Stochastic promoter activation affects Nanog expression variability in mouse embryonic stem cells

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Mouse embryonic stem cells (mESCs) are self-renewing and capable of differentiating into any of the three germ layers. An interesting feature of mESCs is the presence of cell-to-cell heterogeneity in gene expression that may be responsible for cell fate decisions. Nanog, a key transcription factor for pluripotency, displays heterogeneous expression in mESCs, via mechanisms that are not fully understood. To understand this variability, we quantitatively analyzed Nanog transcription and found that Nanog was both infrequently transcribed, and transcribed in a pulsatile and stochastic manner. It is possible that such stochastic transcriptional activation could contribute to the heterogeneity observed in Nanog expression as “intrinsic noise.” To discriminate the effects of both intrinsic noise from other (extrinsic) noise on the expression variability of Nanog mRNA, we performed allele-specific single-molecule RNA fluorescent in situ hybridization in a reporter cell line and found that intrinsic noise contributed to approximately 45% of the total variability in Nanog expression. Furthermore, we found that Nanog mRNA and protein levels were well correlated in individual cells. These results suggest that stochastic promoter activation significantly affects the Nanog expression variability in mESCs.

Results

Establishment of a Nanog-MS2 mESC line. To quantitatively analyze Nanog transcription in mESCs, we applied the MS2 system14,15. The transcribed MS2 sequence derived from MS2 bacteriophage forms a stem-loop structure, which is known to be bound by the MS2 coat protein (MCP) as a dimer (Figure 1a). Therefore, the integration of the 24 tandem MS2 sites into a specific gene of interest and expression of MCP fused with fluorescent protein enables the visualization of mRNA transcription as bright spots in the nucleus16–18. We applied transcription activator-like effector nuclease (TALEN)-mediated targeted integration of the MS2 repeat sequence into the

ESC stem cells are clonal cell lines derived from the pre-implantation epiblast. They are capable of self-renewing in media containing leukemia inhibitory factor (LIF), can differentiate into three germ layers, and display heterogeneity in gene expression. Uncovering the nature of this heterogeneity at the molecular level is important to the understanding of how stem cells modulate their stemness/differentiation balance. Nanog, a key transcription factor for pluripotency, exhibits a broad range of expression levels, and its heterogeneity seems to be related to the stemness/differentiation balance1–4.

The autocrine fibroblast growth factor (FGF)/extracellular signal-regulated kinase (Erk) signaling that induces lineage commitment and inhibits Nanog expression is thought to be a source of Nanog heterogeneity5–7, along with the positive and negative feedback loops in the gene regulatory networks responsible for pluripotency. Recently, it has been reported that Nanog is prone to be monoallelically transcribed (i.e., transcribed from only one copy of Nanog) in mESCs cultured in standard mESC medium containing serum and LIF (serum condition), even though mice have two copies of Nanog9–11. If this effect is a result of an intrinsically stochastic effect on promoter activation, it may also contribute to Nanog heterogeneity12,13.

In this study, to gain insight into the contribution of Nanog transcriptional activity to Nanog heterogeneity, we quantitatively analyzed the transcription dynamics of endogenous Nanog and distribution of Nanog mRNA in a mESC population at single-cell resolution and observed infrequent and stochastic switching on and off of Nanog promoter states. Furthermore, we found that expression noise stemming from such promoter dynamics significantly affected heterogeneity in Nanog expression.

SCIENTIFIC REPORTS | 4 : 7125 | DOI: 10.1038/srep07125
Nanog loci (Figure 1b, c), and established a biallelically targeted mESC line (Figure 1d). Then, selection cassettes were removed by transient expression of Cre recombinase (Figure 1c, d). Southern blot analysis showed the expected band patterns and no random integrations (Figure 1d). We refer to the obtained cell line as Nanog-MS2 (NM) cells. The cell line expressed the undifferentiated embryonic stem-cell markers SSEA1 and Oct4 (also known as Pou5f1) (Figure 1e).

