Index of Supplemental Material

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Supplemental Materials and Methods

Time series collection and staging
Multiple pairs of fish were set together for a maximum of five minutes, after which eggs were collected. Before the canonical onset of ZGA (128-cell, 256-cell, 512-cell), embryos were collected as soon as they displayed the morphological characteristics of the stage. From 1K-cell stage onwards, embryos were collected every five minutes. At the transition of each morphological stage, embryos that were delayed were removed. From the remaining embryos, samples were collected every five minutes until the next stage transition. During sphere stage, embryos were collected every fifteen minutes because repeatedly taking embryos out of the incubator for sample collection prolonged this stage. Until oblong stage, embryonic stages were verified using cell cycle stage distribution analysis (Supplemental Fig. S2). Embryos whose cell cycle stage distribution strongly deviated from that of other embryos of their stage of collection were restaged.

Gene selection
Genes were selected according to the subsequent criteria: (i) absence of maternally provided transcripts at 2/4-cell stage and 256-cell stage according to RNA-seq data (Pauli et al. 2012), (ii) high expression levels at dome and shield stage according to RNA-seq data (Pauli et al. 2012), (iii) transcript length greater than 1100 bp to ensure binding of a sufficient number of smFISH probes for efficient transcript detection, (iv) known gene function to allow the selection of a wide variety of genes, (v) induction at dome stage and absence of maternal transcripts confirmed by (Aanes et al. 2011 and zfin.org), (vi) ubiquitous expression according to whole mount ISH to enable analysis of a large number of cells, (vii) high smFISH probe quality to enable efficient automated transcript detection.

Whole mount in situ hybridization
Whole mount in situ hybridization was performed as described previously (Thisse and Thisse 2008). After staining, embryos were cleared in MeOH and gradually transferred to 87% glycerol for imaging. Samples were imaged in 87% glycerol on a Leica M165C dissecting scope equipped with a Leica MC170 HD camera. The following primers were used for probe cloning:

### Table S1. WM-ISH probe cloning primers

| Gene | Forward primer | Reverse primer |
|------|----------------|----------------|
| aldob | AGCCTGAGATCCTCCAGACG | TGTGGATGCTTGCCAGCCTG |
| apoeb | AGTCCCAGGCTTTCCAAAATG | GCCACGTGTTGGCCCCACTAAC |
| lrwd1 | CGTCTATTAAACGACCCGCATG | GCACACGTGCTGCTGATGATG |
| mex3b | CCCTGAGGCAAGACGAAATAC | CGTTCCATGCAGGTCAAAACC |
| slc25a22 | TCAATCCATGTGATGTGAAAG | AGTGTCTTGACAGCGACCG |
| sox19a | GAATGACCGACGTGAGCCCGTCC | GCCATGGCGGATGGATACTGC |
| tbx16 | GTCTCCATCTTCATCTTCCAGCG | GCTCACCACGACAGATGAG |
| zic2b | TGAACCAACCGAATGTTCCC | TGACCAGTTCGTGCATGGTC |

**Degradation rate analysis**

Embryos were left to develop until sphere stage at which point they were treated with 1:200 flavopiridol (5mg/ml stock) in embryo water to inhibit transcription. After 0, 30, 60, 120, 180 and 240 minutes in flavopiridol, twenty-five embryos were collected and snap frozen in liquid nitrogen. RNA was extracted using the RNeasy kit (Qiagen). DNA was removed using the DNA-free™ Kit (Life Technologies). mRNA was converted to cDNA using the SuperScript® III First-Strand Synthesis System (Thermo Fisher Scientific) kit with random hexamer primers. qPCRs were performed on the Roche Light Cycler 96. Intronic primers were used to confirm transcription inhibition, which was efficient for all genes. Primers to the coding sequence were used to monitor transcript levels over time. For primer sequences, see Table S2. The change in Ct value over time
was used to calculate the degradation rate for each sample and results were averaged over five biological replicates (Supplemental Fig. S11).

Table S2. qPCR primers for transcript half-life analysis

| Gene            | Forward primer | Reverse primer          |
|-----------------|----------------|-------------------------|
| aldob_exonic    | TACCACACAGGGGTTGGA | CAGGCTCTACAATAGGCACC     |
| apoeb_exonic    | CAGCTCAGAAAGATCTTCAGC | CTACGGTGTGGCATCTC       |
| lrwd1_exonic    | TGTATGTGTGAAAGGGTGAC | CTGCGATTCCCTTTCAAGTC     |
| mex3b_exonic    | TCGGCAGAAAGGTGCA | GTAGCGTTCCTTCAGGCT       |
| slc25a22_exonic | TCAATCCATGTGATGTGAAGAG | TCACGATAAACTCTCCCACG   |
| sox19a_exonic   | GAGGATGGACAGCTACGG | CTATAGGACATGGGGTTGAG     |
| tbx16_exonic    | ACCAGCCGTACAGATTTCAC | ATGTTTAGCAGCTCCAGCTA    |
| zic2b_exonic    | CACATGAAGGTTCACAGAG | GTTCTTGGCGGAGTCTTTAA     |
| aldob_intronic  | GGTTACACGTGCCAAGGTAAC | GTTGAGAACTGCCAAAACTAGC   |
| lrwd1_intronic  | CAACAGCTCATCATATTCGCC | TGTATGTGTGAAAGGGTGAC    |
| mex3b_intronic  | TCGGCAGAAAGGTGAAC | AGGATGACAGTTAAAGGTTAAGC |
| slc25a22_intronic | GCGCCATTTTATTGGTCCTTC | GTGGATGGGCACCTTTAAATTAC |
| tbx16_intronic  | TGTCCTTCCTCAAGCTGAAG | CTTACTTGATCAAAGTCG       |
| zic2b_intronic  | GGGATGTGGCAGAGGTGTC | TGGCAAGGTCAAAAGTTATGCG   |

