Appendix S1

Contents

Section A

Two-sample Kolomogorov-Smirnov test

A two-sample Kolmogorov-Smirnov test was used to compare the daughter and granddaughter distributions of median Nanog intensity, with the null hypothesis that the daughter and granddaughters are sampled from the same underlying distribution.

The test is based on the maximal absolute difference in the two cumulative distribution functions:

$$\max\left(|F1(x) - F2(x)|\right)$$

We used a standard 5% significance level to reject null hypotheses. However, as we compared three experiments and are essentially performing three comparisons, we adjusted the p-value using a correction for multiple comparisons to prevent an increase in false positives. The Bonferroni correction method divides the significance level, α, by the number of tests, while the Šidák correction uses the family-wide error rate, $\overline{\alpha} = 1 - (1 - \alpha)^{1/n}$. The table below summarizes the outcome of the K-S tests using the two correction methods, showing that no significant differences are observed between the Nanog intensity distributions of daughters and granddaughters.

Experiment	p	h (Bonferroni) α=0.0167	h (Šidák) α=0.017
1	0.2916	0	0
2	0.1082	0	0
3	0.0284	0	0

Section B

Fitting cell cycle data

Two fits were tested on plots for the fraction of undivided sister cells remaining in Figures 1D and E. The first fit is a simple exponential of the form:

$$B = e^{-\alpha \Delta t}, \qquad (1)$$

where α is the fitting parameter with units of (hours)$^{-1}$ and Δt is the time difference in hours. The second fit, based upon the Eyring-Stover (ES) survivial theory (Murphy et al., 1984) has the form:

$$B = 2(1 - e^{\alpha \Delta t})^{-2}[1 + (\alpha \Delta t - 1)e^{\alpha \Delta t}] \qquad (2)$$

For our LIF data, the exponential fit is rejected in the majority of cases (see the table below). The more reproducible fit of equation 2, implies that more than one control step may be involved in the cell cycle transitions. The goodness of fit was tested using a chi-squared test as discussed in the main text. For the non-related cells in LIF, neither equation fits.

Summary of statistics for cell cycle fits

Movie	sisters		unrelated	
	Exp fit (p)	ES FIT (p)	Exp fit (p)	ES FIT (p)
1	0.46	0.64	1.4x10-9	6.1x10-5
2	0.05	0.68	1.1x10-5	2.4x10-25
3	0.01	0.31	7.5x10-24	6.9x10-10

Movie	LIF		LIF + 2i	
	Exp fit (p)	ES FIT (p)	Exp fit (p)	ES FIT (p)
4	6x10-5	0.01	0.01	0.08
5	0.22	0.62	0.17	0.11
6	0.71	0.85	0.005	0.08

Summary of statistics for comparing cycle times in LIF with LIF + 2i

Movie	KS test		t-test	
	H	p	H	p
4	1	1.9×10^{-17}	1	1.7×10^{-11}
5	1	1.6×10^{-3}	1	3.3×10^{-5}
6	1	6.9×10^{-24}	1	3.1×10^{-25}

Section C

MSD analysis

By analogy with the random walk of diffusing particles, we investigated the fluctuations in Nanog intensity using an MSD (mean squared displacement) analysis. In terms of Nanog intensity, the mean squared deviation is defined as:

$$MSD(\tau) =< (\mathrm{I}(\mathrm{t}+\tau) - \mathrm{I}(\mathrm{t}))^2 >_t \qquad (3)$$

Where I is the Nanog intensity, t is the time and τ is the lag time. The form of the MSD provides information of the magnitude and timescale of fluctuations – a linear MSD describes purely random walk behaviour, while a plateau is suggestive of the value being constrained or corralled.

The data in Figure 3D show the difference between sister intensities remained small for a time after division, suggested that sister intensities may fluctuate in a correlated manner. To investigate this further, we decomposed the two sister intensities into an alternative orthogonal basis set, rotated by 45 degrees from the standard representation:

$$I_s = \frac{1}{\sqrt{2}}(I_1 + I_2)$$
$$I_d = \frac{1}{\sqrt{2}}(I_1 - I_2) \qquad (4)$$

In this notation I_s can be thought of as the summed intensity, measuring fluctuations which are common to both sisters while I_d, the difference intensity, quantifies fluctuations where one sister moves in the opposite direction to the other. Using the theory of the combination of random variables, if fluctuations in I_1 and I_2 are completely independent then MSD(I_s) and MSD(I_d) are expected to be equal, while if the behaviour is correlated then MSD(I_s)>MSD(I_d). The contrasting case of anti-correlated fluctuations would yield MSD(I_d)>MSD(I_s).