Next, to check the cell-to-cell variability of Nanog mRNA copy numbers in the NM cell line, we performed single-molecule fluorescent in situ hybridization (smFISH) using Nanog exonic probes (Figure 2a–c). In the serum conditions used (standard mESC culture medium containing LIF and serum), the mean count of Nanog mRNA copies in NM cells was slightly, but significantly, lower than that of the parental mESC line (133 for the parental mESC line and 77 for the NM cell line) (Figure 2c). However, the degrees of variability between the parental and derived lines were comparable (coefficient of variation [CV] for the parental and NM cell lines were 0.85 and 0.89, respectively). One of the possible causes of the difference in expression is the change in mRNA stability due to insertion of the MS2 repeats. To explore the possibility, we examined the half-lives of Nanog mRNA in NM and the parental mESCs (Supplementary Figure S1), and found that insertion of MS2 repeats slightly destabilizes Nanog mRNA. Therefore, the mean number of Nanog mRNA in NM cells appears lower than that in the parental mESCs.

It has been reported that the cytokine FGF4 is secreted by undifferentiated cells, acts in an autocrine manner, and represses Nanog expression through Erk signaling, suggesting that FGF/Erk signaling is a contributor to the transcription factor heterogeneity. When cell lines were cultured in medium containing FGF/Erk and GSK3β inhibitors (2i conditions), the means of Nanog mRNA copies were increased, as expected (Figure 2c). Furthermore, the variability of these cells was lower than cells grown in the serum condition (CV for parental and NM cell lines in 2i conditions were 0.43 and 0.42, respectively). These findings suggest that the NM cell line displays similar heterogeneity to that of the parent cell line.

Next, to visualize transcriptional activity, we established a cell line that constitutively expresses nuclear localization signal (NLS)-MCP-GFP fusion protein (mNeonGreen) (NM-G cell line). In this cell line, either none, one or two bright fluorescent spots were observed (Figure 2d). To confirm whether these spots corresponded with the Nanog transcription sites, we performed smFISH using a Nanog intronic probe set (Figure 2a, e). Although a majority of the smFISH and NLS-MCP-GFP spot signals coincided in cells cultured in 2i conditions (88%, n = 101), only one type of signal was detected.
in some of the spots. This limitation could be attributed to the distance between the sites of Nanog intrinsic probes and the MS2 repeat (~4.5 kb, Fig. 2a). The RNA Pol II elongation rate in mESCs was reported to be 0.5–4 kb/min\(^2\); therefore, all signals of smFISH and MS2-MCP may not be simultaneously detected at the same position. These findings suggest that the MS2-MCP spot signals indicate the transcription of MS2-integrated Nanog alleles and that the NM-G cell line is useful for quantifying the Nanog transcription dynamics.

**Quantification of Nanog transcription dynamics.** To quantify Nanog transcription dynamics, we performed live imaging of NM-G cells cultured in serum or 2i conditions at 2-min intervals for 4 h (Figure 3a–c). In both conditions, Nanog was transcribed in a pulsatile manner, as reported in other model systems\(^{26,27}\). The transcription frequency over the 4-h time period was higher in 2i conditions than in serum conditions, consistent with previous reports (Figure 3c)\(^{10,11}\).

Consistent with the smFISH data (Figure 2c), transcription frequencies were apparently variable among cells even in 2i conditions (Figure 3a–c). 2i conditions inhibit autocrine FGF/Erk signaling, one potential source of Nanog heterogeneity. This finding suggests that factors other than FGF/Erk signaling may significantly affect the expression variability of Nanog. Furthermore, transcription frequency during the 4-h imaging period seemed to show multimodal distributions in both conditions (Supplementary Figure S2). This suggests the existence of multiple states of Nanog expression that switch at intervals longer than 4 hours, as recently reported\(^{28}\). On the other hand, the distributions of transcriptional signal intensities in the two conditions were comparable (Figure 3d), suggesting that Nanog transcription was regulated not by modulation of the number of transcripts per transcriptional pulse, but rather by transcription frequency\(^{27}\). This kind of transcription dynamics might be explained by the random telegraph model\(^{29–31}\) (Figure 3e). In this model, promoter states stochastically switch “ON”, permitting transcription, and “OFF”, deactivating transcription (Figure 3f). The ON and OFF times are predicted to be exponentially distributed. To determine those parameters from imaging data, transcriptional dynamics of each allele should be separately quantified. Although non-transcribing alleles were not actually traceable, their position is roughly estimated by the somewhat uneven nuclear background signals of NLS-MCP-mNeonGreen and changes in nuclear shape (Supplementary Video S1 and S2). By this qualitative estimation, distributions of ON and OFF time durations were obtained (Figure 3g). The ON and OFF time distributions were well fitted by exponential distributions, consistent with the Poisson stochastic processes expected in the random telegraph model\(^{29–31}\). Furthermore, the mean duration of ON time in 2i conditions was longer than that in serum conditions. Conversely, the mean duration of OFF time in 2i conditions was shorter than that in serum conditions. These results are consistent with the increase in transcription frequency in 2i conditions (Figure 3c). Collectively, these findings suggest that Nanog transcription might be regulated by modulation of promoter states.