smFISH

smFISH was performed as described previously (Stapel et al. 2016). A summary can be found below.

smFISH sample preparation
Embryos were fixed in the chorion in 4% formaldehyde in PBS with 0.1% Tween-20 (PBT) at 4 °C overnight. The next day, embryos were manually dechorionated in PBT and incubated in several changes of fresh 30% sucrose in PBS over the course of two hours before being incubated in 30% sucrose in PBS/OCT (50/50, v/v) at 4 °C for 5 days. Then, embryos were embedded in OCT and blocks were quickly frozen in precooled isopentane at -80 °C. Blocks were wrapped in plastic foil and stored at -80 °C until cryosectioning, but at least overnight. Blocks were sectioned at 8 μm thickness on a Microm HM560 cryostat. Cryosections were attached to selected #1.5 22x22 mm coverslips, that were cleaned by sonicating once in 1:20 mucasol, and twice in 100% EtOH, and were then coated with 1:10 poly-L-lysine in Milli-Q water (Sigma, P8920). Coverslips with sections were stored at -80 °C in 6-well plates sealed with parafilm. Sections were stored at -80 °C for at least one day until start of the smFISH protocol.

**smFISH probes**

48 20-base probes per mRNA were designed using the Stellaris Probe Designer ([https://www.biosearchtech.com/stellarisdesigner/](https://www.biosearchtech.com/stellarisdesigner/)) and checked for binding specificity using BLAST. CAL Fluor Red 610 and Quasar 670 labeled probes were ordered from Biosearch Technologies. For probe sequences see Table S3.

**smFISH**

8 μm cryosections of OCT embedded embryos were postfixied in 4% formaldehyde in PBS for 15 minutes and rinsed twice with PBS. Sections were equilibrated in 70% ice cold EtOH for 5 minutes and incubated in fresh 70% ice cold EtOH at 4 °C for 4-8 hours for permeabilization. Samples were rehydrated in 2x SSC and subjected to a mild proteinase K digestion step with 1:2000 proteinase K (10 mg/ml stock) for 10 minutes with shaking to increase accessibility of RNAs. After two 5-minute washes with shaking in 2x SSC, samples were equilibrated in 10% smFISH wash buffer (10% formamide, 2x SSC) while probe mixes were being prepared. Probes (Biosearch Technologies) were hybridized at a concentration of 75 – 250 nM in 10% hybridization buffer (10% dextran sulfate (w/v) (Sigma D8906), 10% formamide (v/v), 1 mg/ml E. coli tRNA (Roche), 0.02% BSA, 2 mM Vanadyl-ribonucleoside complex (NEB S1402S), 2xSSC). To this end, smFISH
wash buffer was carefully drained from the coverslips as much as possible before coverslips were placed section down on a 90 μl drop of hybridization buffer with probe on a parafilm-coated cell culture dish. Genes were grouped in pairs of two for dual color imaging, based on activation timing and expression pattern. The following probe combinations were used: aldob-CF610 & apoeb-Q670, sox19a-CF610 & slc25a22-Q670, zic2b-CF610 & mex3b-Q670, tbx16-CF610 & lrwd1-Q670. Hybridization was performed at 30 °C for ~16 hours. The next day, coverslips were carefully released from the parafilm with 10% wash buffer. Samples were rinsed with 2 ml 10% smFISH wash buffer and washed twice for 30 minutes with 1 ml 10% wash buffer at 30 °C without shaking. 1:2000 DAPI (1mg/ml stock) and 1:100 phalloidin (Life technologies, A12379) were added to the second wash to stain nucleus and cell outlines, respectively. After the second wash step, samples were placed in GLOX buffer (10 mM TRIS pH7.5, 0.4% glucose, 2x SSC) at 4 °C until mounting, but at least for 20 minutes to reduce background signal. Samples were mounted in freshly prepared GLOX mounting medium (GLOX buffer with 1:50 each of 3.7 mg/ml Glucose oxidase (Sigma G2133), Catalase suspension (Sigma C3515) and Trolox). Samples were sealed with nail polish after excess mounting medium was drained using filter paper, and imaged immediately.

**smFISH imaging**

Samples were imaged in a tile scan with 15% overlap between tiles on a Delta Vision epifluorescence microscope equipped with a 100x 1.4NA oil objective, a Photometrics Cool Snap CCD camera and the following emission filter sets, (435/48, DAPI), (525/36, Alexa Fluor 488), (632/60, CAL Fluor Red 610), (676/34, Quasar 670). Pixel size in the image plane was 0.065 x 0.065 μm. Z-stacks of 19 z-sections were acquired with 0.3 μm spacing. After acquisition, image tiles were stitched with the “Grid/Collection stitching” plugin in Fiji (Preibisch et al. 2009).