MSD for motility

By analogy with the random walk of diffusing particles, we investigated the cell movement using an MSD (mean squared displacement) analysis. The mean squared deviation is defined as:

$$MSD(\tau) =< (\mathrm{I}(\mathrm{t}+\tau) - \mathrm{I}(\mathrm{t}))^2 >_t$$

Where I is the cell co-ordinates, t is the time and τ is the lag time. The form of the MSD provides information of the magnitude and timescale of fluctuations – a linear MSD describes purely random walk behaviour, while a plateau is suggestive of the value being constrained or corralled. Here we find that an active transport fit best describes the movement of cells in both LIF or LIF + 2I conditions.

$$< r^2 >= 4D\Delta t + v^2\Delta t^2$$

Where D is the Diffusion constant in μm²/min and v is the drift term (velocity of drift) in μm/min:

Movie	LIF **D** (μm²/min)	LIF **v** (μm/min)	LIF + 2i **D** (μm²/min)	LIF + 2i **v** (μm/min)
1	1.19 +- 0.11	0.13 +- 0.004	1.23 +-0.04	0.08 +- 0.005
2	0.76 +- 0.03	0.06 +- 0.002	0.69 +- 0.03	0.04 +- 0.003
3	1.43 +- 0.11	0.07 +- 0.007	1.02 +- 0.04	0.09 +- 0.003

Section D

Bootstrapping field-of-view correlations

Cell cycle lengths and Nanog pairs were randomised between field-of-views to test the possibility that the field-of-view correlations between Nanog and cell cycle length were simply enhanced from the individual cell cycle and Nanog correlations. The mean correlation once randomised was 0.11 + 0.19 (Figure S3B), which is within the range of the individual correlation between Nanog and cell cycle lengths of 0.13, for daughters. This can be shown mathematically:

Let F = field-of-view average of X (cell cycle lengths) and G = field-of-view average of Y (Nanog) The mean of F and X are equal ($\mu_F = \mu_x$) as are the mean of G and Y ($\mu_G = \mu_Y$), and the standard deviations of F and G are:

$$\sigma_F = \frac{\sigma_X}{\sqrt{N}} \qquad \sigma_G = \frac{\sigma_Y}{\sqrt{N}}$$

The covariance of F,G is:

$$COV(F,G) = E((F - \bar{F})(G - \bar{G})) \qquad (5)$$

$$COV(F,G) = E\left[\left(\frac{\sum_{i=1}^{N} X_i}{N} - \frac{\mu_x}{N}\right)\left(\frac{\sum_{i=1}^{N} Y_i}{N} - \frac{\mu_Y}{N}\right)\right]$$

$$COV(F,G) = \frac{[\sum(X_i - \mu_x)(Y_i - \mu_Y)]}{N^2}$$

$$COV(F,G) = \frac{COV(X,Y)}{N}$$

$$r_{F,G} = \frac{COV(F,G)}{\sigma_F \sigma_G}$$

$$r_{F,G} = \frac{COV(X,Y)/N}{\frac{\sigma_x}{\sqrt{N}}\frac{\sigma_y}{\sqrt{N}}}$$

$$r_{F,G} = \frac{COV(X,Y)}{\sigma_X \sigma_Y}$$

$$r_{FG} = r_{XY}$$

Therefore, once randomized, the correlation between field average Nanog and cell cycle lengths should be equal to the individual correlation between Nanog and cell cycle length, as we have observed.

Section E

Modelling inheritance of Nanog expression

The simplest model generates two daughters from one mother using the correlation values known experimentally from mother to daughter for Nanog (r = 0.77) and cell cycle length (r = 0.6) separately.

In the derivations which follow, it is assumed that any variables have been normalized such that their distributions have zero mean and unity standard deviation. This simplifies the calculation of the product moment correlation coefficient. If required, the final variables can be converted back to their unnormalized values, however parameters such as the correlation coefficient are invariant to shifting and scaling.

