Stem Cell Reports, Volume *10*

Supplemental Information

Inductive and Selective Effects of GSK3 and MEK Inhibition on Nanog Heterogeneity in Embryonic Stem Cells

Simon Hastreiter, Stavroula Skylaki, Dirk Loeffler, Andreas Reimann, Oliver Hilsenbeck, Philipp S. Hoppe, Daniel L. Coutu, Konstantinos D. Kokkaliaris, Michael Schwarzfischer, Konstantinos Anastassiadis, Fabian J. Theis, and Timm Schroeder

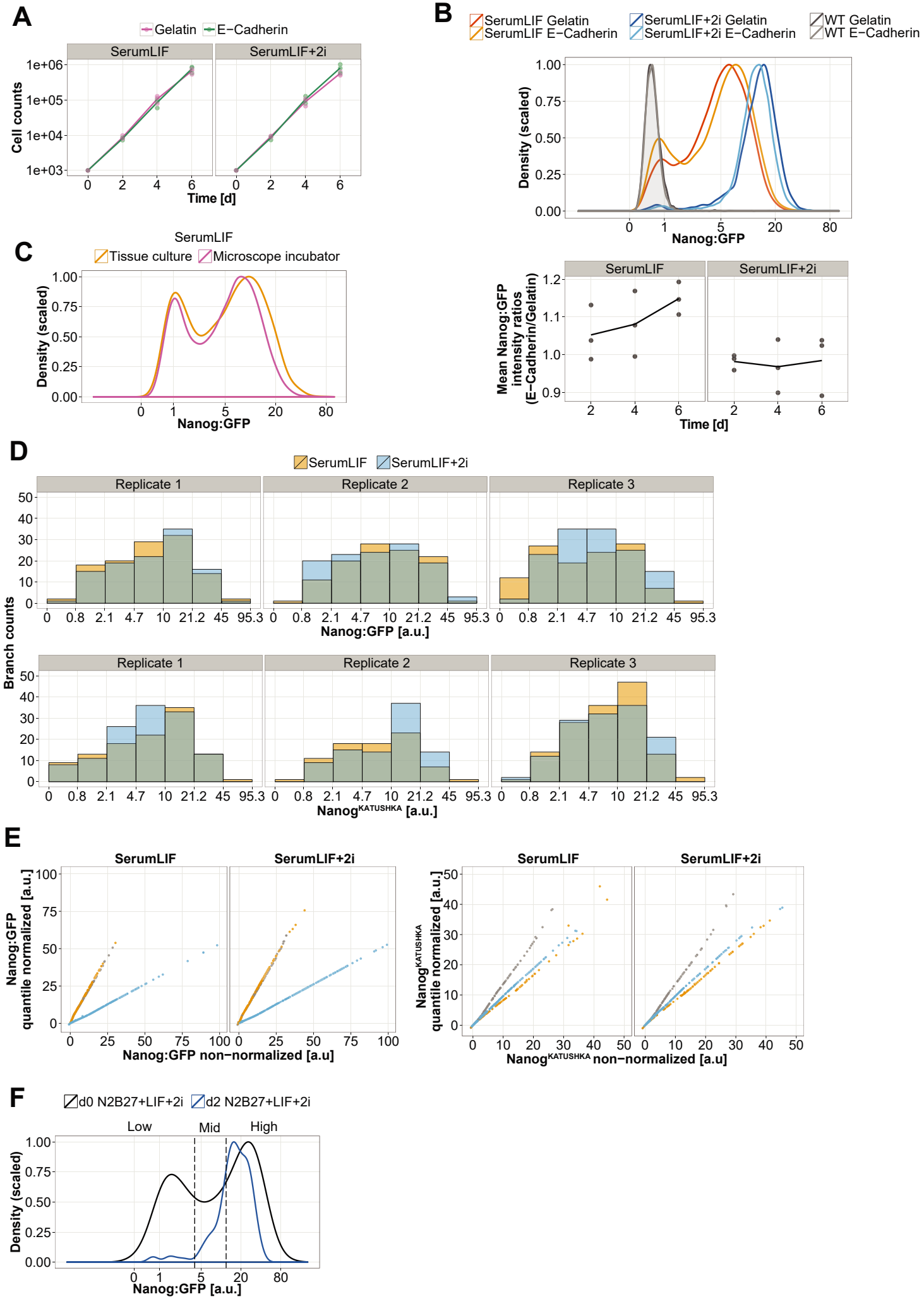

A
Gelatin
E−Cadherin
SerumLIF
SerumLIF+2i
Cell counts
1e+06
1e+05
1e+04
1e+03
0
2
4
6
Time [d]
B
SerumLIF Gelatin
SerumLIF E−Cadherin
SerumLIF+2i Gelatin
SerumLIF+2i E−Cadherin
WT Gelatin
WT E−Cadherin
Density (scaled)
1.00
0.75
0.50
0.25
0.00
0
1
5
20
80
Nanog:GFP
C
SerumLIF
Tissue culture
Microscope incubator
Density (scaled)
Nanog:GFP
Mean Nanog:GFP intensity ratios (E−Cadherin/Gelatin)
1.2
1.1
1.0
0.9
Time [d]
D
SerumLIF
SerumLIF+2i
Replicate 1
Replicate 2
Replicate 3
Branch counts
50
40
30
20
10
0
0
0.8
2.1
4.7
10
21.2
45
95.3
Nanog:GFP [a.u.]
Nanog^KATUSHKA [a.u.]
E
SerumLIF
SerumLIF+2i
Nanog:GFP quantile normalized [a.u.]
100
75
50
25
0
Nanog:GFP non−normalized [a.u]
Nanog^KATUSHKA quantile normalized [a.u.]
Nanog^KATUSHKA non−normalized [a.u]
F
d0 N2B27+LIF+2i
d2 N2B27+LIF+2i
Low
Mid
High
Density (scaled)
1.00
0.75
0.50
0.25
0.00
0
1
5
20
80
Nanog:GFP [a.u.]