**Image processing**

Image analysis was performed as described previously (Stapel et al. 2016) using our freely available analysis tools. All Fiji plugins are available through the Fiji update site MS-ECS-2D (see http://fiji.sc/MS-ECS-2D). The Random Forest classification pipeline for cell segmentation is
available in KNIME (http://tinyurl.com/KNIME-MS-ECS). A summary of the analysis steps can be found below.

**Transcript detection**

First, images were filtered to remove background signal, using tophat filtering. Next, images were smoothed with a Gaussian kernel to remove noise. Transcripts were detected as local maxima in this image and distinguished from the background noise with an intensity threshold, $T_{tx}$. In the histogram of local maxima intensity, $T_{tx}$ was chosen between two sharp peaks corresponding to background signal inside and outside of the embryo respectively, and a lower peak corresponding to the transcripts at higher intensity. Transcripts were segmented using watershed segmentation initiating from the detected maxima. Transcription foci were identified among the regions defined in the transcript segmentation based on their larger size (volume) and intensity compared to single transcripts with the use of thresholds $T_{l_{max}}$ for maximum intensity and $T_{v_{ol}}$ for volume. Transcript detection was implemented in the custom Fiji plugin ‘Transcript analysis’.

**Nuclear segmentation**

Nuclei were segmented based on DAPI staining using a watershed approach. Segmentation was done in 2D on a maximum projection of the original image along the z-axis of the image stack. This strategy could be used because tissue sections of less than one cell layer thick were used in the analysis. This makes it unlikely that multiple nuclei would overlap in the maximum projection. Nuclear segmentation was integrated in the Fiji plugin ‘Transcript analysis’.

**Cell segmentation**

Cell segmentation was based on phalloidin staining. The middle slice of the z-scan acquisition was used for cell segmentation, as an approximation of the cell outline in our thin sections. With a pixel-level classifier, the probability of being on a membrane, as well as a probability of being at the intersection of multiple membranes (i.e., a vertex) was assigned to each pixel. To this end, we trained a two-level cascaded Random Forest classifier from manually segmented training
data. The classification pipeline was implemented in KNIME to enhance ease of use. Based on the output of the classifiers, we traced membranes as highly likely paths between vertices using our custom ‘PathFinder’ plugin in Fiji.

Errors in cell segmentations were corrected manually with the aid of our custom Fiji plugin ‘Cell Annotation’. This plugin was also used for annotation of cell types (DEL, EVL) that were identified based on their position in the embryo, and annotation of nuclear states (interphase, early prophase, mitosis, transition out of mitosis) based on DAPI signal (Supplemental Fig. S2). Furthermore, a small number of cells was marked for exclusion from analysis because they contained holes introduced by sectioning, or non-specific probe signal (characterized by appearance in both smFISH channels).

**Integration of all image analysis components**

Cell, tissue and nuclear masks were used as input to the transcript detection plugin ‘Transcript analysis’ in Fiji. Nuclei were automatically assigned to cell regions with the restriction that each cell region could at most contain one nucleus. If more nuclei were partially present within one cell region, the largest nucleus was used and the smaller nucleus was excluded from analysis. Transcripts and transcription foci were assigned to cells and if applicable to nuclei. This information was used to determine the transcript density in each cell and each nucleus.

**Data analysis**

Data analysis was performed in R version 3.2.3 “Wooden Christmas-Tree” in R Studio version 0.99.491 with the additional libraries “ggplot2”, “plyr”, “rJava”, “reshape2” and “XLConnect”. Details of the analyses can be found below.

**Selection of cells to analyze**

Segmented cells spanned a wide variety of sizes. The smallest cells were excluded in all analyses because measurement of transcript density in these cells is sensitive to small fluctuations in transcript levels. To determine the threshold for cell exclusion, the size of the smallest cell in the
sections that contained a nucleus was determined at each stage. All cells smaller than this were excluded from the analysis. In addition, cells that were more than 1.7 fold larger than the median cell size were excluded, as they were likely to be the result of incorrect segmentations and represent more than one cell. The cell size thresholds were determined for all embryos and all time series data combined and the same thresholds were used for all embryos of one stage in all acquisitions of the time series.

Assessment of positional bias

Images of embryos at sphere and dome stage were divided in 20 bins of 480 pixels width (X) or 10 bins of 480 pixels height (Y) (Supplemental Fig. S6). Sphere and dome stage were selected because from sphere stage onwards asymmetries in gene expression can clearly be detected between ectoderm and mesendoderm progenitors for some genes. Transcript density was calculated for DEL cells in each bin and plotted to assess positional bias (Supplemental Fig. S6). Substantial positional bias was only detected for tbx16 in the X dimension, with higher expression in the margin of the embryo. To exclude any positional bias from affecting the results for this gene, the outermost five bins on either side of the embryo were excluded from tbx16 analysis for embryos of all stages.

