

Transcription of functionally related constitutive genes is not coordinated

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Expression of an individual gene can vary considerably among genetically identical cells because of stochastic fluctuations in transcription. However, proteins comprising essential complexes or pathways have similar abundances and lower variability. It is not known whether coordination in the expression of subunits of essential complexes occurs at the level of transcription, mRNA abundance or protein expression. To directly measure the level of coordination in the expression of genes, we used highly sensitive fluorescence *in situ* hybridization (FISH) to count individual mRNAs of functionally related and unrelated genes within single *Saccharomyces cerevisiae* cells. Our results revealed that transcript levels of temporally induced genes are highly correlated in individual cells. In contrast, transcription of constitutive genes encoding essential subunits of complexes is not coordinated because of stochastic fluctuations. The coordination of these functional complexes therefore must occur post-transcriptionally, and likely post-translationally.

Proper execution of cellular processes is mediated through various proteins working together in complexes to perform specific tasks¹. A crucial task for cells is to coordinate the expression of genes that encode these functionally related proteins to ensure proper complex stoichiometry. Considerable progress has been made in identifying genes encoding functional complexes and in characterizing transcriptional networks that co-regulate their expression^{2–6}. These transcriptional networks describe regulator-gene interactions that allow a cell to coordinate the expression of proteins needed to facilitate biological functions such as the optimal assembly of multi-protein complexes^{7–9}.

The expression of a gene, however, involves random interactions between molecules present in small numbers per cell. Most proteins are produced from fewer than ten copies of mRNA, which in turn are produced from just one or two copies of a gene per cell^{4,10}. Therefore, the process of gene expression is subject to stochastic fluctuations and can lead to considerable differences in the level of expression between genetically identical cells¹¹. Several studies have used fluorescent protein reporters to track protein levels in single cells so as to gain a comprehensive understanding of sources of variation in expression, which are generally classified into extrinsic and intrinsic components^{12–15}. Extrinsic variation arises from cell-to-cell differences in global factors such as transcriptional activators, metabolic status or cell cycle stage. Intrinsic variation, on the other hand, arises from inherently random fluctuations in molecular events such as production or destruction of mRNAs and proteins.

Initial experiments in yeast, largely limited to induced genes, suggested that cell-to-cell differences in expression were mostly due to extrinsic sources^{16,17}. However, recent studies aimed at a broader set of genes reported a more substantial contribution from intrinsically

random fluctuations, especially for proteins with low or intermediate abundance^{18–20}. These high-throughput studies also noted protein-specific differences in variation. Particularly, essential genes encoding subunits of multi-protein complexes were characterized by low variation²¹. Moreover, a proportional relationship between expression variance and mean suggested that variation in protein levels arises from fluctuations in mRNA levels due to random production and decay of mRNAs or random activation and inactivation of the gene promoter^{13,18}. Therefore, direct measurements of mRNA abundance are crucial to understanding how individual cells co-regulate the expression of functionally related proteins.

Although ensemble methods such as northern blots and reverse-transcription PCR are inadequate for measuring mRNA abundance in individual cells, technological advances in detecting single mRNAs have made it possible to measure mRNA abundance as well as transcriptional activity in single cells^{22–25}. Indeed, a recent study in the yeast *Saccharomyces cerevisiae* showed that random fluctuations in mRNA abundance of constitutive genes arise from single, uncorrelated transcription-initiation events²⁴. Constitutive genes, which are expressed throughout the cell cycle without requiring additional stimuli when cells are grown in rich media, account for two-thirds of the yeast genome. Studying how their expression is coordinated in the presence of stochastic fluctuations is therefore instrumental to understanding how cellular systems work. In particular, how does an individual cell coordinate the expression of functionally related genes and produce the stoichiometry required for a multi-protein complex in the presence of stochastic fluctuations?

To address this question, we used a highly sensitive fluorescence *in situ* hybridization (FISH)-based approach to count the mRNAs of

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multiple functionally related and unrelated genes simultaneously in single *S. cerevisiae* cells. We hypothesized that mRNA abundances of essential genes encoding proteins in the same complex or pathway would be more correlated than those of functionally unrelated genes. We show that cells transcribe induced genes in a highly coordinated manner. However, transcript levels of constitutive genes encoding essential subunits of multi-protein complexes, such as the proteasome, RNA polymerase II or the general transcription factor TFIID, are not correlated any more than functionally unrelated genes. Finally, our modeling results show that synchronizing effects of cell division account for weak correlations observed among transcript levels of all constitutively active genes.

RESULTS

We used a previously described FISH-based approach to detect nascent mRNAs at the site of transcription in the nucleus as well as mature mRNAs in the cytoplasm for two genes in single *S. cerevisiae* cells^{22,24}. Multiple oligodeoxynucleotide probes, each labeled with five fluorescent dye molecules, hybridized to nascent transcripts at the transcription site in the nucleus and mature transcripts in the cytoplasm. Spectrally distinct fluorescence signals from individual mRNAs of each gene, labeled with either cyanine 3 or cyanine 3.5 dye, were detected with a spot-detection algorithm and counted²⁶. This approach allowed us to simply fix cells and generate single-cell expression profiles of endogenous genes without the need for any genetic perturbations. Single-cell mRNA abundances were then used to calculate pairwise correlation coefficients (r), representing the degree of coordination between two genes with extremes of +1 (most correlated), 0 (uncorrelated) and -1 (most anti-correlated).