Data sets for daughters can be produced by a linear combination of the mother data and a Gaussian random variable; the relative weight of the mother contribution determines the strength of the correlation. If:

$$d_i = \alpha M + \beta Z ,$$

Where Z = N(0,1), the constraint $\alpha^2 + \beta^2 = 1$ ensures that the resulting distribution has a standard deviation of unity. The correlation coefficient between mother and daughter is then given by:

$$r_{md} = \mathrm{cov}\left(M, \alpha M + \sqrt{1-\alpha^2}\,Z\right)$$
$$r_{md} = \alpha\,\mathrm{cov}(M, M) + \sqrt{1-\alpha^2}\,\mathrm{cov}(M, Z)$$
$$r_{md} = \alpha$$

Therefore daughter data is generated from the mother values as follows:

$$d1 = r_{cmd}M + \sqrt{1 - r_{cmd}^2}Z_{d1} \qquad (6)$$
$$d2 = r_{cmd}M + \sqrt{1 - r_{cmd}^2}Z_{d2}$$

Where M is the mother cell cycle lengths, r_{cmd} is the correlation between mother to daughter cell cycle lengths experimentally calculated and Z_{d1} and Z_{d2} are random variables generated. The correlation between sisters can be calculated from:

$$r_{cdd} = \frac{cov(d1 \cdot d2)}{\sigma_{d1}\sigma_{d2}} \qquad (7)$$

The only non-zero term in the above equation is cov(M,M)=1, leaving

$$r_{cdd} = r_{cmd}^2$$

Performing 100 repetitions of the model and using 16 experimental mother cells (a typical starting number of mother cells) produces an average correlation between the new daughter Nanog sister pairs of 0.59 $\pm$0.05 and an average correlation between cell cycle sister pairs of 0.36 $\pm$ 0.09. However, experimentally these correlations are 0.91 $\pm$ 0.01 and 0.69 $\pm$ 0.007. One possibility is that stability of Nanog reporter levels and cell cycle lengths between mothers to daughters may be enhanced by environmental regulation to generate unexpectedly high correlations between daughters. Alternatively, this may arise, if the state of the mother cell changes between the point at which the median density applies and the time of division, and this changed state is then transmitted to the daughter cells.

Section F

Constraining the model: sister pairs

In order to replicate the field-of-view correlations experimentally observed, the model must also contain the correlations between sister pairs for both Nanog and cell cycle length.

Therefore, as described above, we add an intermediate state after the mother state, from which daughter data is independently generated:

If r_{MI} is the correlation between mother and intermediate state, and r_{Id} is the correlation between intermediate state and daughter 1 or 2, following the same process of covariance calculation as in section D yields:

$$r_{dd} = r_{Id}^2$$

$$r_{Md} = r_{MI} r_{Id}$$

These results can be rearranged to give the values of r_{MI} and r_{Id} required for the desired mother-daughter and daughter-daughter correlations:

$$r_{MI} = \frac{r_{Md}}{\sqrt{r_{dd}}} \qquad (8)$$

$$r_{Id} = \sqrt{r_{dd}}$$

Using this framework, data for the current generation can be produced from the previous generation values, matching the values of mother-daughter and daughter-daughter correlations to those observed experimentally, to test if the observed Nanog-cell cycle correlation could arise from the relatedness of cells in the field-of-view. Starting with one mother cell (this is the most extreme case, all cells would arise from one original cell), 10 generations are produced for each of the seven fields-of-view (replicating experiment 3), with cell numbers doubling each time (1, 2, 4, 8, 16 etc); this whole process is repeated 1000 times. An average Nanog and Cell cycle length is calculated for every field. From this an average correlation value is produced for each generation. The correlation value of average Nanog reporter and cell cycle of fields-of-view decreased rapidly to 0. So even in the extreme case of starting with one mother cell the strong correlation between mother to daughter for Nanog and cell cycle length is not solely sufficient to produce such a strong correlation (0.60) at the field-of-view level.

Section G

Constraining the model: Cell Cycle and Nanog

Experimentally, there is a low correlation between Nanog levels and cell cycle length which is not included in model B.