Presence of maternal transcripts

To determine whether any transcripts were maternally provided, transcript densities were determined for the three stages prior to the canonical onset of ZGA, at 128-cell, 256-cell and 512-cell stage. No significant increase in transcript density was observed from 128-cell to 256-cell stage for any of the genes (Student’s t-test). This suggests that the transcripts that are present at 128-cell and 256-cell stage have a maternal origin. A small increase in transcript densities was detected for lrwd1 and tbx16 from 256-cell stage to 512-cell stage, but not for the other genes. Thus, small amounts of transcription may occur prior to 1k-cell stage, but most transcripts observed at this time have a maternal origin.
Transcript density calculation

Transcript density was calculated per cell by dividing transcript count over the cell volume in \( \mu m^3 \). To calculate the mean of median transcript density, the median transcript density was determined for each embryo, and then the mean of these medians was calculated across embryos.

Activation rate

To determine the activation rate of each gene during ZGA, the proportion of cells that had activated zygotic transcription was determined for each stage. Cells were classified as having activated transcription of a specific gene when i. the transcript density for the gene was higher than the maternally provided transcript density, or ii. transcription foci for the gene were present in the nucleus. The threshold for detection of zygotic transcripts over maternally provided transcripts for each gene was set at two times the 95\(^{th}\) percentile of the transcript density at either 128-cell or 256-cell stage, whichever was higher. To minimize the chance that transcription foci were not detected as a result of sectioning, we only analyzed those nuclei with the 25\% largest projected surface which we have previously shown are centered in the cryosection (Stapel et al. 2016). The proportion of cells with zygotic transcripts for each embryo at each stage was fit with a Hill equation to determine the time of 50\% activity \( (K) \) for each gene using non-linear model fitting in R Studio:

\[
F(x) = \frac{(x - x_0)^n}{k^n + (x - x_0)^n}
\]  

(1)

Here, \( x \) is the time in minutes from the start of the time series, \( x_0 \) is the time at which zygotic transcripts were detected in more than one embryo (to exclude incidental activation events, staging errors and detection errors). \( K \) is the time from \( x_0 \) until 50\% of the cells contain zygotic transcripts. \( n \) denotes the steepness the curve. The data was only fitted for \( x \geq x_0 \).

Extrinsic noise
The presence of extrinsic noise was assessed by analyzing the co-occurrence of transcription foci of two genes with similar expression domains and timing of activation in one nucleus. In the absence of extrinsic noise, the observed co-occurrence of transcription foci is equal to the co-occurrence that is expected based on the proportion of cells with transcription foci for each individual gene (pAB = pA * pB). In the presence of extrinsic noise, the observed co-occurrence is higher (pAB > pA * pB). Genes that were activated at the same time and in the same cell types (DEL/EVL) were paired and their expression was imaged at each stage of the time series using dual-color smFISH. The proportion of nuclei with transcription foci was determined for each individual gene and the observed and expected co-occurrences were calculated for each sample. To minimize the chance that transcription foci were not detected as a result of sectioning, we only analyzed those nuclei with the 25% largest projected surface which we have previously shown are centered in the cryosection (Stapel et al. 2016). Data was only included if the minimum number of nuclei in the sample was 10 or higher and if at least 5 nuclei contained transcription foci. To determine the contribution of cell cycle state to extrinsic noise, the analysis was repeated for interphase cells only. A significant decrease in the amount of extrinsic noise after cell cycle stage correction was detected for three out of four tested gene combinations (Student’s t-test). Interphase cells were identified based on chromatin morphology and were annotated using the ‘Cell annotation’ plugin in Fiji.

**Intrinsic noise**

The presence of intrinsic noise was assessed by analyzing the correlation in size between transcription foci (as a measure for their timing of activation) at the two alleles of a gene in a single nucleus. In the absence of intrinsic noise, the size of these two foci is correlated. In the presence of intrinsic noise, the size of these two foci is correlated to a lesser extent. Since maximum foci size decreased over time in accordance with the decrease in cell size, foci size correlation was determined at each stage individually. At each stage, nuclei with two transcription foci were identified and the Pearson correlation coefficient was determined in RStudio.
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Mathematical model of transcription activation
Supporting information

1 Stochastic model

To gain a quantitative understanding of transcript variability during genome activation, we developed and analyzed a minimal mathematical model accounting for stochastic acquisition of transcriptional competence during ZGA, cell cycle variability, as well as random production and degradation of RNA. Our core model describing transcription dynamics during early embryonic development is based on a stochastic birth-and-death process of the form

\[
G \xrightarrow{c_1(t)} G + X \\
X \xrightarrow{c_2} \emptyset,
\]

with symbols $G$ and $X$ corresponding to DNA and RNA, respectively. We assume the birth-rate $c_1(t)$ to be zero before acquisition of transcriptional competence during ZGA and whenever cells are in mitosis. Furthermore, we assume that $c_1(t)$ is proportional to the cell volume, which stochastically decreases due to continued cell division. This is in agreement with our observation that intensity of transcription foci normalized by the cell volume is largely stable over time (Supplemental Fig. S10). Altogether, transcription is modeled to happen at a stochastic rate $c_1(t) = k_b A(t) Z(t) \Omega(t)$, with $k_b$ as a rate constant, $\Omega(t)$ as the cell volume, $A(t) \in \{0, 1\}$ as the activation process describing when genes reach transcriptional competence and $Z(t) \in \{0, 1\}$ modeling inhibition of transcription during mitosis. Details about $A(t)$, $Z(t)$ and $\Omega(t)$ are described in the following Sections 1.1 and 1.2.