Highly coordinated transcription of galactose network

We used a network of galactose-inducible (GAL) genes to validate our method of quantifying the level of coordination in individual cells. Transcription of *GAL1*, *GAL10* and *GAL7* is activated through de-repression of a common transcription factor Gal4p upon induction with galactose^{27,28}. Gal4p operates through four binding sites in the upstream activating sequence (UAS) of the *GAL1-GAL10* divergent promoter (Fig. 1a). Gal4p also activates

the transcription of *GAL7* through two binding sites in a similar, but distinct, UAS from the *GAL1-GAL10* UAS.

We first examined whether nascent GAL transcripts were present in a coordinated manner at the site of transcription in the nucleus. Pairwise analysis of transcription sites for these three genes revealed various modes of transcription (Fig. 1b–d). GAL genes were tightly repressed in cells grown in 2% (w/v) raffinose. Only $6 \pm 2\%$ of cells were actively transcribing either *GAL1* or *GAL10*, and these had on average less than one transcript in the cytoplasm. After induction with 2% (w/v) galactose for 15 min, a majority ($60 \pm 4\%$) of cells were actively transcribing both *GAL1* and *GAL10* (Fig. 1d). However, a small fraction of cells were transcribing only *GAL1* ($12 \pm 2\%$) or *GAL10* ($7 \pm 1\%$). The remaining $21 \pm 3\%$ of cells did not have a transcription site for either gene. A pairwise analysis of two genes with similar but distinct UAS (*GAL1* and *GAL7*) showed a similar fraction of cells with both genes in the ‘off’ state (Fig. 1c). However, a slightly smaller percentage of cells were actively transcribing both genes ($44 \pm 5\%$) compared to *GAL1-GAL10*.

Because transcription sites in the nucleus describe only the earliest stages of coordination in gene expression, we next compared cytoplasmic expression profiles in a pairwise manner (Fig. 2). As expected, the three induced genes showed expression profiles with similar means after 15 min of induction with 2% (w/v) galactose. However, the expression level varied between individual cells in the population. For example, *GAL1* expression ranged between 0 and 40 mRNAs per cell with a mean of 9.2 transcripts (Fig. 2b, right histogram). *GAL10* expression in the same population ranged between 0 and 40 mRNAs per cell with a mean of 7.6 (Fig. 2b, top histogram). Notably, we found that *GAL1* and *GAL10* transcript levels within the same cell were highly correlated (Fig. 2b, heat map). A correlation coefficient r of 0.88 ± 0.01 was calculated from the joint distribution of *GAL1* and *GAL10* mRNAs per cell. The same pairwise measurement between *GAL7* and *GAL1*, two genes with distinct Gal4p binding sites,

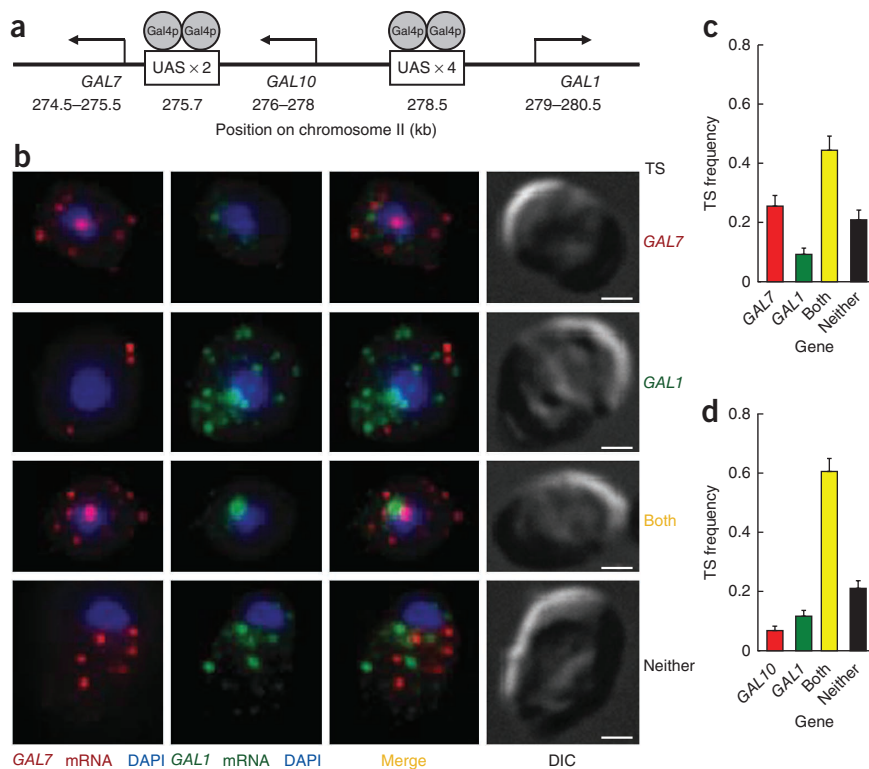
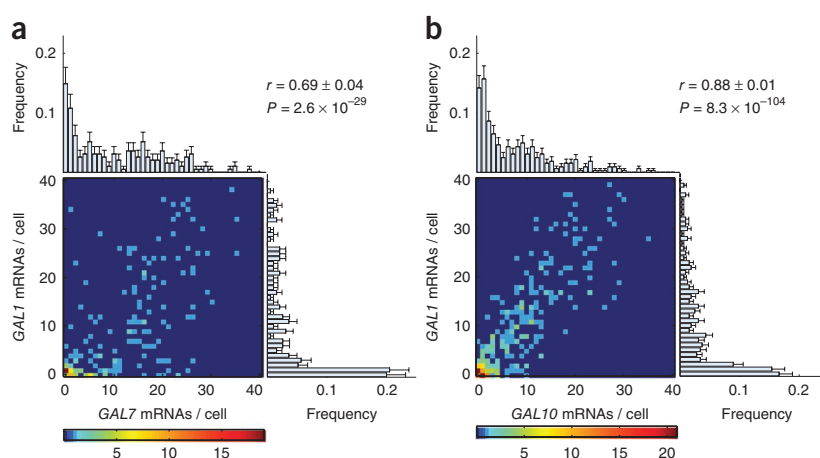


