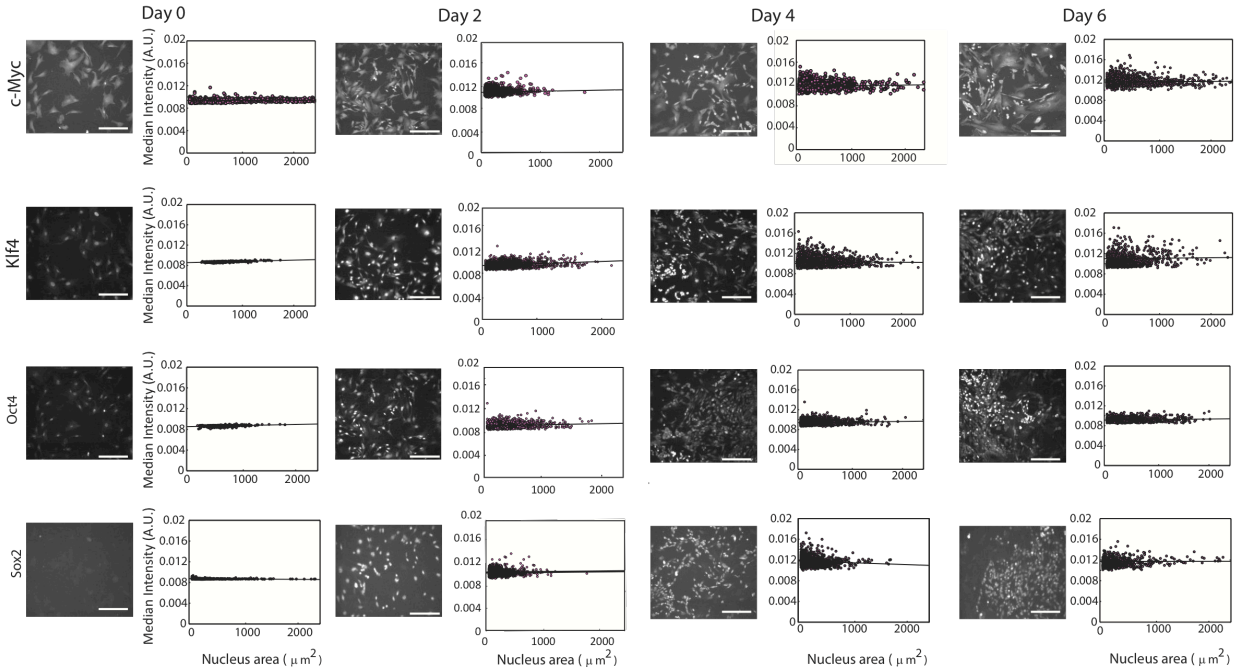

**SI Figure S6. Cell replating disturbs reprogramming up to day 6.** CFP labeled MEFs were replated onto gelatin or feeder coated plates during the reprogramming protocol (day 2,4,6,8 or 12 after OKSM induction). **(a)** Reprogramming efficiency (as colony counts at final day) vs. day of replating. The black dashed line shows the efficiency in a no-split control. **(b)** Day of first colony appearance as a function of replating day (gelatin coated wells). Replating at days earlier than 6 results in both lower efficiency and delayed appearance of colonies. From day 8 onward, colonies are formed soon after replating, and at much higher numbers than control, likely reflecting splitting of lineages containing committed cells into multiple colonies.



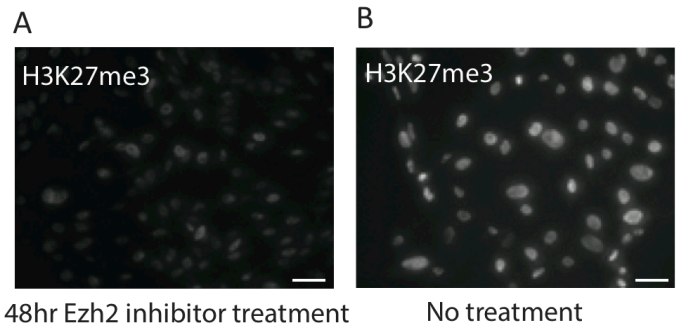

**SI Figure S7. OKSM levels do not limit reprogramming in NGFP inducible MEFs. (a)** Histograms of OKSM levels in polycistronic OKSM Oct4-GFP inducible MEFs [4] (red) or NGFP inducible MEFs (blue), at day 2 or 4 after dox induction. Oct4, Sox2, c-Myc and Klf4 were immunostained and quantified as in **Figure 3F**. **(b)** Reprogramming efficiency for the NGFP inducible MEFs, with the addition of OK or OKSM factors. Inducible NGFP MEFs were infected with viral vectors containing extra copies of either inducible O,K or O,K,S,M factors prior to induction by doxycycline. Shown are mean and standard deviation for imaged colony counts over 4 replicate wells. Similar results were obtained in two independent experiments.