As in section E, where sisters are more correlated than independent generation from the mother values would give, an intermediate state is created from which the two daughters are generated. However, since we wish to maintain a correlation between cell cycle length and Nanog intensity, these variables are generated simultaneously from a correlated bivariate distribution.

$$I_c = r_{cmI} m_c + \sqrt{1 - r_{cmI}^2}\, Z_c$$

$$I_N = r_{NmI} m_N + \sqrt{1 - r_{NmI}^2}\, Z_N$$

$$d_c = r_{cId} I_c + \sqrt{1 - r_{cId}^2}\, W_c$$

$$d_N = r_{NId} I_N + \sqrt{1 - r_{NId}^2}\, W_N$$

Where Z_c and Z_N, and W_c and W_N are correlated random variables with correlation coefficient r_Z and r_w respectively. The values of r_Z and r_w (that is, the value of the correlation for the random mixing variables) are calculated so as to maintain the value of the correlation coefficient between cell cycle length and Nanog from one generation to the next:

$$r_{cN} = r\left(I_c, I_N\right)$$

$$r_{cN} = r\left(r_{cmI} m_c + \sqrt{1 - r_{cmI}^2}\, Z_c, r_{NmI} m_N + \sqrt{1 - r_{NmI}^2}\, Z_N\right)$$

$$r_{cN} = r_{cmI} r_{NmI} r\left(m_c, m_N\right) + \sqrt{1 - r_{cmI}^2}\,\sqrt{1 - r_{NmI}^2}\, r\left(Z_c, Z_N\right)$$

$$r_{cN} = r_{cmI} r_{NmI} r_{cN} + \sqrt{1 - r_{cmI}^2}\,\sqrt{1 - r_{NmI}^2}\, r_Z$$

$$r_Z = \frac{r_{cN} - r_{cmI} r_{NmI} r_{cN}}{\sqrt{1 - r_{cmI}^2}\,\sqrt{1 - r_{NmI}^2}} \qquad (9)$$

When adding single cell Nanog reporter-cell cycle correlations in to the model (Figure S3C) we observe that the correlation between fields will plateau at the individual cell cycle length and Nanog correlation for granddaughters of 0.14 (Figure S3D). Again this implies the field-of-view effect is not caused by a small number of related cells at the beginning of image acquisition.

Section H

Alternative Background Correction Method

Background was estimated for each pixel by accumulating and averaging pixel intensities for frames when no cell was present. The decision whether a cell is present in the pixel was made

by smoothing with a Gaussian kernel and applying a threshold. In densely populated regions, there may be insufficient samples of a pixel to accurately calculate the background intensity; pixels with fewer than 40 samples throughout a movie were filled in using an iterative diffusion algorithm. This yields a map of the estimated background intensity at each xyz location of movies, allowing the position-dependent background intensity to be subtracted from cell intensity measurements.