Figure 1 Highly coordinated transcription of genes in the galactose network. (a) Schematic diagram of the organization of three GAL genes and their promoters on chromosome II. (b) Nascent transcripts at the transcription site (TS) in the nucleus and individual transcripts in the cytoplasm detected with single-mRNA FISH. *GAL7* mRNA (red) and *GAL1* mRNA (green) were detected in the same cell with cyanine 3- and cyanine 3.5-labeled probes, respectively. DAPI (blue) was used to demarcate the nucleus. Differential interference contrast (DIC) images are shown in the last column. The scale bars are 1 μ m. (c) Fraction of cells showing each of the four different modes of transcription shown in b. Fraction of 196 cells with active transcription sites for only *GAL7* (red), only *GAL1* (green), both genes (yellow) and neither gene (black). (d) The same pairwise analysis of *GAL10* and *GAL1* transcription sites in 309 cells. Error bars indicate s.e.m.

Figure 2 Correlation between cytoplasmic mRNA abundance of GAL genes in individual cells. **(a)** Heat map of number of *GAL7* and *GAL1* mRNAs in 195 individual cells. The color of each point indicates the number of cells observed at that value as specified by the color bar at the bottom. The marginal histograms represent the frequency of *GAL7* mRNAs per cell (top) and *GAL1* mRNAs per cell (right) across the entire population. The expression of *GAL7* (top) ranged between 0 and 40 mRNAs per cell with a mean (μ_{GAL7}) of 10.6 ± 0.7 and a s.d. (σ_{GAL7}) of 9.9 transcripts. The expression of *GAL1* (right) ranged between 0 and 40 mRNAs per cell with $\mu_{GAL1} = 9.0 \pm 0.7$ and $\sigma_{GAL1} = 11.1$. The correlation (r) between transcripts of these two genes in the same cell was 0.69 ± 0.04 . **(b)** Pairwise correlation between the number of *GAL10* and *GAL1* transcripts in 325 cells. Marginal histograms: $\mu_{GAL10} = 7.6 \pm 0.5$, $\sigma_{GAL10} = 8.1$ (top); $\mu_{GAL1} = 9.2 \pm 0.5$, $\sigma_{GAL1} = 10.4$ (right). Error bars indicate s.e.m.



yielded a slightly lower r value (0.69 ± 0.03) (Fig. 2a). The lower correlation was consistent with a slightly lower probability of both promoters actively transcribing at the same time. Nevertheless, coordinate activation of transcription sites in the nucleus and high correlation coefficients between cytoplasmic mRNAs indicate that expression of GAL transcripts is highly coordinated in individual cells.

Anti-correlated mRNAs of cell cycle stage-regulated genes

Progression through the cell cycle requires orchestrated expression of specific proteins at well-defined time intervals. Many genes have been shown by ensemble measurements to be transcribed only within specific windows during the cell cycle²⁹. We therefore expected that within single cells, genes expressed during different stages of the cell cycle would be anti-correlated: that is, their expression would

be mutually exclusive. We measured pairwise correlations between mRNA abundance for a network of cell cycle stage-regulated genes (Fig. 3a). The expression of transcriptional activator *NDD1* peaks during the S phase and is essential for expression of its target genes, *SWI5* and *CLB2*, during the G2/M phase^{30,31}. To measure the expression profiles of these genes, we used differential interference contrast (DIC) images to divide 503 asynchronous cells into three different cell cycle stages based on morphology: G1, S and G2/M. As expected, *SWI5* and *CLB2* expression is off during most of the cell cycle but peaks sharply during the G2/M phase. *NDD1* expression, on the other hand, is broader and peaks during the S phase (Fig. 3b). Because the expression of *NDD1* and its target genes peak during different stages of the cell cycle, we expected the number of *NDD1* mRNAs to be anti-correlated with the numbers of *SWI5* or *CLB2* mRNAs within the same