Figure S1. Related to Figure 1. NG4 (Nanog:GFP) mESCs proliferate similarly on gelatin and E-Cadherin coated plates. Means are shown as lines and individual experiments as points (n=3) (B) Similar Nanog:GFP distribution on gelatin and E-Cadherin coating (measured by flow cytometry). A representative measurement after 4 days (top) and a time-course of mean fluorescence intensity ratios (bottom) are shown. Means are shown as lines and individual experiments as points (n=3 experiments) (C) Nanog:GFP distributions are similar when cells are incubated in a tissue culture incubator or at the microscope incubator on E-Cadherin as measured by flow cytometry after 2 days. Mean fluorescence intensity ratios (tissue culture/microscope) were 0.96 +/- 0.14 (mean +/- sd, n=4 experiments). One representative experiment is shown. (D) Branch counts of each bin of the Nanog distribution at movie start (d0) in SerumLIF or SerumLIF+2i for individual replicate experiments. Branches were tracked at comparable numbers between replicate experiments, and the Nanog d0 distributions in SerumLIF or SerumLIF+2i within each experiment were similar. (E) Quantile normalization of replicate experiments did not introduce any relevant changes in the relationship between single cells within an experiment. Original and normalized data points are perfectly correlated (R>0.99) within each experiment, showing that Nanog quantifications got transformed in a virtually linear way. Quantifications at d0 and d2 are shown. Max. 11 data points per cell line with high values were removed from the plot to improve the visualization of the main population without changing any conclusions. Different colors correspond to different replicate experiments within each cell line. (F) Culture for 2 days in serum-free N2B27+LIF+2i medium leads to a more homogeneous mid/high Nanog:GFP distribution (quantification by image analysis; n=238 branches pooled from two independent experiments). The data set was biased as described in Figure 1D and gates are the same.

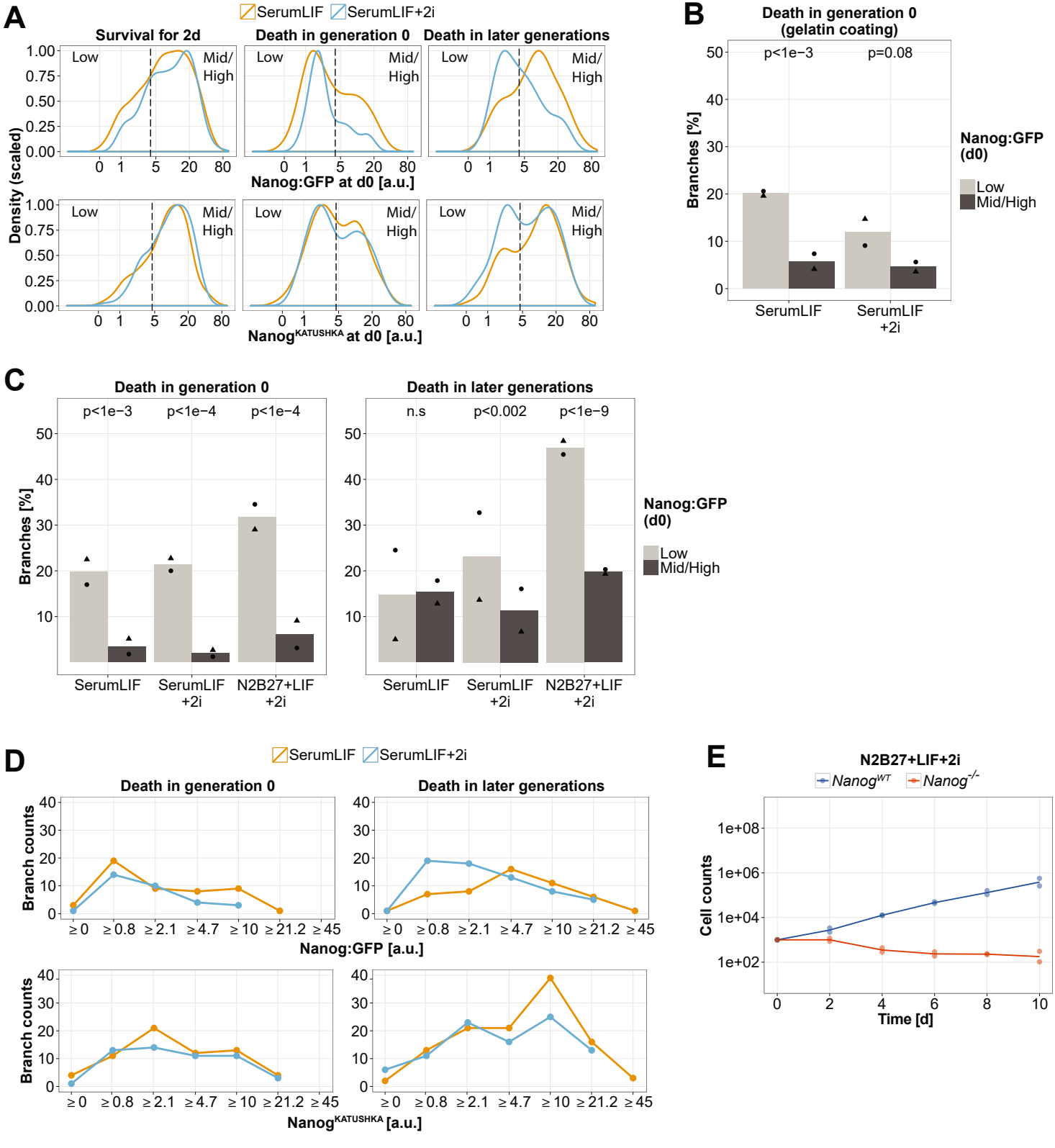
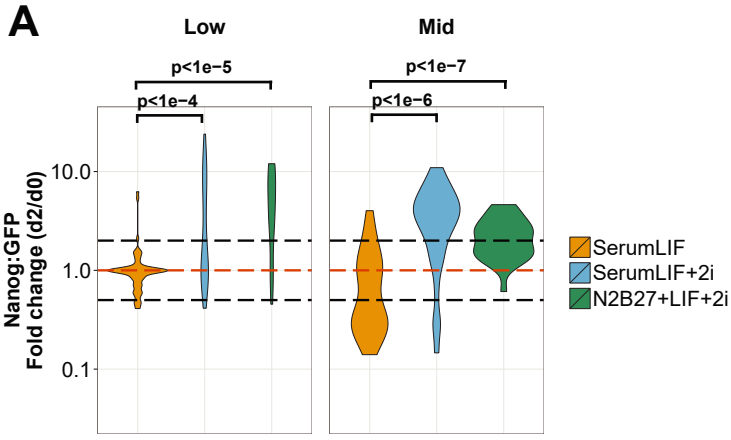