1.1 Model of transcription activation

To account for stochasticity in the acquisition of transcriptional competence during ZGA, we assume that the time $T^*$ that passes until a gene can be activated is randomly distributed across cells. In particular, we assume that $T^*$ follows a Gamma distribution with rate and inverse shape parameters $\rho$ and $\phi$, i.e., $T^* \sim \Gamma(\rho, \phi)$. Depending on the parameter $\rho$, this distribution can capture the whole spectrum between unsynchronized ($\rho = 1$) and highly synchronized ($\rho \gg 1$) acquisition of competence during ZGA. The activation process $A(t)$ affecting the rate $c_1(t)$ can be defined as $A(t) = \sigma(t - T^*)$ with $\sigma(t)$ as the Heaviside step function given by

\[
\sigma(t) = \begin{cases} 
1, & \text{if } t \geq 0 \\
0, & \text{otherwise}.
\end{cases}
\]

1.2 Cell cycle model

Since we found cell cycle stage variability to be a major determinant of transcript variability, we accounted for it explicitly in our analysis, by modeling how cells stochastically transition between interphase and mitosis. Since the cell cycle lengthens dramatically during the considered time window [Kane and Kimmel 1993], we allow the cell cycle duration to increase with the number of cell cycles to reflect the lengthening of the cell cycle after ZGA in the zebrafish embryo. In the following we denote by
$T_n$ the duration of the $n$-th cell cycle. The total duration $T_n$ consists of a mitotic part $t_m$ at the end of the cell cycle, which has been shown to be constant across different cell cycles [Kane and Kimmel 1993]. The remaining period – corresponding to interphase – lengthens over time and is divided into a fixed (i.e., deterministic) part $t_a$ and an additional stochastic part $T_r$, which randomly varies between cells according to an exponential distribution with mean $\lambda$. This random part allows the cells to desynchronize over time as is apparent in our data and known from the literature (Supplemental Fig. S2 and [Kane and Kimmel 1993]). The average total cell cycle length for the $n$-th generation is given by $\mu_n = \lambda + t_a + t_m$, whereas we assume that the ratio $\alpha = t_a/(\lambda + t_a)$ is constant over different cell cycles such that $t_a = \alpha(\mu_n - t_m)$ and $\lambda = (1 - \alpha)(\mu_n - t_m)$. Under the above assumptions, the cell cycle dynamics can be described as a renewal process $Z(t) \in [0,1]$ that captures the switching between interphase (i.e., $Z(t) = 1$) and mitosis (i.e., $Z(t) = 0$). The time that the cell spends in interphase $T_I = t_a + T_r$ is distributed according to a shifted exponential distribution $T_I \sim p(t) = \sigma(t - t_m) e^{-\lambda(t-t_m)}$, with $\sigma(t)$ as defined above. The time the cell spends in mitosis is assumed to be deterministic, which can be formally described as a random variable $T_m$ distributed according to a Dirac-delta distribution, i.e., $T_M \sim p(t) = \delta(t - t_m)$ (Fig. A1). The cell volume $\Omega(t)$ is assumed to remain constant within one cell cycle and divided by a factor of two after mitosis. This is consistent with the fact that the total embryo volume remains roughly constant across the considered developmental stages [Joseph et al. 2017].

In order to compare the cell cycle model to our measurements, we require the probability distribution of finding a cell in mitosis / interphase at different time points, denoted by $P(z, t \mid \theta_{cc}) = P(Z(t) = z \mid \theta_{cc})$ with $z \in \{0,1\}$. The set $\theta_{cc}$ collects the parameters of the cell cycle model, i.e., $\theta_{cc} = \{t_0, t_a, \alpha, \mu_j, \ldots, \mu_N\}$, with $j$ and $N$ as the number of the first and last considered cell cycle, respectively. The additional parameter $t_0$ describes the time lag between the time zero in the model (where all cells are assumed to be at the beginning of interphase) and the first measurement time point (corresponding to 1K-cell stage). Since $Z(t)$ involves non-exponential waiting-times, the probability distribution $P(z, t \mid \theta_{cc})$ does not satisfy a conventional master equation such as frequently used to describe Markovian networks. In particular, the static time-delays of our model, would give rise to a time-delay master equation, which is numerically expensive to simulate. We therefore resorted to a Monte Carlo approach, where individual transitions between interphase and mitosis are stochastically simulated and subsequently averaged to determine $P(z, t \mid \theta_{cc})$ at different time points.