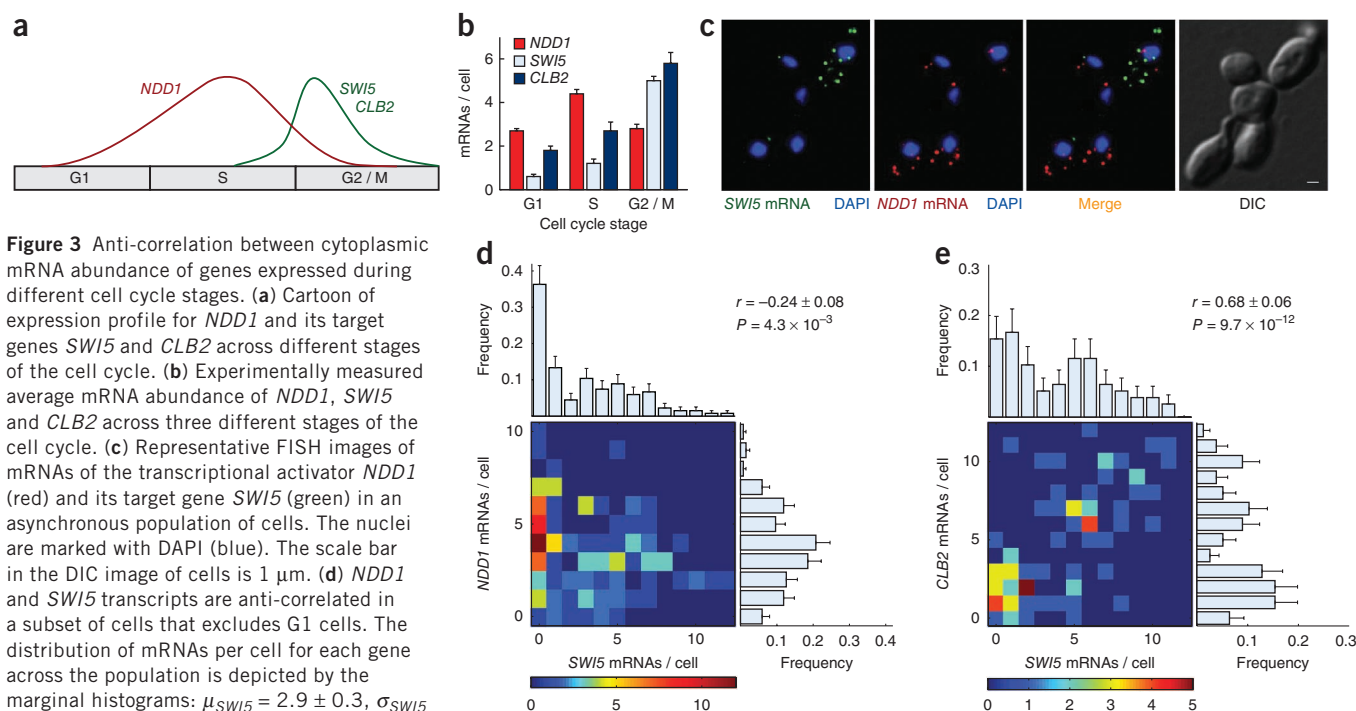


Figure 3 Anti-correlation between cytoplasmic mRNA abundance of genes expressed during different cell cycle stages. **(a)** Cartoon of expression profile for *NDD1* and its target genes *SWI5* and *CLB2* across different stages of the cell cycle. **(b)** Experimentally measured average mRNA abundance of *NDD1*, *SWI5* and *CLB2* across three different stages of the cell cycle. **(c)** Representative FISH images of mRNAs of the transcriptional activator *NDD1* (red) and its target gene *SWI5* (green) in an asynchronous population of cells. The nuclei are marked with DAPI (blue). The scale bar in the DIC image of cells is 1 μ m. **(d)** *NDD1* and *SWI5* transcripts are anti-correlated in a subset of cells that excludes G1 cells. The distribution of mRNAs per cell for each gene across the population is depicted by the marginal histograms: $\mu_{SWI5} = 2.9 \pm 0.3$, $\sigma_{SWI5} = 2.9$ (top); $\mu_{NDD1} = 3.6 \pm 0.3$, $\sigma_{NDD1} = 2.1$ (right). **(e)** *SWI5* and *CLB2* mRNAs, expressed during the same cell cycle stage, are highly correlated in a subset that excludes G1 cells. Marginal histograms: $\mu_{SWI5} = 4.1 \pm 0.4$, $\sigma_{SWI5} = 3.4$ (top); $\mu_{CLB2} = 4.7 \pm 0.4$, $\sigma_{CLB2} = 3.4$ (right). Error bars indicate s.e.m.

cell. On the other hand, we predicted that mRNA levels of the transcription factors *SWI5* and cyclin *CLB2* would be highly correlated because their expression peaks during the same cell cycle stage.

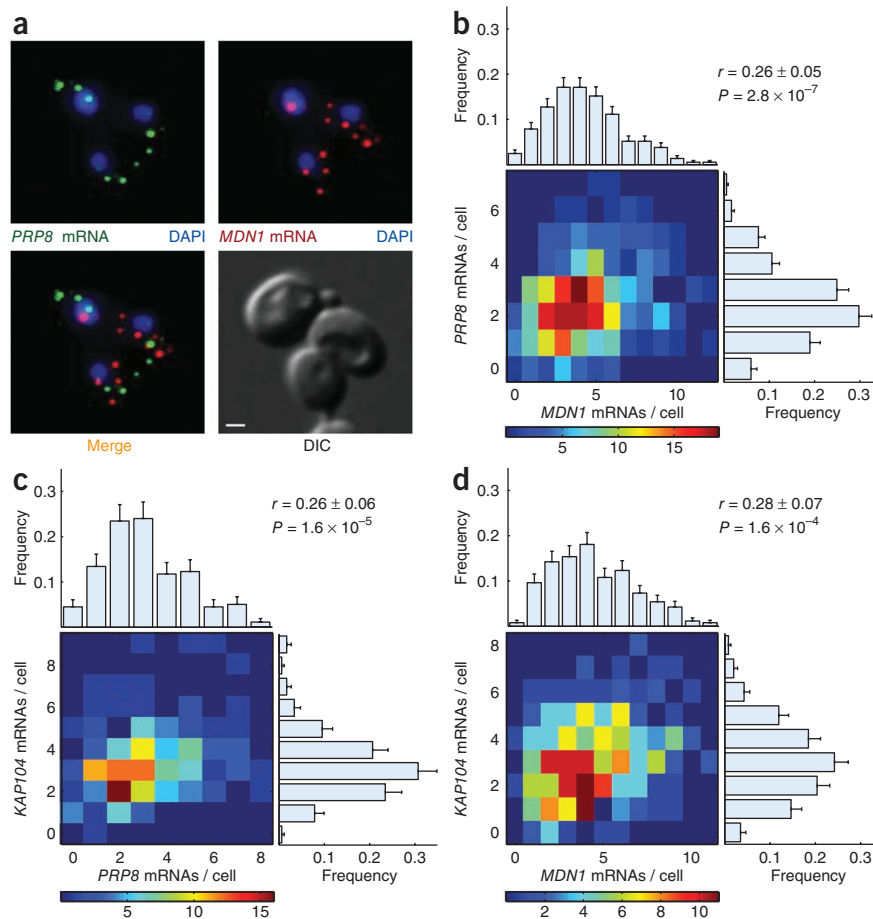
Figure 3c shows representative FISH images, with some cells in G1, where transcription of both *NDD1* and *SWI5* is essentially off, and other cells in stages (S to M), where they are expressing either *NDD1* or *SWI5*. Transcript distributions for cells in G1 showed that *NDD1* expression ranged between 0 and 8 mRNAs per cell and *SWI5* ranged between 0 and 11 mRNAs per cell, with more than 90% of cells expressing only 0 or 1 mRNA (**Supplementary Fig. 1a,b**). Because mRNAs of these genes are not expressed during the G1 phase of the cell cycle, we used DIC images to exclude unbudded G1 cells from our analysis. As expected, mRNA levels of *NDD1* and *SWI5* in the remaining cells were weakly anti-correlated ($r = -0.26 \pm 0.08$) (**Fig. 3d**). For comparison, we used the same approach to measure the pairwise correlation between *SWI5* and *CLB2*, two target genes of *NDD1* that are activated during the same cell cycle stage. Pairwise measurements between *SWI5* and *CLB2* showed similar expression profiles for both genes (**Fig. 3e** and **Supplementary Fig. 1b,c**). Moreover, their transcript abundances in individual cells were highly correlated ($r = 0.68 \pm 0.06$), as expected.