A
SerumLIF
SerumLIF+2i
Survival for 2d
Death in generation 0
Death in later generations
Low
Mid/
High
Density (scaled)
Nanog:GFP at d0 [a.u.]
Nanog^KATUSHKA at d0 [a.u.]
B
Death in generation 0
(gelatin coating)
p<1e−3
p=0.08
Branches [%]
Nanog:GFP
(d0)
Low
Mid/High
SerumLIF
SerumLIF
+2i
C
Death in generation 0
Death in later generations
p<1e−3
p<1e−4
p<1e−4
n.s
p<0.002
p<1e−9
Branches [%]
Nanog:GFP
(d0)
Low
Mid/High
SerumLIF
SerumLIF
+2i
N2B27+LIF
+2i
D
SerumLIF
SerumLIF+2i
Death in generation 0
Death in later generations
Branch counts
≥0
≥0.8
≥2.1
≥4.7
≥10
≥21.2
≥45
Nanog:GFP [a.u.]
Nanog^KATUSHKA [a.u.]
E
N2B27+LIF+2i
Nanog^WT
Nanog^-/-
Cell counts
Time [d]

Figure S2. Related to Figure 2. (A) 2i induces cell death preferentially in Nanog low ESCs. Nanog distributions at d0 (see Figure 1D) were divided into surviving branches, branches dying in generation 0, or branches dying in later generations. The dashed line represents the threshold between low and mid/high compartments chosen for statistical analysis in Figures 2A and S2B-C. (B) Nanog low cells have higher death rates than mid/high cells before the first cell division also on gelatin coating (compare to Figure 2A). (C) 2i induces death of Nanog low cells in later generations also in serum-free N2B27+LIF+2i medium. Additionally, Nanog low cells also have higher death rates than mid/high cells before the first cell division in this medium (as in serum-containing medium). The experiments have been performed in addition to Figure 2A, but including N2B27+LIF+2i as extra condition. In (B) and (C), p-values were determined with the Cochran-Mantel-Haenszel Chi-Squared Test and means (n=2 experiments) are shown as bars. (D) Absolute branch counts in bins used to calculate fractions of dying cells. No data points for bins without branches are shown. (E) The *Nanog*$^{-/-}$ cell population does not expand in serum-free N2B27+LIF+2i medium, but can be maintained for at least 10 days. Means are shown as lines and individual experiments as points (n=2 experiments). Also see Figure 2E, but absolute cell counts differ due to different cell splitting procedures (Also see Supplemental Experimental Procedures). In A and D, data were pooled from three independent experiments.


A
Low
Mid
p<1e−5
p<1e−4
p<1e−7
p<1e−6
Nanog:GFP
Fold change (d2/d0)
10.0
1.0
0.1
SerumLIF
SerumLIF+2i
N2B27+LIF+2i