In order to infer the cell cycle parameters $\theta_{cc}$, we fitted the stochastic cell cycle model to the experimentally measured fraction of mitotic cells using a Bayesian approach. We denote by $y_i = \{l_i, k_i\}$ the measurements taken at time $t_i$, consisting of the number of mitotic cells $l_i$ and the total number of acquired cells $k_i$. The likelihood of observing $y_i$ is given by a binomial distribution

$$L(y_i \mid \theta_{cc}) = P(l_i \text{ out of } k_i \text{ cells in mitosis at time } t_i \mid \theta_{cc})$$

$$= \binom{k_i}{l_i} P(z = 0, t_i \mid \theta_{cc})^{l_i} (1 - P(z = 0, t_i \mid \theta_{cc}))^{k_i - l_i}.$$  \hspace{1cm} (3)

The likelihood of observing an entire trajectory of measurements $y = \{y_1, \ldots, y_J\}$ is correspondingly given by the product of the likelihood terms for the individual time points, i.e.,

$$L(y \mid \theta_{cc}) = \prod_{i=1}^{J} L(y_i \mid \theta_{cc}).$$  \hspace{1cm} (4)

The Bayesian posterior distribution over the cell cycle parameters $\theta_{cc}$ reads

$$p(\theta_{cc} \mid y) \propto L(y \mid \theta_{cc}) p(\theta_{cc}).$$  \hspace{1cm} (5)

with $p(\theta_{cc})$ as the prior distribution over $\theta_{cc}$. For all experiments, we considered flat priors on the positive orthant. We used a Markov chain Monte Carlo scheme with log-normal proposal distributions to sample from (5). Point estimates of $\theta_{cc}$ were subsequently determined by choosing the parameters $\theta_{cc}^*$ with maximal posterior probability (i.e., maximum a-posterior estimation). The measurements used for fitting start at the end of cell cycle $j = 10$ (transition to 1K-cell), where most cells are in mitosis, and end during cell cycle $N = 13$ (dome). The resulting model fits are shown in (Fig. A1) and the corresponding parameter estimates for DEL and EVL are listed in Table A1.
1.3 Computing the mean and variability of transcript densities

In order to efficiently determine the mean transcript densities and their variability we resorted to a semi-analytical approach that exploits the analytical simplicity of our birth-and-death transcription model. In particular, if one assumes the cell cycle stage and time of transcriptional competence to be known, one can formulate a conditional master equation describing the stochastic production and degradation of transcripts. In the following, we denote by $P(x,t)$ the transcript copy number for a given cell at time $t$. With $Z_T = \{ Z(t) \mid t \leq T \}$, $A_T = \{ A(t) \mid t \leq T \}$ and an initial cell volume $\Omega(0)$, the conditional probability distribution over the transcripts $P(x,t) = P(X(t) = x \mid A_T, Z_T, \Omega(0) = \omega_0)$ satisfies the master equation

$$\frac{d}{dt} P(x,t) = k_o A(t) Z(t) \Omega(t) P(x - 1,t) + c_2 (x + 1) P(x + 1,t) - (k_o A(t) Z(t) \Omega(t) + c_2 x) P(x,t). \quad (6)$$

From this distribution, equations for the first and second order conditional moments can be obtained by multiplying (6) with $x$ and $x^2$ and summing over all $x \in \mathbb{N}$. The resulting equations are given by

$$\frac{d}{dt} \nu_1(t) = k_o A(t) Z(t) \Omega(t) - c_2 \nu_1(t)$$

$$\frac{d}{dt} \nu_2(t) = k_o A(t) Z(t) \Omega(t) + (2k_o A(t) Z(t) \Omega(t) + c_2) \nu_1(t) - 2c_2 \nu_2(t), \quad (7)$$

with $\nu_1(t) = \mathbb{E} [X(t) \mid A_T, Z_T, \Omega(0) = \omega_0]$ and $\nu_2(t) = \mathbb{E} [X(t)^2 \mid A_T, Z_T, \Omega(0) = \omega_0]$. Eq. (7) is a linear ordinary differential equation which can be solved in closed form, meaning that it can be evaluated at very low computational cost. The unconditional moments of the transcript densities $S(t) = X(t) \Omega(t)^{-1}$ can then be obtained by dividing $\nu_1(t)$ and $\nu_2(t)$ by $\Omega(t)$ and $\Omega(t)^2$, respectively and averaging over

Table A1: Estimated cell cycle parameters $\theta_{cc}$ for DEL and EVL cells.

| Parameter | Value   | Unit |
|-----------|---------|------|
| $t_0$     | 10.2008 | min  |
| $t_m$     | 10.8553 | min  |
| $a$       | 0.4657  | -    |
| DEL       |         |      |
| $\mu_{10}$| 17.5032 | min  |
| $\mu_{11}$| 24.8373 | min  |
| $\mu_{12}$| 31.2222 | min  |
| $\mu_{13}$| 49.8363 | min  |
| EVL       |         |      |
| $\mu_{10}$| 19.4517 | min  |
| $\mu_{11}$| 29.2885 | min  |
| $\mu_{12}$| 40.4934 | min  |
| $\mu_{13}$| 86.9417 | min  |
different realizations of $A_T$, $Z_T$ and $\Omega(0)$. From the resulting moments $\mathbb{E}[S(t)]$ and $\mathbb{E}[S(t)^2]$ one can then determine the variance or the squared coefficient of variation of transcript density as

\[
\text{Var}[S(t)] = \mathbb{E}[S(t)^2] - \mathbb{E}[S(t)]^2
\]

\[
\text{CV}[S(t)]^2 = \frac{\text{Var}[S(t)]}{\mathbb{E}[S(t)]^2}.
\]