These experiments show that mRNA expression can be highly correlated or anti-correlated within single cells, and they confirm that counting of single mRNAs provides a very precise approach for quantifying a wide range of coordination in transcript abundance.

Weakly correlated functionally unrelated constitutive genes

After validating our method with genes whose abundance was expected to be positively or negatively correlated, we turned our attention to a common class of genes, the housekeeping genes. Previous single-cell measurements of mRNA abundance for constitutive genes have shown that cell-to-cell variation can be described by a Poisson distribution and arises from intrinsically stochastic fluctuations in transcription initiation²⁴. However, the extent to which these random fluctuations affect a cell's ability to coordinate mRNA levels of multiple genes, and of the entire transcriptome in general, is not known.

Figure 4 Correlation between cytoplasmic mRNA abundance of functionally unrelated constitutively active genes. **(a)** Representative FISH images of mRNAs of two functionally unrelated genes, *PRP8* (green) and *MDN1* (red), are shown along with the DIC image of cells. The nuclei are marked with DAPI (blue). The scale bar is 1 μm . **(b)** Heat map of number of *MDN1* and *PRP8* transcripts in 369 cells. The correlation (r) between transcripts of these two genes in the same cell was 0.26 ± 0.05 . The distribution of mRNAs per cell for each gene across the population is depicted by the marginal histograms: $\mu_{MDN1} = 4.3 \pm 0.1$, $\sigma_{MDN1} = 2.4$ (top); $\mu_{PRP8} = 2.5 \pm 0.1$, $\sigma_{PRP8} = 1.4$ (right). **(c)** Pairwise correlation between *PRP8* and *KAP104* in 179 cells. Marginal histograms: $\mu_{PRP8} = 3.1 \pm 0.2$, $\sigma_{PRP8} = 1.8$ (top); $\mu_{KAP104} = 3.3 \pm 0.2$, $\sigma_{KAP104} = 1.5$ (right). **(d)** Correlation between *MDN1* and *KAP104* in 260 cells. Marginal histograms: $\mu_{MDN1} = 4.4 \pm 0.1$, $\sigma_{MDN1} = 2.4$ (top); $\mu_{KAP104} = 3.1 \pm 0.1$, $\sigma_{KAP104} = 1.6$ (right). Error bars indicate s.e.m.

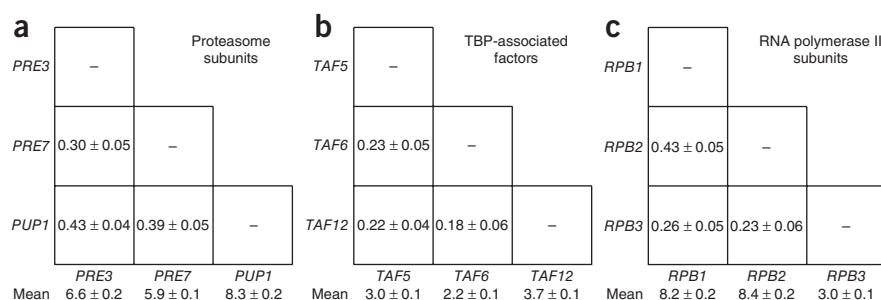


We began by measuring pairwise correlation coefficients between mRNAs of three functionally unrelated constitutive genes: *MDN1* (ribosome biogenesis), *PRP8* (pre-mRNA splicing) and *KAP104* (nucleocytoplasmic transport). Representative FISH images of *MDN1* and *PRP8* mRNAs within single cells are shown in **Figure 4a**. The three genes show similar expression profiles, with variation that can be described by a Poisson distribution, consistent with previously described uncorrelated transcription initiation of constitutive genes (**Fig. 4b–d**, histograms on top and right)²⁴. As such, we predicted that transcript levels of these unrelated genes, without any known regulatory pathways in common, would be essentially uncorrelated ($r \sim 0$). Indeed, we observed a weak correlation ($r = 0.26 \pm 0.05$) between the number of *MDN1* and *PRP8* transcripts in a cell (**Fig. 4b**, heat map). Pairwise comparison of these two genes against *KAP104* also yielded the same result (**Fig. 4c,d**). These results suggest that global or extrinsic factors lead to weak correlations between transcripts of functionally unrelated constitutive genes within a cell.