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Figure 2 | Nanog-MS2 mESC line is useful for quantifying the Nanog transcription dynamics. (a) An illustration of the Nanog genomic locus and positions of smFISH probes used in this study. (b) Single-molecule fluorescent in situ hybridization (smFISH) analysis. Nanog mRNAs in a cell were visualized using Nanog exonic smFISH probes. Nuclei were counter-stained with Hoechst33342. Scale bar, 10 μm. (c) Distributions of Nanog mRNAs in mESC lines cultured in either serum (Ser) or 2i conditions (2i). The number of Nanog mRNAs in parental C57BL/6 (B6) or NM cell lines (NM) was counted by smFISH using Nanog exonic probes. In the box plot, center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; outliers are represented by dots; crosses represent sample means. n = 129, 109, 102, and 119 sample points. Statistical significance of differences was assessed by a two-sample Kolmogorov-Smirnov test (**P < 0.01). Red dots represent the mean counts of Nanog transcripts predicted by the random telegraph model (see the main text). (d) Bright fluorescent spots as observed in NM-G cells. The dashed lines delineate individual nuclei of cells. The white arrowheads point out bright fluorescent spots assumed to be Nanog transcription sites. Scale bar, 5 μm. (e) Results of smFISH analysis in NM-G cells. A representative image shows the overlap of fluorescent spots of nuclear localization signal (NLS)-MS2 coat protein (MCP)-mNeonGreen (mNG) and Nanog intronic smFISH probes, indicating that the MS2-MCP spot signals coincide with Nanog transcription sites. Scale bar, 5 μm.
mRNA, the degradation rate of MS2-integrated and wild-type (WT) Nanog means of OFF and ON time durations, respectively (Figure 3g). The copy number of can be predicted as the mean quantity of mRNA at steady state of was subtracted by the mean fluorescent intensity of each nucleus (n = 171 and 565 sample points). (e) A schematic presentation of the random telegraph model. “OFF” and “ON” depicts the OFF and ON states of promoters, respectively. OFF to ON and ON to OFF transition rates were defined as α and β, respectively. mRNA is assumed to be transcribed from only ON state promoter at rate ε. mRNA is degraded at rate δ. (f) A schematic representation of fluorescence intensity dynamics of an allele. (g) Distributions of ON and OFF time of Nanog transcription in cells cultured in serum or 2i conditions. Each probability distribution was fitted with an exponential function. Mean values are displayed in each graph.

If the Nanog transcription kinetics could be explained by the random telegraph model, the mean quantity of mRNA at steady state can be predicted as $<mRNA> = \gamma / ((\alpha + \beta)(\alpha / \beta))$ (Figure 3e). Here, $\gamma$ is the copy number of Nanog gene; $\alpha$ and $\beta$ are the reciprocal of the means of OFF and ON time durations, respectively (Figure 3g). The degradation rate of MS2-integrated and wild-type (WT) Nanog mRNA, δ, was determined to be 0.00348 and 0.00245 min⁻¹, respectively (Supplementary Figure S1). A least square fit of the random telegraph model to the experimentally obtained means of Nanog transcripts in NM and parent cells cultured in serum and 2i conditions reveals that the transcription rate at ON state is $2.11 \text{ min}^{-1}$ (Figure 2c). In both cell types cultured in serum conditions, the fitted means of Nanog mRNAs were considerably lower than those of the experimentally observed values. Although we performed live imaging at 2-min intervals, the estimated mean duration of ON time in serum conditions was shorter than 2 min (1.64 min, Figure 3g). Therefore, it is possible that not all the transcriptional pulses had been detected and that we underestimated the transcriptional frequency in serum conditions. To confirm whether the value of ε is realistic or not, we performed further quantitative analysis. The value of ε could be predicted from the number of nascent mRNAs remaining at each transcription site. When we imaged the NM-G cells at higher magnification, not only transcriptional bright spots but also other relatively weak and fast-moving signals, which are assumed to be individual mRNAs, were observed (Supplementary Video S3 and Figure S3).