Supplementary Figures

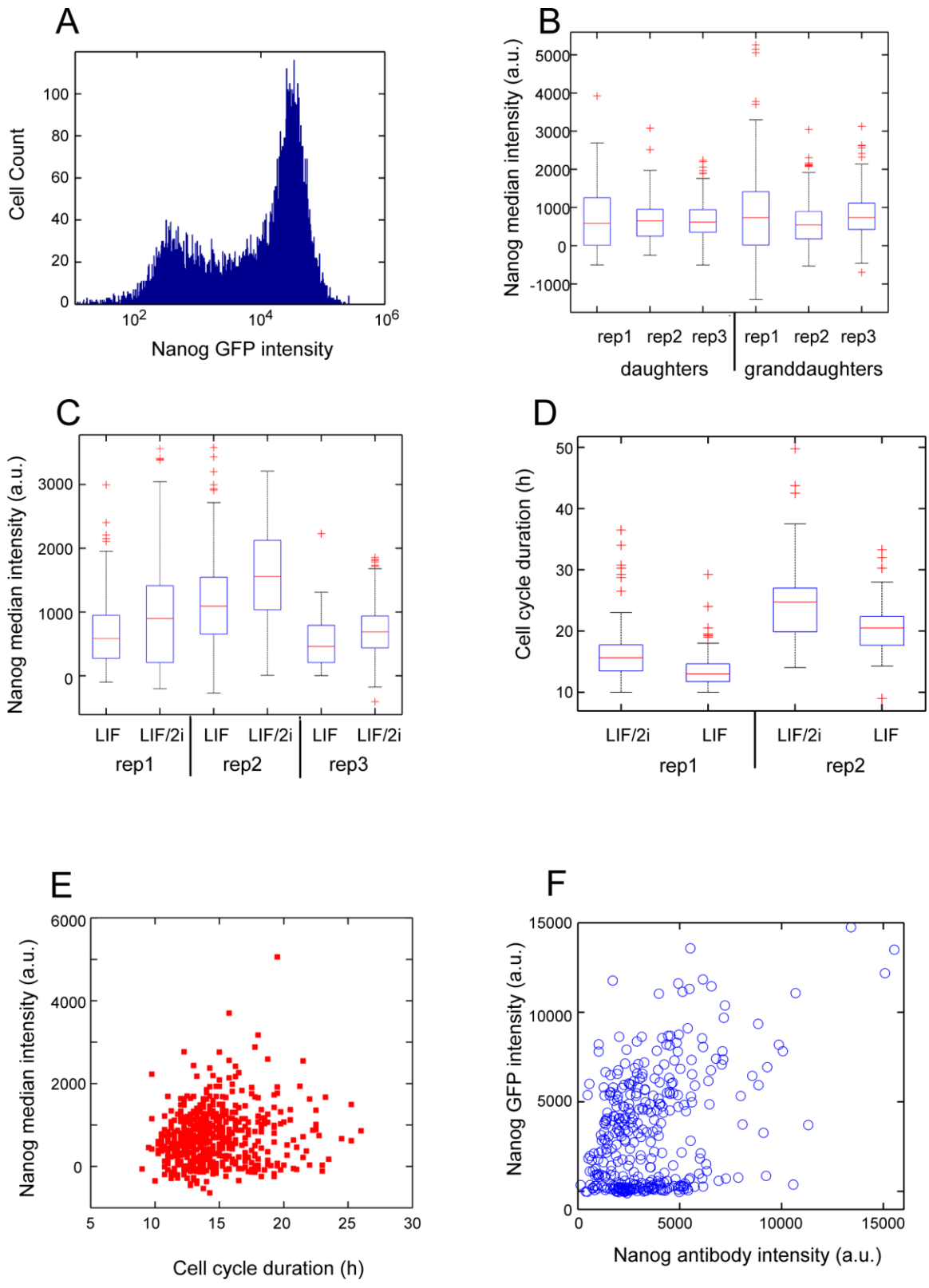

Figure S1

Heterogeneous Nanog expression and cell cycle behaviour

A) Flow cytometry data showing bimodal distribution of GFP expression driven by the Nanog promoter under LIF culture conditions. B) Distributions of Nanog expression from 3 experiments for daughters and granddaughters. The median value was taken from each cell from its entire cycle. A two-sample Kolmogorov-Smirnov test showed that the distribution of Nanog expression levels was unchanged between daughters and granddaughters. C) Distributions of Nanog expression levels from all 3 experiments in LIF or in LIF after the addition of 2i. D) Cell cycle durations of cells after 5 passages in 2i, compared to similar culture age controls. 2 independent experiments (2i n=167 cells, LIF n=119). E) Median GFP expression from the Nanog gene plotted against cell cycle duration for all complete cell cycles from 3 independent experiments for granddaughter cells (n=632). F) Comparing GFP expression and Nanog protein expression in TNGA cells by immunofluorescence (349 cells total). Cells were fixed in 4% paraformaldehyde, and stained with a rabbit polyclonal to the Nanog protein (Abcam ab80892; 1 in 100) and Cy3 conjugated anti-rabbit secondary. Images were captured on a Perkin Elmer Vox spinning disc microscope. Excluding the low Nanog population gave a correlation between GFP and Nanog antibody staining of 0.56 (226 cells).