Functionally related genes are only weakly correlated

Previous studies have suggested that optimal complex assembly depends on the presence of equal levels of protein subunits in a cell⁷. Furthermore, proteins in the same complex or pathway tend to have similar mean abundances and lower variability between individual cells^{8,18,20,21}. This would suggest that mRNA expression of genes encoding subunits of multi-protein complexes should also be coordinated to facilitate efficient complex assembly. However, whether transcript levels of constitutively expressed functionally related genes within a cell are more correlated than those of functionally unrelated genes is not known.

Figure 5 Correlation between cytoplasmic mRNA abundance of essential genes encoding subunits of multi-protein complexes. (a) Mean abundance and pairwise correlation coefficients for transcripts of three genes encoding β -subunits of the proteasome 20S core particle. (b) Correlation coefficients for three genes encoding TATA binding protein associated factors involved in transcription initiation. (c) Correlation between three genes encoding subunits of RNA polymerase II. Errors indicate s.e.m.



For comparison, we measured pairwise correlations among several groups of genes encoding subunits of multi-protein complexes (Fig. 5). The complexes we investigated were constitutive and essential and required rigid stoichiometry between subunits. For all genes, the variance of transcript distributions was equal to the mean transcript abundance, which is characteristic of fluctuations due to uncorrelated stochastic processes. Surprisingly, mRNAs of genes encoding β -subunits of the stable proteasome core complex were no more correlated than mRNAs of functionally unrelated genes (Fig. 5a and Supplementary Fig. 2). TBP-associated factor (TAF) genes encoding subunits of the general transcription factor TFIID, essential for initiating RNA polymerase II transcription, also showed pairwise correlation coefficients in the same range (Fig. 5b and Supplementary Fig. 3). Finally, transcripts of three genes (*RPB1*, *RPB2* and *RPB3*) encoding core subunits of RNA polymerase II were only weakly correlated, just like functionally unrelated genes (Fig. 5c and Supplementary Fig. 4). These results suggest that coordination of both functionally related and unrelated genes is subject to a balance between two opposing processes: global factors simultaneously affecting all constitutively active genes in a cell (correlated process) and stochastic fluctuations independently affecting individual genes (uncorrelated process).

One alternative possibility is that the lack of strong correlation between mRNAs of functionally related genes is due not to stochastic fluctuations, but rather to gene-specific differences in regulation. Although this is an unlikely possibility, because genes such as *PRE3* and *PUP1* are only weakly correlated despite being regulated through a common transcriptional activator Rpn4p, the results thus far do not explicitly rule it out^{32,33}.

Two alleles of the same gene are also weakly correlated

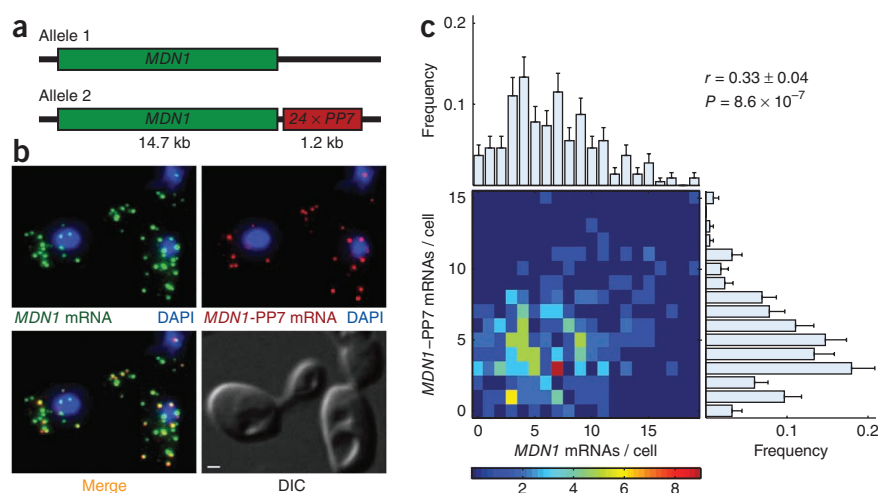
To determine whether two genes dependent on the same transcription factor are any more correlated in their expression than two unrelated