From the comparison of individual mRNA signals and transcriptional spot signals, we estimate that there are $2.79 \pm 0.55$ [mean ± standard deviation, n = 15] nascent NLS-MCP-mNeonGreen-tagged mRNAs per transcription site. The sum length of an MS2 repeat and the 3’ untranslated region of Nanog mRNA is 2.55 kb; therefore, each nascent mRNA is roughly estimated to be transcribed by RNA Pol II at 946 ± 184-bp intervals. Accordingly, the RNA Pol II elongation rate was estimated to be 1.99 ± 0.39 kb/min. Recently, the RNA Pol II elongation rate was reported to be 0.5–4 kb/min (mean and median are 1.793 and 1.824 kb/min, respectively) in mESCs (Supplementary Figure S4), suggesting that the estimated ε seems to be a realistic value.

In the random telegraph model, mRNAs are transcribed in bursts during promoter switches from an inactive to active state, and the average size of the transcriptional bursts should be described as $\alpha / \beta$ (30). Because of the increase in the mean ON time duration in 2i conditions, the burst size in 2i conditions is larger than that in serum conditions (3.46 and 6.69 for serum and 2i conditions, respectively). Collectively, these suggest that Nanog transcription can be explained by the random telegraph model.

Stochastic promoter activation significantly contributes to expression variability of Nanog. Heterogeneity in gene expression is induced by several factors (32). One factor is the presence of stochasticity that is inherent to the biochemical process of gene expression, called the ‘intrinsic noise’. Therefore, the stochastic promoter activation observed in Nanog transcription could be a result of intrinsic noise. On the other hand, other effects, including variability of cellular components (such as RNA Pol II or other regulatory molecules), asynchronous cell cycle, and heterogeneous

Figure 3 | Transcription dynamics of Nanog in mESCs. (a, b) NM-G cells cultured in serum (a) and 2i conditions (b) were imaged at 2-min intervals for 4 h. Each line of the color-coded graph represents the transcription dynamics of each cell. (c) Distribution of transcription frequencies over a 4-h period for NM-G cells cultured in serum or 2i conditions [(total counts of transcribed alleles in each cell at 121 time points)/242] (n = 62 and 56 for serum and 2i conditions, respectively). (d) Distribution of fluorescence intensity at the transcription site in cells cultured in serum or 2i conditions. Each value was estimated by the mean fluorescent intensity of each nucleus (n = 171 and 565 sample points). (e) A schematic presentation of the random telegraph model. “OFF” and “ON” depicts the OFF and ON states of promoters, respectively. OFF to ON and ON to OFF transition rates were defined as $\alpha$ and $\beta$, respectively. mRNA is assumed to be transcribed from only ON state promoter at rate $\varepsilon$. mRNA is degraded at rate $\delta$. (f) A schematic representation of fluorescence intensity dynamics of an allele. (g) Distributions of ON and OFF time of Nanog transcription in cells cultured in serum or 2i conditions. Each probability distribution was fitted with an exponential function. Mean values are displayed in each graph.
inter-cellular signaling also affect gene expression variability and are called 'extrinsic noise'. These two types of noise can be discriminated by two-reporter assays. If different reporters are integrated into each allele, distribution of each reporter enables the determination of how intrinsic noise contributes to the total expression variability in the population.

To determine the type of noise that is dominant in the expression of Nanog, we established another cell line (NMP) in which repeats of MS2 and PP7 were integrated immediately upstream of each allele of the endogenous Nanog stop codon (Figure 4a). PP7 is used to visualize mRNAs. Southern blot analysis showed the expected band patterns and no random integrations (Figure 4b). The derived cells express the undifferentiated embryonic stem-cell markers, SSEA1 and Oct4 (Figure 4c). To quantify Nanog transcription dynamics on each allele, we introduced NLS-MCP-mNeonGreen and NLS-PP7 coat protein (PCP)-red fluorescent protein (RFP), which binds to the PP7 RNA stem loop. However, a tendency toward nucleoli localization of NLS-PCP-RFP prevents quantification of the transcription dynamics of Nanog-PP7. Using Nanog exonic and MS2 probes, we performed smFISH analysis of NMP cells cultured in serum and 2i conditions (Figure 2a and 5a). Among the Nanog probe-positive spots, those with an MS2 probe signal intensity above the threshold value were assumed to be mRNAs expressed from the Nanog-MS2 allele; otherwise, mRNAs were considered to be expressed from the Nanog-PP7 allele (Figure 5b, Supplementary Figure S5). To confirm the accuracy of this methodology, we also performed the smFISH using MS2 and PP7 probes in NMP cells cultured in 2i conditions (Supplementary Figure S5). The distribution of mRNA counts was similar to that obtained using Nanog exonic and MS2 probes (Figure 5b), suggesting that the method is reasonably accurate.