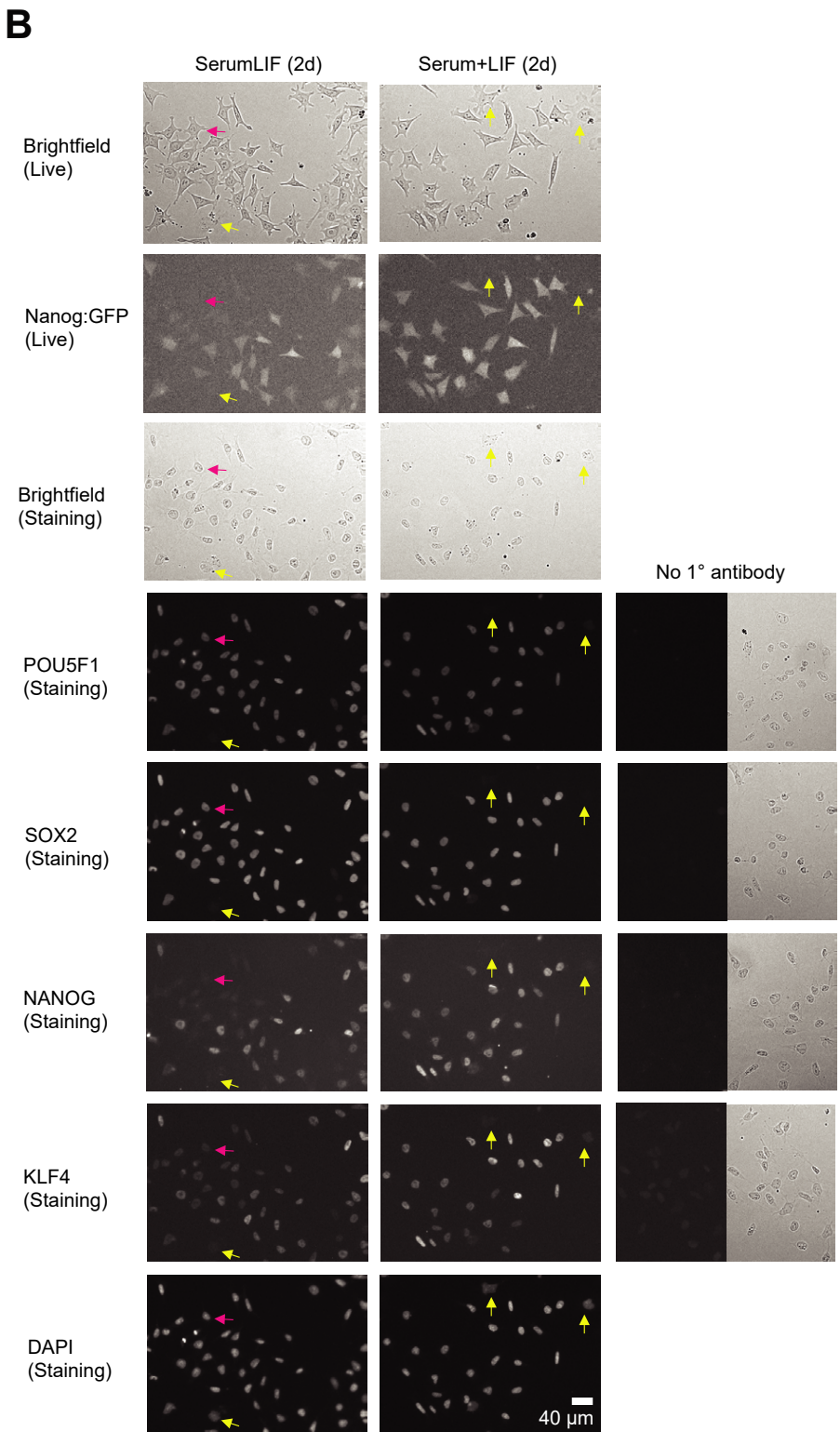

B
SerumLIF (2d)
Serum+LIF (2d)
Brightfield
(Live)
Nanog:GFP
(Live)
Brightfield
(Staining)
No 1° antibody
POU5F1
(Staining)
SOX2
(Staining)
NANOG
(Staining)
KLF4
(Staining)
DAPI
(Staining)
40 μm

Figure S3. Related to Figure 3. (A) 2i induces Nanog in low and mid cells (at d0) also in serum-free N2B27+LIF+2i medium. The red dashed line indicates Nanog maintenance, black dashed lines indicate 2-fold changes. P-values were calculated using the Wilcoxon rank sum test. The experiments have been performed in addition to Figure 3B, but including N2B27+LIF+2i as extra condition. Data were pooled from two independent experiments. (B) Cells with large cell area (yellow arrows) often lack expression of pluripotency markers POU5F1, SOX2, KLF4 and endogenous NANOG protein. Morphologically normal Nanog:GFP low cells, which are only present at larger numbers in SerumLIF conditions (magenta arrows), also express low levels of NANOG protein, but normal POU5F1 and SOX2 levels. Representative immunostaining at the end of a 2d experiment is shown. Right column shows individual control stainings, where one 1° antibody was missing for each control and the corresponding brightfield images. Display settings (black/white points) have been set individually for each fluorescent channel but kept constant for all conditions and the staining control.