The overall model of transcription was fitted to the experimental single-molecule measurements using a moment-based inference approach [Zechner et al. 2012] to determine the model parameters. In particular, we used this technique to match the time-series of experimental transcript density means and squared coefficients of variation with those obtained from the model using a Bayesian approach. For a more detailed explanation of this scheme the reader shall refer to the original study from [Zechner et al. 2012]. Since the cell cycle model has been calibrated in a preceding step, the model comprises four unknown parameters, i.e., the rate of transcription $k_\rho$, the mRNA degradation rate $c_2$ as well as the parameters $\rho$ and $\phi$ describing the probability distribution of the time it takes to reach transcriptional competence. Additionally, we estimated the initial mean $\mathbb{E}[S(0)]$ and squared coefficient of variation $\text{CV}[S(0)]^2$ of the transcript density (i.e., due to maternally provided RNA). The degradation rate was measured experimentally (Supplemental Fig. S11) and provided to the inference method in the form of an informative prior distribution. In particular, we assumed Gamma-type prior distributions whose mean was adjusted to the experimentally measured value of the degradation rate. The squared coefficient of variation of the prior distributions was set to one third such that $\text{CV}[S(0)]^2$ as the measured degradation rate. Furthermore, the distribution over initial cell volumes $\Omega(0)$ was determined experimentally and provided to the model (Supplemental Fig. S10). As in Section 1.2, we used a Markov chain Monte Carlo technique to draw samples from the posterior distribution and extracted MAP estimates from these samples. The resulting model parameters are shown in Table A2.

| Parameter | $\mathbb{E}[T^+|h] = \rho/\phi$ | $\text{Std}[T^+] = \sqrt{\rho/\phi}$ | $k_\rho$ | $c_2$ |
|-----------|-------------------------------|---------------------------|----------|--------|
| apoeb     | 0.741937                      | 0.400439                  | 0.113717 | 0.019867 |
| slc25a22  | 1.088551                      | 0.543304                  | 0.373899 | 2.215407 |
| mex3b     | 1.273525                      | 0.695762                  | 0.152475 | 0.176630 |
| lrwd1     | 1.037986                      | 0.406869                  | 0.191756 | 0.026058 |
| aldob     | 1.302461                      | 0.587520                  | 0.127748 | 0.251547 |
| sox19a    | 1.133046                      | 0.516908                  | 0.344161 | 0.948035 |
| tbx16     | 1.238316                      | 0.455831                  | 0.246472 | 0.219969 |

### 1.4 Model-based decomposition of transcriptional noise

Once calibrated, the mathematical model of transcription can be used to quantify and dissect variations due to differences in the timing of the acquisition of transcriptional competence between cells, cell cycle variability and intrinsic fluctuations of RNA production and degradation (Figs. 4D and Supplemental Fig. S13). It can be shown by the law of total variation that the variance of transcript density across cells is given by

\[
\text{Var}[S(t)] = \mathbb{E}[\text{Var}[X(t) | A_T, Z_T] | A_T] + \mathbb{E}[\text{Var}[X(t) | A_T, Z_T] | A_T] + \mathbb{E}[\text{Var}[X(t) | A_T, Z_T] | A_T] + \mathbb{E}[\text{Var}[X(t) | A_T, Z_T] | A_T].
\]

Note that the inner conditional moments $\mathbb{E}[X(t) | A_T, Z_T]$ and $\text{Var}[X(t) | A_T, Z_T]$ in (10) correspond to the moments from (7) averaged over the initial cell volume $\Omega(0)$. The individual terms on the right-hand-side of (10) were determined by forward-simulation of the model.
1.5 Perturbation experiment

The results from our model suggest that transcript variability is inversely correlated with the amount of time that cells spend in interphase. To test this assumption, we modelled a situation in which cell cycle length was kept constant at 15 min, whereas all other parameters remained unchanged. Note that as a consequence of the constant cell cycle length, every cell undergoes more cell cycles than in the unperturbed situation, which would – due to continued division – lead to unreasonably small cell volumes. To prevent that, we assumed that the cell volume reduces only during the first 4 cell cycles (cycles 10-13) and stays roughly constant during the subsequent cell cycles. In the perturbed situation, noise at the end point of the simulation was found to be around 1.2 – 1.6 times higher than in the unperturbed situation (Supplemental Fig. S14), confirming the importance of cell cycle lengthening in noise reduction.
References