Intrinsic noise \( \sigma_{\text{int}}^2 \), extrinsic noise \( \sigma_{\text{ext}}^2 \), and total noise \( \sigma_{\text{tot}}^2 \) (sum of intrinsic and extrinsic noises) can be calculated using obtained allele-specific mRNA distribution data. Consistent with other reports, the total noise (heterogeneity in Nanog expression) in serum conditions was larger than that in 2i conditions (Figure 5b). Surprisingly, intrinsic noise contributed to approximately 45% of the total noise in both conditions, sug-
In Supplementary Table S1.

The biological meaning of heterogeneity is not fully understood, but also in pre-implantation embryonic inner cell mass (ICM) cells36. We have demonstrated that the stochastic promoter activation signifies that intrinsic noise significantly affects expression variability of Nanog in mESCs. Nanog expression variability is observed not only in mESCs cultured in serum conditions (Figure 2c and 5b), the Fano factor is the ratio between the variance and the mean of the mRNA copy number distribution, and is a key parameter used to quantify the deviation from Poisson statistics. For a complete Poisson distribution, the Fano factor equals 1. Distribution of Nanog mRNA transcripts in NMP cells were non-Poissonian (Fano factor $\approx 1$), suggesting that heterogeneity in Nanog expression is relatively high in both conditions. This observation is consistent with a previous report by Abranches et al. on the expression dynamics of the Nanog protein; using a bacterial artificial chromosome transgenic reporter and smFISH analysis, significant variability was observed in Nanog expression in mESCs cultured in 2i conditions37. Furthermore, we found that intrinsic noise derived from stochastic promoter activation significantly affects expression variability. Recently, Singer et al. reported that expression levels of genes, including Nanog, fluctuate between cells due not only to stochastic gene expression, but also to transitions between states with different gene activation potential39. This is consistent with our observation of multimodal distribution of transcription frequency over 4 h (Supplementary Figure S2). To compare the Nanog transcription dynamics among cells belonging to different states, we divided the cells into two groups according to their transcription frequency (lower or higher; Supplementary Figure S6). However, the distributions of ON and OFF time of Nanog expression between the groups did not show statistically significant differences in any of the culture conditions (Supplementary Figure S6). Singer et al. suggested that metastable states are correlated with DNA methylation in mESCs34. However, inhibition of DNA methylation by DNA methyltransferase inhibitor 5-AzaC (our study) or inactivation of all three DNA methyltransferases16,35 scarcely affects Nanog expression. Further analysis is necessary to understand what mechanisms determine the various Nanog expression states.

In our system, insertion of MS2 repeats into the Nanog locus slightly destabilizes Nanog-MS2 mRNA (Supplementary Figure S1). In NMP cells, in which MS2 and PP7 repeats were integrated into the Nanog locus, Nanog-MS2 and Nanog-PP7 mRNA showed similar expression levels (Supplementary Figure S5), suggesting that
integration of PP7 also affects Nanog mRNA degradation rate. Therefore, WT Nanog mRNA stays intact longer than Nanog-MS2 (or -PP7) mRNA in cells. This might mask the cell-to-cell expression variability in Nanog mRNA in WT mESCs; in other words, it is possible that intrinsic noise of Nanog mRNA in WT mESCs is lower than that in NMP cells. However, it has been recently reported that considerable intrinsic noise in Nanog mRNA expression seemed to exist in hybrid mESCs without integration of reporter genes. Therefore, it is possible that intrinsic noise has a considerable effect on Nanog expression variability in WT mESCs as well as in NMP cells.