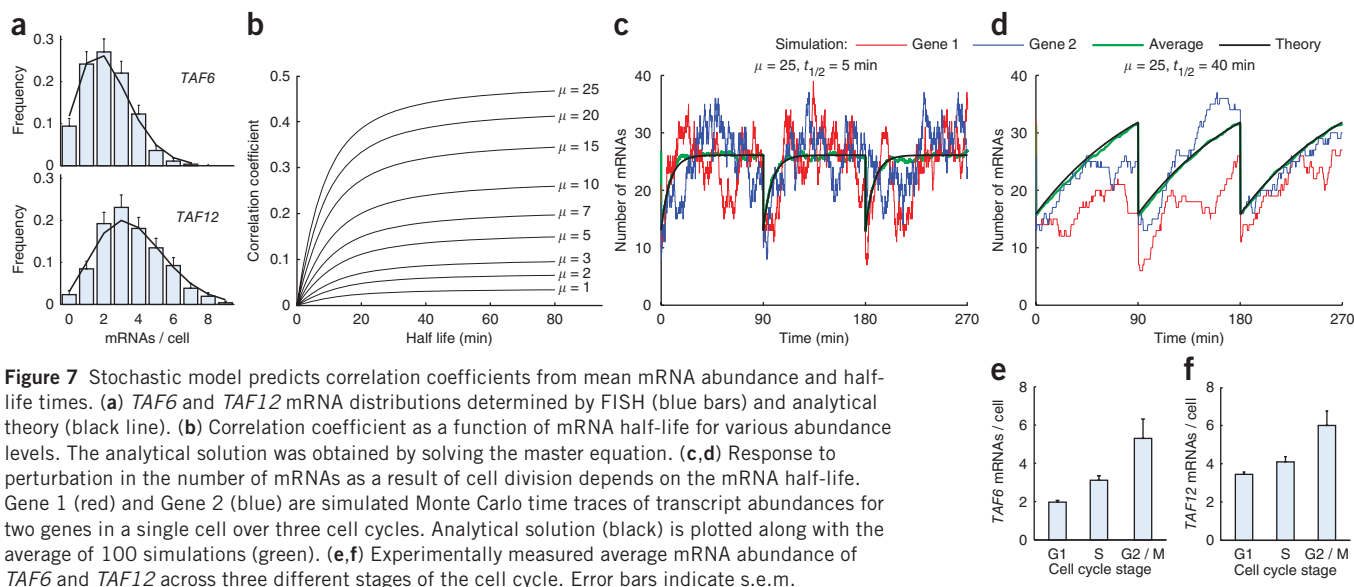
genes, we measured the correlation between transcripts produced by each allele of *MDN1* in diploid cells. The two endogenous alleles have identical promoters and would be affected identically by gene-regulatory signals within the same cell. However, stochastic fluctuations in the transcription of each allele are independent and would lead to differences in expression between the two alleles. To distinguish between transcripts from the two alleles, we inserted RNA hairpins from bacteriophage PP7 in the 3' untranslated region of one of the two *MDN1* alleles (Fig. 6a). Whereas *MDN1* coding sequence probes would hybridize to transcripts from both alleles, the probes for RNA hairpins would only hybridize to transcripts from one of the two alleles (Fig. 6b).

The expression of each *MDN1* allele in diploid cells was similar to previously reported measurements²⁴ from haploid cells (Supplementary Fig. 5). Each allele expressed between 1 and 15 mRNAs per cell, with a mean around 5 transcripts (Fig. 6c, histograms on top and right). In the absence of intrinsic fluctuations, a cell would have an equal number of transcripts from each allele ($r = 1$). However, constitutive genes in yeast are subject to stochastic fluctuations, leading to uncoordinated transcription initiation at each allele. As a result, we found only a weak correlation ($r = 0.33 \pm 0.06$) between transcripts from two alleles of *MDN1* (Fig. 6c, heat map). In summary, mRNA levels of unrelated genes, functionally related genes and even two alleles of the same gene with identical promoters are only weakly correlated.

To verify that this observation reflects stochastic fluctuations in the transcriptional activity of a gene and not another process (for example, mRNA decay), we also measured the distribution of nascent mRNAs at the transcription site (Supplementary Fig. 6). Indeed, the number of nascent mRNAs present at the transcription site for functionally related genes was not correlated any more than for functionally unrelated genes (Supplementary Fig. 7).

Figure 6 Correlation between transcripts from two alleles of a constitutively active gene, *MDN1*, in diploid cells. (a) Schematic diagram of the PP7 array inserted in the 3' untranslated region of one of the two endogenous *MDN1* alleles. (b) Transcripts from both alleles were detected with cyanine 3-labeled probes hybridizing to the coding region of *MDN1* (green). Transcripts from Allele 2 (yellow) were distinguished with colocalizing signals from cyanine 3.5-labeled probes against 11 binding sites in the 24 × PP7 array (red). The scale bar in the DIC image is 1 μ m. (c) Heat map of number of transcripts from two *MDN1* alleles in 217 diploid cells. The correlation coefficient (r) between transcripts from two alleles in the same cell was 0.33 ± 0.04 . The distribution of mRNAs per cell for each allele across the population is depicted by the marginal histograms: $\mu_{MDN1} = 6.6 \pm 0.3$, $\sigma_{MDN1} = 4.0$ (top); $\mu_{MDN1-PP7} = 4.8 \pm 0.3$, $\sigma_{MDN1-PP7} = 2.8$ (right). Error bars indicate s.e.m.





Modeling reveals that weak correlations arise from cell division

To understand the source of weak correlations, we modified the mathematical framework based on a gene activation and inactivation model to obtain an exact solution for joint mRNA distributions^{34,35}. In this model, a gene randomly switches between an active 'on' state and an inactive 'off' state, likely corresponding to chromatin modifications³⁶. Because our investigation was limited to genes transcribing 'constitutively' (independent initiations distributed in time), rather than in bursts (multiple initiations during infrequent on states), we assumed that genes were always in the on state. We validated this assumption by quantifying the number of nascent mRNAs as a direct measure of transcriptional activity for all genes considered in this study²⁴ (**Supplementary Fig. 6**). Accordingly, in our model, individual transcripts initiate independently and with a constant probability over time. Two variable parameters needed to describe the mRNA distribution of each gene, initiation rate (k_i) and decay rate (k_d), were calculated from the experimentally measured mean transcript number (μ) and previously reported half-life measurements ($t_{1/2}$), respectively (**Supplementary Table 1**). In addition, a binomial process was used to divide transcripts from the mother cell between two daughters at cell division³⁷.