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### Table S3. smFISH probe sets

| Table | Probe Set 1 | Probe Set 2 | Probe Set 3 |
|-------|-------------|-------------|-------------|
| S3.   | S3.         | S3.         | S3.         |
| aldob-CalFluor610 | apoeb-Quasar670 | mex3b-Quasar670 |
| ctggacagacgtagttgagac | ccacaagagctctcatgctt | gatattccccctctgagttgc |
| aatgctgcattatagggct | ttaaacctgccaagggcagaag | tggagaaagcaaatattcgccag |
| gaatacaggtgcaagagttgag | catcagctgtacaccgctcaaat | aatatcagcttacctccccaat |
| tgtgacgccatatgattttta | agacacatatcccaagaaacag | aacacggcagtttttaacagc |
| taaaatctgtacggccaag | ctcgcttattgcttggag | ataggacacatgatagatgcttg |
| ctgggaactggttgtaatct | gaggcccttgatgttttgcac | cgctgtggagagcagtgactgt |
| ttctctgccgctgtaagagag | ggtgctcgattttcttcagtg | cccagagagaaagcagattccc |
| caatgcgtcagcagacatg | cgtgcggctcgagtttggagta | gttgtgtgactaacagtcgac |
| caagatcgctgaaagggct | gcgtgcatcaggtgtagatc | cccatgaaagccgaagccct |
| cttggtcgtgctatgtggag | gtcgcggatcttctgggagta | ggtgtgtgactaacagtcgac |
| agttcgctgtgatgttggag | ggtgctcgattttcttcagtg | cccatgaaagccgaagccct |
| catgcgtgctggcaagacatc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| acaggtccagacctttagctt | gtcgctgtgctgttggagta | ggtgtgtgactaacagtcgac |
| cttctcttagttgcaagacatc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| tggagatgtgataaaggc | cgcctgctaccagcttttcag | cgtgcggctcgagtttggagta |
| cctccagatagatgtaaaggc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| ccccttaagcttaggttaggttc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| catgcgtgctggcaagacatc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| acaggtccagacctttagctt | gtcgctgtgctgttggagta | ggtgtgtgactaacagtcgac |
| cttctcttagttgcaagacatc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| tggagatgtgataaaggc | cgcctgctaccagcttttcag | cgtgcggctcgagtttggagta |
| cctccagatagatgtaaaggc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| ccccttaagcttaggttaggttc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| catgcgtgctggcaagacatc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| acaggtccagacctttagctt | gtcgctgtgctgttggagta | ggtgtgtgactaacagtcgac |
| cttctcttagttgcaagacatc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| tggagatgtgataaaggc | cgcctgctaccagcttttcag | cgtgcggctcgagtttggagta |
| cctccagatagatgtaaaggc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| ccccttaagcttaggttaggttc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| catgcgtgctggcaagacatc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| acaggtccagacctttagctt | gtcgctgtgctgttggagta | ggtgtgtgactaacagtcgac |
| cttctcttagttgcaagacatc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| tggagatgtgataaaggc | cgcctgctaccagcttttcag | cgtgcggctcgagtttggagta |
| cctccagatagatgtaaaggc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| ccccttaagcttaggttaggttc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| catgcgtgctggcaagacatc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| acaggtccagacctttagctt | gtcgctgtgctgttggagta | ggtgtgtgactaacagtcgac |
| cttctcttagttgcaagacatc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| tggagatgtgataaaggc | cgcctgctaccagcttttcag | cgtgcggctcgagtttggagta |
| cctccagatagatgtaaaggc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| ccccttaagcttaggttaggttc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| catgcgtgctggcaagacatc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| acaggtccagacctttagctt | gtcgctgtgctgttggagta | ggtgtgtgactaacagtcgac |
| cttctcttagttgcaagacatc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| tggagatgtgataaaggc | cgcctgctaccagcttttcag | cgtgcggctcgagtttggagta |
| cctccagatagatgtaaaggc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| ccccttaagcttaggttaggttc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| catgcgtgctggcaagacatc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| acaggtccagacctttagctt | gtcgctgtgctgttggagta | ggtgtgtgactaacagtcgac |
| cttctcttagttgcaagacatc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| tggagatgtgataaaggc | cgcctgctaccagcttttcag | cgtgcggctcgagtttggagta |
| cctccagatagatgtaaaggc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| ccccttaagcttaggttaggttc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| catgcgtgctggcaagacatc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| acaggtccagacctttagctt | gtcgctgtgctgttggagta | ggtgtgtgactaacagtcgac |
| cttctcttagttgcaagacatc | cgtgcggctcgagtttggagta | ggtgtgtgactaacagtcgac |
| sox19a-CalFluor610                                                                 | slc25a22-CalFluor610                                                                 | tbx16-CalFluor610                                                                 |
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| cgggttcgtctgtttggtgg persisted                                                                         | acctcttggtctgcttatc                                                                         | catatatcacaagctgttc                                                                         |
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| cgggttcgtctgtttggtgg persisted                                                                         | acctcttggtctgcttatc                                                                         | catatatcacaagctgttc                                                                         |
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| cgggctgttctttttgtgg                                                                         |acctcttggtctgcttatc                                                                         | catatatcacaagctgttc                                                                         |
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| tgggttgattttctgttggtgg persisted                                                                         | acctcttggtctgcttatc                                                                         | catatatcacaagctgttc                                                                         |
| cgggttcgtctgtttggtgg persisted                                                                         | acctcttggtctgcttatc                                                                         | catatatcacaagctgttc                                                                         |
| gagggtgtcttttccgc                                                                         | acctcttggtctgcttatc                                                                         | catatatcacaagctgttc                                                                         |
| cgggctgttctttttgtgg                                                                         |acctcttggtctgcttatc                                                                         | catatatcacaagctgttc                                                                         |
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