Although pulsatile transcriptional events or “transcriptional bursting” has been reported in several model systems, the underlying molecular mechanisms are still elusive. One of the models of transcriptional bursting is the chromatin-based model. In this model, the efficiency of transcription depends on the absence of nucleosomes, which compete with the binding of transcription factors immediately upstream of the transcription start site (TSS). Therefore, the promoter activation timescale depends on relatively slower nucleosome turnover. Recently, it has been reported that nucleosome occupancy immediately upstream of the Nanog TSS is inversely correlated with Nanog expression level, implying that the stochastic promoter activation of Nanog may originate from the relatively slow nucleosome dynamics.

Figure 6 | Nanog protein heterogeneity originates from Nanog mRNA heterogeneity. C57BL/6 mESCs cultured in serum (a, b) and 2i conditions (c, d) were subjected to smFISH using Nanog exon probes and followed by immunofluorescence (IF) using Nanog antibody. (a, c) Nuclei were counterstained with Hoechst33342 (Hoechst). Dashed lines represent edges of cell membranes of nondividing cells. Scale bar, 20 µm. (b, d) Scatter plot of fluorescence intensities of IF (Nanog protein) and smFISH (Nanog exon probes).

Another potential source of transcriptional bursting includes DNA conformation changes involving efficient transcription. Some genes are regulated via long-range interaction between promoters and enhancers; because such long-range interactions seem to be variable among cells, the regulatory mechanism could be a source of expression variability. It has been reported that the Nanog promoter region is associated with several regions genome-wide, suggesting that genome-wide stochastic association between Nanog promoters and enhancers may underlie transcriptional bursting. Further investigation is needed to understand the molecular mechanism of the Nanog promoter activation.

In summary, Nanog transcription dynamics were quantitated using the MS2 system in mESCs. We found that the promoter activation occurs in a pulsatile and stochastic manner. Furthermore, allele-specific smFISH analysis revealed that intrinsic noise considerably contributes to the Nanog heterogeneity. Therefore, we conclude that stochastic processes of promoter activation might be a key source of the intrinsic noise, and hence significantly affect Nanog expression variability. The combination of the MS2 system and smFISH analysis seems to be useful for evaluating stochastic promoter activation and expression variability at single-cell resolution. The techniques used in the present report will help further the understanding of the molecular basis of allelic expression in mESCs and their heterogeneity.
Methods

Cell culture. Bruce 4 C57BL/6 mESC (Merk Millipore, Billerica, MA) and C57BL/6 D-MEM (Wako Chemicals, Osaka, Japan) were transfected with 1 mg/mL mCherry expressed in a C57BL/6 mESC (Wako Chemicals, Osaka, Japan) using Lipofectamine 2000 (Life Technologies, Gaithersburg, MD) according to the manufacturer’s instructions. After 12 h, the cells were transferred to 10-cm plates, incubated for 24 h, and then subjected to puromycin selection (1 mg/mL). Homologous recombination was performed by PCR and Southern blotting. Then, to excise the selection cassette flanked by loxP sites, 500 ng of Cre expression vector (pCAG-Cre) (Addgene plasmid 13775) was transfected into the obtained clone (NanogΔ35-Δ68/Δ53-Δ68). The genotype of the resultant ganciclovir-resistant ESC (NanogΔ35-Δ68/Δ53-Δ68) was confirmed by Southern blotting. For constitutive expression of NLS-MCP-mNeonGreen, pPB-LRS-CAG-MCP-mNeonGreen-ires-GFP plasmid was transfected with pmCMV-hyPβase® into NM cells, and G418-resistant clones were selected (1000 μg/mL). To generate the targeting vectors (NMP cells), 1 μg of pTV-mNanog-HPP was transfected with 250 ng of TERT expression vector (pCAG-Cre) into 60 cm2 of 96-well plates, incubated for 12 h, and then subjected to puromycin selection (1 mg/mL). The resultant puromycin-resistant NMP cell was confirmed by Southern blot. Subsequently, to excise the selection cassette flanked by loxP sites, pCAG-Cre was transfected into the obtained clone (NanogΔ35-Δ68/Δ53-Δ68). The genotype of the resultant gancilovir-resistant NMP cell was confirmed by Southern blot. Southern blots were performed as described previously.