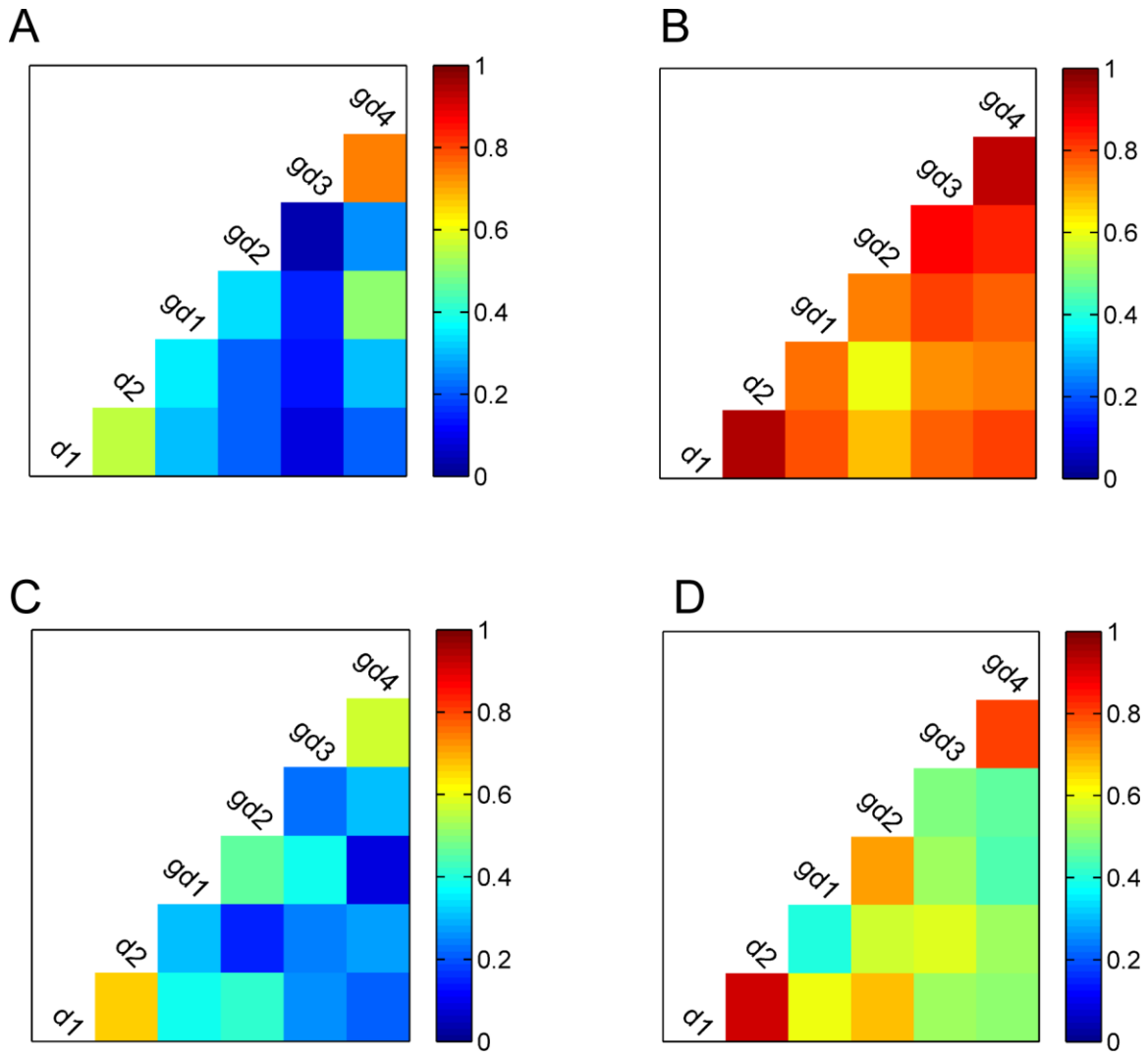

Figure S2

Intergenerational correlations in cell cycle and Nanog dynamics

Intergenerational correlation heatmaps from independent experimental repeats for cell cycle durations (A and C) and Nanog (B and D). These panels are the replicates of Figure 3B and C.