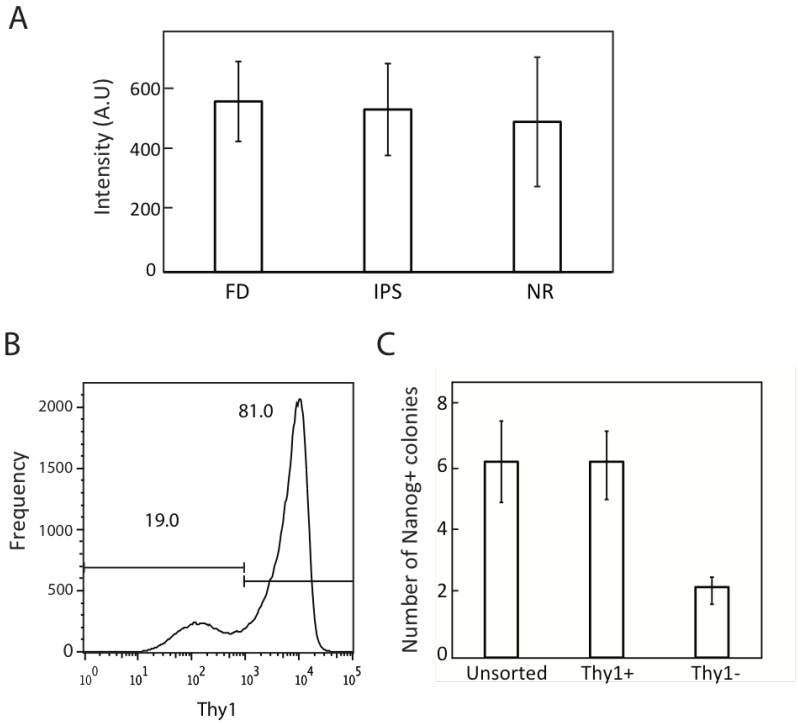

**SI Figure S8. Nucleus size correlates with reprogramming response.**
Nucleus size in H2B-CFP labeled inducible MEFs was measured on days 0, 2 and 3 of an imaged reprogramming experiment. Final lineage fates were determined as in **Figure 2**. Nucleus size mean and standard deviation (over 10-30 cells) are shown for NR, FD and iPS lineages for each day. Nuclei of iPS/FD cells are significantly smaller than those of NR cells (P=4.2E-9, 2.4E-7, 7.0E-6 for days 0,2,3, respectively), and decrease in size over the first few days of reprogramming. Cells of different final fates were randomly picked from 3 independent imaging experiments.