Immunostaining. Immunostaining was performed on fixed cells (4% paraformaldehyde in BBS [50 mM BES, 200 mM NaCl, 1.5 mM Na2HPO4, 2H2O with 1 mM CaCl2, for 15 min]), washed, and blocked for 30 min in BSA-RSA buffer (BBS with 0.5% BSA, 0.1% Triton, and 1 mM CaCl2). Cells with primary antibodies were incubated overnight at 4°C in the following dilutions: anti-Nanog (1:500; MILC-51, ebioscience, San Diego, CA), anti-Oct4 (1:500; ab19857, Abcam, Cambridge, MA), anti-SSEA-1 (1:250, ab16285, Abcam). Cells were washed and blocked in BSA-RSA and then incubated with Hoechst33342 (1:1000, Life Technologies) and Alexa-conjugated secondary antibodies (1:500; Life Technologies). Images were acquired using an Olympus IX83 microscope (Olympus, Tokyo, Japan) with a CFI Plan 1.40 NA, and an iXon3 EMCCD camera (Andor, Belfast, UK), with laser illumination at 405 nm, 561 nm, and 637 nm, and were analyzed using Metamorph software (Universal Imaging Corporation, West Chester, PA); 101 z planes per site spanning 15 μm were acquired. Images were filtered with a one-pixel diameter 3D median filter and subjected to background subtraction via a rolling ball radius of 5 pixels, using ImageJ software. Detection and counting of smFISH signals were performed using Imaris software (Bitmap, Zurich, Switzerland) as described by Yang et al. Mixtures of Nanog exonic and intronic probes were conjugated with CY3. Fluor Red 590 were obtained from BioSearch Inc (Novato, CA) and used at 0.25 μM. MS2 probes conjugated with Cy5, and PP7 probes conjugated with Cy3 were obtained from Operon Technologies Inc. (Alameda, CA) and used at 0.5 μM each. Probe sequences are shown in Supplementary Table S2. To estimate mRNA half-life, NM and C57BL6 mESCs were cultured in 2i medium containing actinomycin D (5 μg/mL) for 2, 4, 7, and 6 h, and subjected to smFISH analysis using Nanog exonic probes.

smFISH-immunostaining. Trypsinized cells were transferred onto μ-Slide 8-well (Ibidi, Martinsried, Germany) coated with Laminin-511 and cultured for 1 h at 37°C and 5% CO2. Then, cells were fixed and subjected to smFISH as described above using Nanog exonic probes. After the image acquisition, cells were subjected to immunostaining, as described in the immunostaining section above, using the anti-Nanog antibody and Alexa Fluor 647 goat anti-rat IgG (1:500; Life Technologies). The images were acquired using an Olympus IX83 microscope with a CSU-W1 confocal unit, 60X Olympus oil-immersion objective of 1.42 NA; 101 z planes per site spanning 15 μm were acquired. smFISH images were filtered using ImageJ, with a one-pixel diameter 3D median filter, and subjected to background subtraction via a rolling ball radius of 2 pixels. Further fluorescence subtraction (100, 16-bit pixel unit) for almost complete removal of background intensity and maximum-intensity projection was subsequently performed. Immunostained images were filtered with a two-pixel diameter 3D median filter, and subjected to fluorescence subtraction (300, 16-bit pixel unit) for almost complete removal of background intensity and maximum-intensity projection, using ImageJ. The freehand selection tool of ImageJ was used for measurements of integrated signal intensity in each cell.

Live imaging. Trypsinized cells were transferred onto μ-Slide 8-well coated with Laminin-511 and cultured overnight at 37°C and 5% CO2. The next day, after the medium was changed, cells were subjected to live imaging by an Olympus IX83 microscope with a CSU-W1 confocal unit and a 60X Olympus oil-immersion objective of 1.42 NA for quantification of transcriptional dynamics. Fluorescence intensity was measured using an Olympus IX83 microscope equipped with a 488-nm laser, a stage-top microscope incubator (5% CO2; 37°C; Tokai Hit, Shizuoka, Japan), and an ASI MS-2000 piezo stage (ASI, Lyon, France), and were analyzed using Metamorph software; 41 z planes per site, spanning 12 μm, were acquired with a 2-min interval time for 4 h. Acquired images were filtered with a one-pixel diameter 3D median filter and subjected to fluorescence subtraction (300, 16-bit pixel unit) for almost complete removal of background intensity and maximum-intensity projection, using ImageJ. For observation of individual mRNA spots, a 100X Olympus oil-immersion objective of 1.40 NA was used. See Supplementary Video S3 and Figure S3 for details.

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