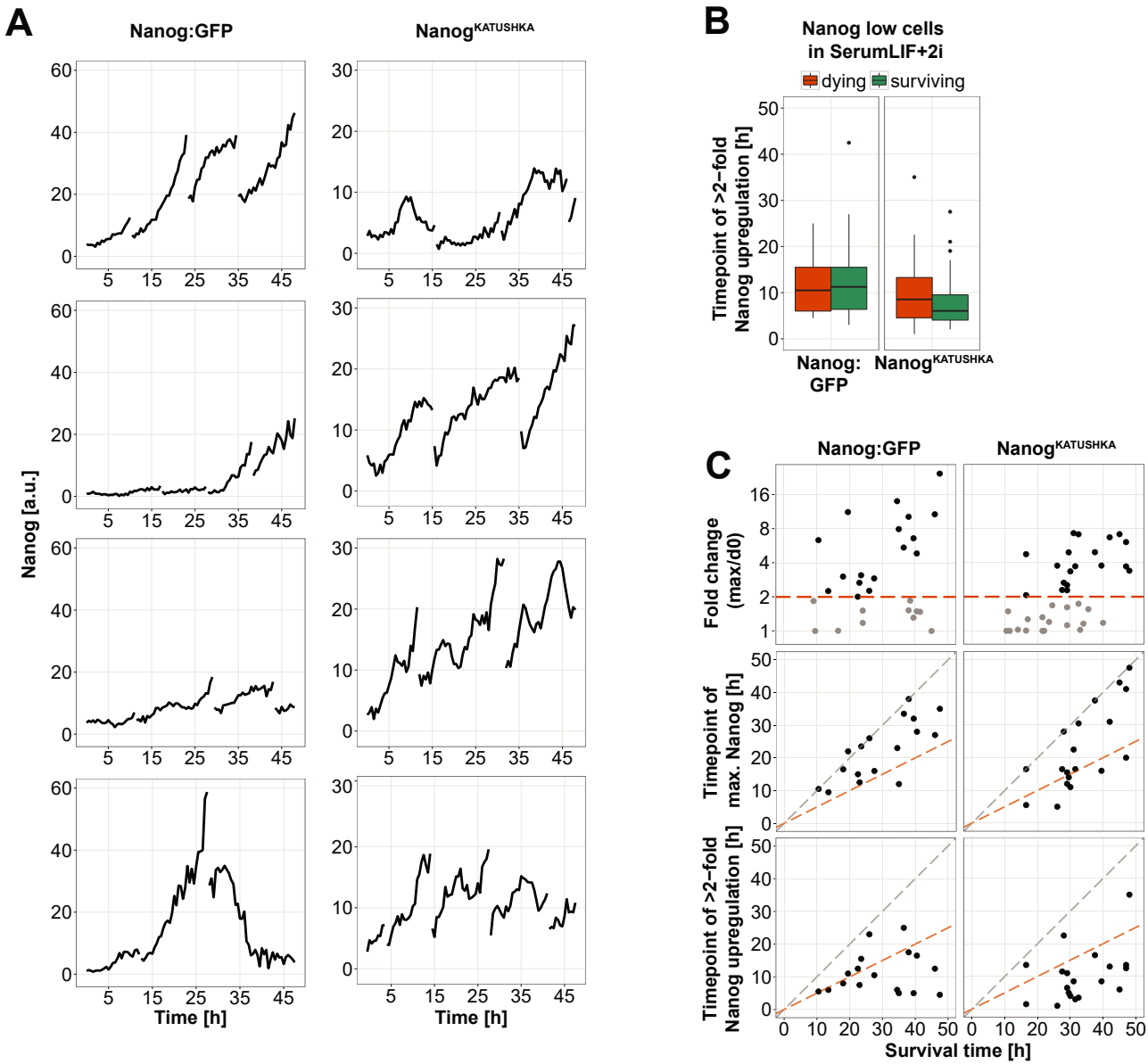

A
Nanog:GFP
Nanog^KATUSHKA
Nanog [a.u.]
Time [h]
Time [h]
B
Nanog low cells
in SerumLIF+2i
dying
surviving
Timepoint of >2−fold
Nanog upregulation [h]
Nanog:
GFP
Nanog^KATUSHKA
C
Nanog:GFP
Nanog^KATUSHKA
Fold change
(max/d0)
Timepoint of
max. Nanog [h]
Timepoint of >2−fold
Nanog upregulation [h]
Survival time [h]

Figure S4. Related to Figure 4. (A) Example branches of 2i induced Nanog upregulation in Nanog low cells show variable onset times, maximum Nanog levels, stabilities and occurrences of fluctuations. Qualitatively, fluctuations were more typical for Nanog^{KATUSHKA} than Nanog:GFP cells. (B) Nanog upregulation in low cells in SerumLIF+2i occurs in a similar time-range in dying and surviving branches (n=17 dying and n=32 surviving branches for Nanog:GFP, and n=19 dying and n=29 surviving branches for Nanog^{KATUSHKA}). The dataset was filtered on dying branches that upregulated Nanog >2-fold until death and surviving branches that upregulated Nanog >2-fold from d0 to d2. Cells that died in generation 0 were excluded from analysis. (C) Parameters extracted from Nanog low cells that upregulated Nanog and died in SerumLIF+2i later than generation 0 (see Figure 4B). Cells that survive longer, upregulate Nanog stronger (top), maximum Nanog levels mostly occur in the second half of the survival time (middle), but Nanog upregulation mostly occurs in the first half of the survival time (bottom). The horizontal red line (top) marks 2-fold upregulation, the diagonal grey line marks the 100% survival time and the red diagonal line the 50% survival time (middle and bottom). In B and C, data were pooled from three independent experiments.

Supplemental Legends

Video S1. Cell death and ceased proliferation in a Nanog:GFP colony after 2i treatment

Video S2. Upregulation of Nanog:GFP after 2i treatment

Video S3. Upregulation of $Nanog^{KATUSHKA}$ after 2i treatment

Video S4. Cell death, ceased proliferation and Nanog upregulation of individual cells in a Nanog:GFP colony after 2i treatment

Supplemental Experimental Procedures

Cell Lines

Mouse ESC lines NG4 (Schaniel et al., 2009), containing a BAC based transcriptional reporter producing GFP from an ectopic Nanog locus and R1 $Nanog^{KATUSHKA}Klf4^{VENUS}$ producing a Nanog - fluorescent protein (KATUSHKA) fusion (Filipczyk et al., 2013) and a Klf4 - fluorescent protein (VENUS) fusion from one endogenous allele each (unpublished) as well as $Nanog^{-/-}$ ESCs (TβC44Cre6; Chambers et al., 2007) were used for this study. Cells were routinely tested negatively for mycoplasma using PCR. NG4 cells were confirmed by genotyping, subsequent sequencing and antibiotics resistance. TβC44Cre6 cells were confirmed for absence of NANOG by immunostaining and presence of a cytoplasmic GFP signal by flow cytometry and imaging.