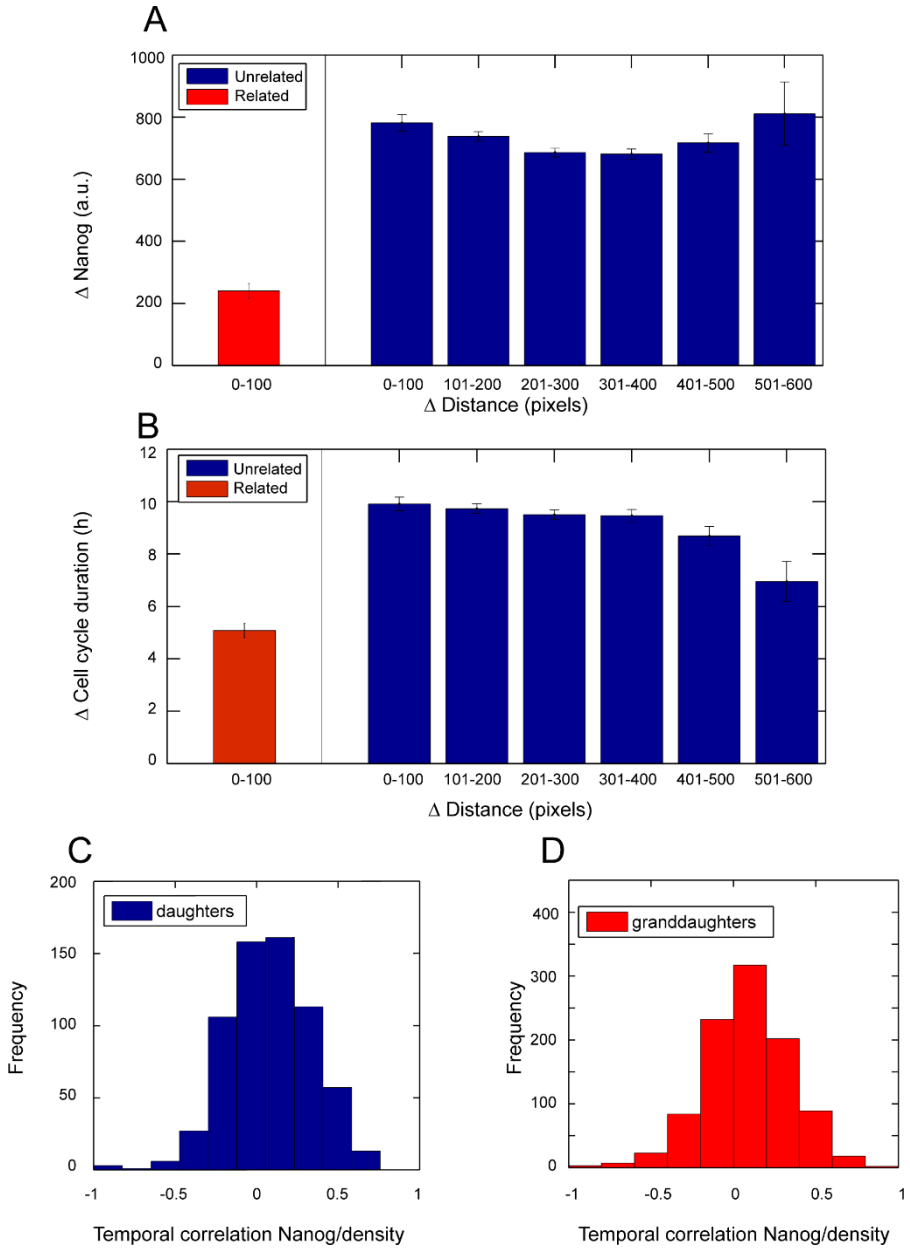

Figure S3

Difference in A) Nanog and B) Cell cycle lengths as a function of distance between cells for related (red) and unrelated (blue) cells (combining three independent experimental repeats). This data is related to the data in Figures 4A-D which show data from one repeat only. C) Histograms of measured correlation values, from individual cells, between local density and Nanog reporter intensity, at each time point of movies, for daughters and D) granddaughters.

Table S1. Summary of correlation and related *P*-values for Fig. 2A-C.

LIF	**Correlation**	***P*-value**
Nanog vs cycle	0.20	8×10^{-7}
Rate Nanog vs cycle	0.14	1×10^{-3}
Nanog (first 5 h) vs cycle	0.20	2×10^{-6}

LIF + 2i	**Correlation**	***P*-value**
Nanog vs cycle	0.19	1×10^{-6}
Rate Nanog vs cycle	0.13	9×10^{-4}
Nanog (first 5 h) vs cycle	0.12	3×10^{-3}

Data are from three pairwise experiments comparing LIF with 2i/LIF. Correlation values from multi-generation lineages are described in the main text.

Table S2. Correlation values (and associated p values) between density, cycle duration, Nanog and diffusion coefficient (motility) of daughters in LIF or LIF/2i relating to Fig. 5.

LIF	**Cell Cycle**	**Nanog**	**Diffusion Coefficient**
Density	0.25, 1×10^{-9}	0.32, 5×10^{-17}	-0.11, 4×10^{-3}
Cell cycle		0.20, 8×10^{-7}	-0.12, 3×10^{-6}
Nanog			-0.18, 2×10^{-6}

LIF + 2i	**Cell Cycle**	**Nanog**	**Diffusion Coefficient**
Density	0.05, 0.19	0.14, 0.0002	-0.07, 0.05
Cell cycle		0.19, 1×10^{-6}	-0.22, 2×10^{-8}
Nanog			-0.26, 1×10^{-12}

Multi-generation data are described in the main text.