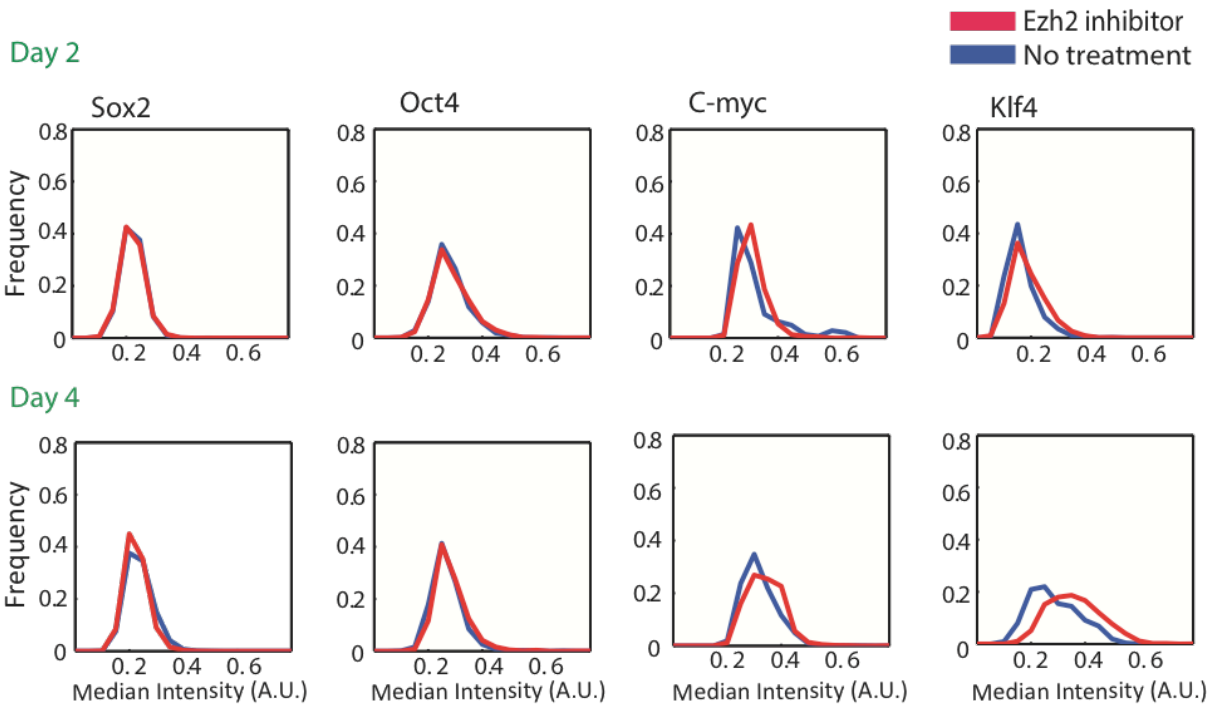

**SI Figure S9. OKSM levels in individual cells during the early days of reprogramming.** Correlations between level of c-Myc, Klf4, Oct4 or Sox2 and morphological response on day 0, 2, 4 or 6 of reprogramming are shown. Each plot shows size of nuclear immunostaining signal for a given factor within induced cells (x-axis) against their median fluorescence intensity (y-axis). A representative immunostaining field is shown next to each plot. Scale bars: 200 μm.




**SI Figure S10. Ezh2 inhibitor treatment for 48 hours decreases global H3K27me3 levels.**
Staining with an anti H3K27me3 antibody after 48 hours of Ezh2 inhibitor treatment **(a)** or after no treatment **(b)** demonstrates the global action of the drug on H3K27me3 levels. Scale bars: 50 μm.




**SI Figure S11. Thy1 expression at Day 0 is not predictive of lineage fate. (a)** MEFs were live-stained for Thy1 on Day 0 of OKSM induction. Lineages were tracked and their fates determined as in Figure 2. Shown are mean day 0 Thy1 levels for each lineage fate group (FD: *n*=10, iPS: *n*=7, NR: *n*=9). No significant difference in Thy1 expression is found between groups. **(b)** Cells were sorted by Thy1 level. Thy1+, Thy1- and unsorted cells were plated separately and reprogrammed. Efficiency was estimated by Nanog staining. Shown are mean and standard deviation for imaged colony counts over 4 replicate wells. Thy1+ cells have the same efficiency as unsorted cells, while Thy1- cells produce far less reprogramming colonies.




**SI Figure S12. Ezh2 inhibitor pre-treatment does not modulate OKSM levels during early reprogramming.** Shown are histograms of OKSM levels in Ezh2 pre-treated (red) or no treatment (blue) inducible MEFs, at day 2 or 4 after dox induction. Oct4, Sox2, c-Myc and Klf4 were immunostained and quantified as in **Figure 3F**.

| Well number | Cells count on day of plating | Cells count on day of + dox | iPS positive? |
|---|---|---|---|
| 1 | 26 | 61 | - |
| 2 | 27 | 65 | - |
| 3 | 28 | 63 | - |
| 4 | 28 | 115 | - |
| 5 | 29 | 80 | + |
| 6 | 29 | 90 | - |
| 7 | 31 | 65 | - |
| 8 | 32 | 170 | + |
| 9 | 32 | 67 | - |
| 10 | 33 | 80 | + |
| Total | 29.5 (±2.4) | 85.6 (±33.9) | 3/10 |

**SI Table S1.** Cell counts and reprogramming outcomes for a group of 10 wells with similar initial cell count. A "+" in iPS outcome denotes at least one GFP+ colony in the well at the time of counting. "Total" denotes average +/- standard deviation for counts, and fraction of positive wells. This groups is highlighted as a dashed box in **Figure 1B**.

**SI Movie S1. Tracing paired lineages over a two week reprogramming period.** The movie shows the CFP channel of a pair of lineages traced to a single MEF of origin. The first cell division occurred 8 hours after imaging. From that division on, the paired lineages (marked red and green) were traced. At 18:40 hours the green lineage divided again (still before dox induction) and its sub-lineages were tracked separately. Dox was added at Day 0. The final GFP+ colonies are composed of cells from different sub-lineages, some of which are merged from several sub-lineages.

**SI Movie S2. Tracing lineages over a two week reprogramming period after Ezh2 inhibition pre-treatment.** The movie shows the CFP channel of one sample well in a 96-well plate. Each lineage pair is annotated using the same color. The pair annotated purple on the bottom left is a mixed pair, with one of the sub-lineages not responding and dying off on day 2.