ESC Culture

ESCs were cultured in DMEM (Catalogue number: 31053-044, Gibco, CA, USA) supplemented with 2mM GlutaMAX (Catalogue number: 35050-038, Gibco, USA), 100U Penicillin/Streptomycin (Catalogue number: 15140-122, Gibco, CA, USA), 1% Non-essential amino acids (Catalogue number: 11140-035, Gibco, CA, USA), 1mM Sodium Pyruvate (Catalogue number: S8636, Sigma, MO, USA), 50uM β-mercaptoethanol (Catalogue number: M6250, Sigma-Aldrich, USA) and 10% FCS (Catalogue number: 2602P250915, PAN, Aidenbach, Germany) (SerumLIF) on 0.1% porcine gelatin (Sigma, Catalogue number: G1890-100G). Leukemia Inhibitory Factor (LIF) was isolated from Cos-7 cells overexpressing the pDR10, DIA/LIF production plasmid and titrated for addition into serum medium to ensure robust ESC self-renewal. Before experiments, cells were cultured for 1-2 passages on Recombinant Human E-Cadherin coated cell culture plates (StemAdhere, 07170, STEMCELL Technologies; diluted in 25x vol. PBS with Ca2+, 14040-091, Thermo Fisher; 1h incubation at 37°C) in SerumLIF. PD0325901 (10mM in DMSO, Catalogue number: S1036, Selleckchem, TX, USA) and CHIR99021 (15mM in DMSO, Catalogue number: 4423, R&D systems, MN, USA) were added at final concentrations of 1μM and 3μM, respectively, into SerumLIF medium for SerumLIF+2i or into serum-free N2B27 medium (NDiff® 227, Y40002, Takara Bio) that has been equally supplemented with LIF for N2B27+LIF+2i.

Proliferation curves of cell populations

ESCs were cultured at a density of ca. 9000 cells/cm^2, except TβC44Cre6 at 18000 cells/cm^2, on gelatin coated plates (or E-Cadherin where noted) at 37°C and 5% CO2 and split every 2 days as single cells. In serum-containing cultures, cells were split using trypsin and in serum-free N2B27+LIF+2i culture with accutase (A11105-01, Thermo Fisher) which was diluted with N2B27 after incubation and removed by a centrifugation step. Cell counts were determined using hemocytometers.

Generation of a constitutive Nanog expression plasmid

The *Nanog* coding sequence was cloned as a 2A construct into a piggybac vector that has been modified to express iRFPnuc (targeted to the nucleus) from the CAG promoter using the In-Fusion system (Catalogue number: 638911, Takara Bio). The resulting construct (CAG.iRFPnuc-P2A-Nanog) is supposed to express iRFPnuc and NANOG proteins in equal abundances.

Generation of stable transgenic mESC lines

R1 $Nanog^{KATUSHKA}Klf4^{VENUS}$ ESCs were transduced with lentiviral particles based on the pRRL backbone (MOI <10) to express a fluorescent nuclear label (iRFPnuc) from the CAG promoter. For $Nanog^{-/-}$ rescue cells, TβC44Cre6 ESCs were co-transfected with the constitutive Nanog expression plasmid (see above) and a PB-transposase plasmid (courtesy of Prof. Pentao Liu) using Lipofectamine 2000 to allow stable integration into the genome. After at least 3 passages, cells were sorted for iRFP positive cells using a FACS Aria III (BD Biosciences, CA, USA). The resulting polyclonal cultures were used for experiments to reduce clone-specific effects.

Time Lapse Imaging

Imaging was performed using a Zeiss Axio Observer Z1 microscope (Zeiss, Germany), equipped with definitive focus module, SpectraX light engine (Lumencor Inc., USA), 10x Fluar objective (Zeiss, Germany), motorized stage, transmitted VIS-LED (Zeiss, Germany) and an AxioCamHRm camera (1388x1040 pixel resolution). All hardware was controlled with a VBA module remote controlling Zeiss AxioVision 4.8.2 microscope control software. 3000 cells per channel were seeded either in SerumLIF, SerumLIF+2i or N2B27+LIF+2i in StemAdhere (or gelatin for Figure S2B) coated μ-Slides VI 0.4 (Catalogue number: 80606, Ibidi, Germany), overlaid with silicon oil (Catalogue number: 85419, Sigma-Aldrich, USA) and gassed with 5% CO_2 and 5% O_2 on the microscope stage with a self-developed incubation system and cultured at 37°C using automated temperature control. Images were

acquired either in the brightfield channel for 50ms; in the GFP channel for 500ms with max. 30% cyan light and the filter sets 489038-9901-000 (Zeiss, Germany) or F56-019 (AHF, Germany); in the KATUSHKA channel for 500ms with max. 50% green light and the filter sets F36-508 or F66-008 (AHF, Germany) or in the iRFP channel for 400ms with max. 53% red light and the filter set F36-524 (AHF, Germany). Cells were imaged continuously at 30 min intervals for min. 2 days. The first time-point was acquired ~1-3h after cell seeding. All experiments have been performed in triplicates.

Flow cytometry

Analysis of reporter ESCs by flow cytometry has been performed in parallel to time lapse experiments at cell seeding and after 2 days or in parallel to cell counting on a BD LSR Fortessa (BD Biosciences, CA, USA). Cells have been cultured as described under "Time Lapse Imaging" and "Proliferation curves of cell populations", respectively, but were incubated in a conventional tissue culture incubator with 20% O_2 and 5% CO_2. In Figure S1C, cells that were incubated as described in "Time-Lapse Imaging" are compared to cells that were cultured in parallel under the same conditions except in a tissue culture incubator. Flow cytometry data have been gated on non-debris (FSC-A/SSC-A) and singlets (FSC-A/FSC-W) using FlowJo (OR, USA). To obtain comparable scales, intensity values from flow cytometry (Figure 1A) have been divided by the median Nanog intensity of SerumLIF+2i (d2) conditions in the imaging data set (Figure 1D) for each cell line separately. For visualization of gelatin/E-Cadherin (S1B) and movie/incubator (Figure S1C) comparisons, flow cytometry data have been also scaled to Nanog:GFP time-lapse data.

Immunostaining

At the end of time-lapse experiments of NG4 cells (48h for the experiment shown in Figure S3B, 63h for one other of total three replicates), immunostaining was performed: Cells were fixed with 4% formaldehyde solution for 5-10 minutes at room-temperature (RT) (Catalogue number: HT5014, Sigma, Falcon, NJ, USA). Fixation was quenched with 100mM Glycin in PBS for 5-10 minutes, washed 3x with 0.2% Triton X-100 solution in PBS and blocked for ~1h with 5% donkey serum (Catalogue number: 017-000-121, Jackson ImmunoResearch, USA) in 0.1% Triton-X solution at RT. Primary antibodies goat anti-POU5F1 (previously known as OCT4, 1:300, Catalogue number: sc-8628, Santa Cruz, USA), rabbit anti-NANOG (1:200, ab80892, Abcam, Cambridge, UK), rat anti-SOX2 (1:700, Catalogue number: 14-9811-80, eBioscience, California, USA) and mouse anti-KLF4 (1:500, Catalogue number: ab75486, Abcam, Cambridge, UK) were incubated in blocking buffer either over-night at 4°C, for 2h at RT or for 1h at 37°C. Fluorescence minus one control stainings were done in parallel. Cells were washed 3x and secondary antibodies (donkey anti-goat IgG Alexa Fluor 555, A-21432; donkey anti-mouse IgG Alexa Fluor 647, A-31571; donkey anti-rat IgG Alexa Fluor 488, A-21208 (all 1:1200, Invitrogen, California, USA) and donkey anti-rabbit IgG Brilliant Violet 421 (1:400, 406410, Biolegend, CA, USA) were applied in blocking buffer for ~40 minutes at RT. Cells were washed again 3x. Images were acquired on the Zeiss microscope setup with a custom designed filter set (F49-547, F48-562, F49-577, AHF, Germany) or 43HE (489043-9901-000, Zeiss, Germany) to detect Alexa Fluor 555, a Cy5 filter set (F46-006, AHF, Germany) or Cy5.5 filter set (F36-524, AHF, Germany) for Alexa Fluor 647 channel, a 38HE filter (489038-9901-000, Zeiss, Germany) set or 44 filter set (000000-1114-459, Zeiss, Germany) for Alexa Fluor 488 channel and a DAPI filter set (F36-513, AHF, Germany) for Brilliant Violet 421 and DAPI channels. After re-staining the blue (Nanog) channel with DAPI, another round of imaging was performed. All experiments were performed in triplicate. Display settings (black/white points) of fluorescent images have been set individually for each channel, but kept constant for all conditions and the staining control.

For quantitative immunostaining of Nanog levels in NG4, *Nanog*$^{-/-}$ and *Nanog*$^{-/-}$ rescue cell lines (Figure 2D), cells were seeded as described for time-lapse imaging in SerumLIF and incubated at 37°C with 5% CO_2. The Nanog antibody, protocol and filter cubes were used as described here, except donkey anti-rabbit IgG Alexa Fluor 555 (A-31572, Life Technologies) as 2° antibody and Hoechst33342 as DNA staining were used. Images were acquired on a Nikon Ti-E microscope equipped with an Orca Flash 4.0 (Hamamatsu) camera. Experiments have been performed in duplicates.

Single-Cell Tracking

Manual single-cell tracking was performed using the custom made image analysis software tTt (Eilken et al., 2009; Filipczyk et al., 2015; Hilsenbeck et al., 2016; Rieger et al., 2009). The cells at the movie start were intentionally selected in a way to enrich for Nanog low expressing cells. To increase the number of independent data points in the analysis, only one randomly selected sister cell was tracked after each cell division for 48h until the end of the experiment or until the selected cell died. Thus, this tracking scheme produced one branch per tree. While tracking it was ensured that the randomly selected progeny was derived from the correct colony. Only living cells which divided, were well attached to the surface or were observed during the process of dying (e.g. got fragmented or detached) were chosen as starting cells. For the example trees (Figure 3A) and example movies, whole trees were analyzed and all individual cell lineages checked for correctness.

Cell quantification

Cells and their fluorescence intensities were quantified as described previously (Filipczyk et al., 2015). For Nanog^{KATUSHKA}, cell nuclei were segmented in the iRFPnuc channel using qTfy and for the cytoplasmic Nanog:GFP reporter whole cell contours were segmented. For quantification of immunostaining, cells were segmented in the Hoechst channel. To ensure most accurate quantifications at day 0 and day 2, the segmentations of all tracked cells were manually checked and corrected for the five first and five last time-points or discarded if corrections were not possible. For the full-length analyses of Nanog low branches in SerumLIF+2i, segmentations of all time-points were manually curated (Figures 4B-D and S4). Mean Nanog levels of the five first and five last time-points were

calculated for each tracked cell at day 0 and day 2, respectively. Cell area in μm^2 was calculated considering camera properties, TV-adapter and objective magnification.

Data normalization of replicate time-lapse experiments

To pool data for Nanog quantifications, Nanog:GFP or $Nanog^{KATUSHKA}$ intensities were normalized between replicates using a quantile normalization method (Bolstad, 2014) which makes the Nanog distributions of replicates identical and allows to keep absolute values in a similar range as before. For each replicate, all automatically generated single-cell Nanog quantifications of SerumLIF and SerumLIF+2i conditions mixed were used for normalization. To ensure that quantile normalization did not introduce any relevant changes in the relationship between single-cells within an experiment, we compared original and normalized data for all manually corrected quantifications (time points at d0 and d2) and found perfect correlations for each of all possible combinations of d0/d2 time-points, experiments, conditions and cell lines (Figure S1B, all individual $R^2>0.99$), showing that Nanog quantifications got transformed in a virtually linear way. For 2 additional experiments that include N2B27+LIF+2i (Figures S2C, S3A) and 1 experiment that includes gelatin coating (Figure S2B) as conditions, background corrected Nanog:GFP intensity values were multiplied with a scaling factor to have scales and gates comparable to the other data. The scaling factors were calculated by dividing the median of all quantile normalized data from the 3 previous experiments by the median of all SerumLIF and SerumLIF+2i data on E-Cadherin coating from each additional experiment separately. One further experiment that contained only data on gelatin coating, scaling was applied in the same way, but using gelatin coating data from the other experiment as reference.

Data visualization

As a standard in this study the plot axes showing Nanog intensities or cell area were transformed with an inverse hyperbolic function (asinh, arcsinh). This allowed us to plot negative values which can occur after image normalization and at the same time to visualize values spanning several orders of magnitude. Only in Figures 3A, 4A, 4B, S1E and S4A linear scales have been used. In Figures 2B, S1D and S2D, the Nanog intensity distribution was binned into bins equally spaced in the asinh scale (precise to 1 digit). In Figures 1A, 1D, 2D, 2F, S1B, S1C, S1F and S2A, the smooth density estimate of each distribution was scaled to a maximum value of 1. To emphasize accumulations of data points in Figure 2C, a two-dimensional density estimate was used which defined the size of (non-opaque) points on a grid. RStudio, ggplot2 (Wickham, 2009) and Adobe Photoshop have been used to analyze time-lapse data and generate figures.

Statistical analysis

All statistical tests have been performed using RStudio. To calculate Spearman correlation values for the Nanog levels at d0 and cell generation of a branch at d2, Nanog intensity values have been asinh transformed. The Cochran-Mantel-Haenszel Chi-Squared Test has been used for Figures 2A, S2B and S2C which allows testing of data from classical 2x2 tables that have been repeated several times. Pooled Nanog distributions from three replicate experiments (each cell line separately) are used in Figures 1D, 2B, 2C, 2F, 3B, 3C, 3D, 4A, 4C, 4D, S2A, S2D, S4B, S4C. Pooled Nanog distributions from two additional experiments were used in Figures S1F and S3A. Means of three replicate experiments and individual data points are shown in Figure 2A where the shapes of data points refer to the replicates. Data from two additional experiments are shown the same way in Figures S2B and S2C. Also in Figures 2E, S1A, S1B (bottom) and S2E means and individual data points are shown. Means and standard errors of the mean (SEM) of three replicates are shown in Figure 4E. Representative plots are shown in Figures 2D, S1B (top) and S1C.

Calculation of Nanog fold changes

Related to Figures 3B and S3A. Nanog intensity levels below a threshold (1.5 [a.u.]) were set to 1.5 [a.u.] to exclude fold changes within the noise level as determined by visual inspection of cell lineage quantifications. Of note, this threshold is much lower than the Nanog low gate (4 [a.u.] or 4.5 [a.u.] for Nanog:GFP and $Nanog^{KATUSHKA}$, respectively.

Analysis of subpopulation time-course data

Related to Figure 4A. For this analysis, automated quantifications have been used for the non-manually corrected time-points and curves were smoothed with RStudio using smoothing splines (smoothing parameter = 0.75).

Analysis of Nanog dynamics in single-cell branches

Related to Figures 4B-D and S4. Manually curated Nanog quantifications over time of Nanog low cells in SerumLIF+2i were smoothed for each cell separately with RStudio using smoothing splines (smoothing parameter = 0.7). Original data were used for cells with less than 4 data points. Then, all Nanog means of 5 subsequent time-points (=2.5h) were calculated throughout the length of a branch, also spanning cell divisions. The following parameters could then be extracted: Maximum Nanog and its fold change compared to day 0 (mean of the first 5 time-points); the time-point of maximum Nanog (the last time-point of the 2.5h span was used) and the first time-point where the Nanog fold-change compared to day 0 was greater than 2 (the first time-point of the 2.5h span was used).

Supplemental References

Bolstad, B.M., 2014. preprocessCore: A collection of pre-processing functions. R package version 1.28.0.

Wickham, H. (2009). ggplot2: elegant graphics for data analysis (Springer New York).