We used this framework to obtain exact analytical solutions for mRNA distributions and pairwise correlations between different genes in a cell by solving the master equation (see Online Methods). As an example, **Figure 7a** shows that distributions predicted by our model (black line) are in excellent agreement with measured distributions for *TAF6* and *TAF12* mRNAs (blue bars). Our model predicted an r value of 0.1 between *TAF6* and *TAF12* mRNAs within the same cell, consistent with the experimentally measured r value of 0.18 ± 0.06 . Next, we used our model to calculate pairwise correlation coefficients for a wide range of mRNA mean and half-life times (**Fig. 7b**). We found that the correlation between mRNAs of constitutive genes increases with mean abundance. Furthermore, longer half-life buffers the mRNA abundance in a cell against fluctuations, leading to a higher correlation.

Next, we did Monte Carlo simulations with a fixed transcript mean but different half-life times. **Figure 7c** shows the simulated time traces for two genes (red and blue lines) with a mean of 25 transcripts per cell and half-life of 5 min. The average of 100 simulated time traces (green line) is plotted along with the exact analytical solution (black line)

to the master equation (see Online Methods). The results show that transcripts with short half-lives reach their steady-state value (k_i/k_d) soon after cell division. On the other hand, the time constant ($1/k_d$) to reach steady-state transcript levels is much longer for two genes with longer half-lives ($t_{1/2} = 40$ min) (**Fig. 7d**). As a result, mean transcript levels of both genes are moving toward their steady-state values during the entire 90-min cell cycle. To verify that mean mRNA abundance increases with time during the cell cycle, we divided the cells into three different cell cycle stages based on morphology: G1, S and G2/M. As expected, the mean mRNA abundance increased as cells progressed through the cell cycle (**Fig. 7e,f** and **Supplementary Fig. 8**).

Because our model suggests that the observed correlations are simply due to the synchronizing effects of cell division, we predicted that correlations would decrease in cells with extremely long cell cycles. To test this prediction, we measured the correlation coefficient between *MDN1* and *PRP8* in cells with a doubling time of 14 h (**Supplementary Fig. 9**). The cells were grown in a chemostat in minimal medium supplemented with limiting concentrations of glucose to achieve the desired doubling time³⁸. As predicted by our model, *MDN1* and *PRP8* mRNA levels were uncorrelated ($r = 0.05 \pm 0.05$) in these cells, in contrast to the weak correlation ($r = 0.26 \pm 0.05$) observed in cells with a 90-min cell cycle (**Fig. 4b** and **Supplementary Fig. 9b**).

In summary, our model shows that cell division is a global factor that affects transcripts of all genes by perturbing them from their steady state levels. After each cell division, transcripts of all genes begin to accumulate until their abundance, on average, doubles before the next division (**Fig. 7c–f** and **Supplementary Fig. 8**). Notably, weak correlations between transcripts of functionally related or unrelated genes arise from the fact that in an asynchronous population, some cells at the beginning of the cell cycle have fewer transcripts compared to other cells near the end of the cell cycle. Beyond this effect of sampling an asynchronous population on the measured correlation, we do not observe any coordination in the expression of functionally related or unrelated genes.

DISCUSSION

In this study, we have combined single-mRNA counting with mathematical modeling to provide fundamental insights into how an individual cell accomplishes what is thought to be one of its most crucial tasks—coordinating gene expression.

Our results reveal that cells transcribe temporally induced genes in a highly coordinated manner. Although there was a large variation in the magnitude of response to galactose between individual cells, the transcript levels of GAL genes within a cell were highly correlated (Fig. 2). These results confirm that measurements at the mRNA level are consistent with those obtained in studies where reporter proteins were used to show that variation in protein levels of induced genes is largely due to cell-to-cell differences in common upstream regulators^{16,17,39}. Moreover, the correlation between transcripts of GAL genes within individual cells was independent of the galactose concentration used for induction (Supplementary Fig. 10). A recent assay for quantifying nucleosome occupancy showed that promoter activation upon galactose induction corresponds to the removal of nucleosomes flanking the UAS of GAL genes and coincides with recruitment of the transcriptional machinery to GAL promoters⁴⁰. We note that a slightly lower correlation between *GAL1* and *GAL7* compared to *GAL1* and *GAL10*, despite common upstream regulation, most likely underscores the importance of chromatin remodeling³⁵. If two promoters were activated independently, the probability of both promoters being 'on' would equal the product of their individual probabilities. However, the probability of a cell transcribing both *GAL1* and *GAL10* is higher than the product of their individual probabilities (Fig. 1c). This result is consistent with the fact that the rate-limiting step of activating the *GAL1* and *GAL10* promoters through nucleosome removal is mediated by a single UAS common to both promoters. On the other hand, the probability of *GAL1* and *GAL7* switching on together is slightly lower, because their promoters are activated independently through derepression of Gal4p at similar but distinct UAS. In order to decouple the *GAL1* and *GAL10* promoters, we introduced independent rate-limiting steps in the activation of these two genes. In wild-type cells, histone H2A variant H2A.Z destabilizes the +1 and -1 promoter nucleosomes and is thought to promote gene activation by exposing the transcription start site⁴¹. We found that deletion of *HTZ1*, the gene encoding H2A.Z, led to decreased expression of *GAL1* and *GAL10* and reduced the correlation between these two genes to a value closer to the correlation between *GAL1* and *GAL7* (Supplementary Fig. 11). These results suggest that common upstream regulation through transcription factors as well as chromatin structure provides a robust way to maintain equal numbers of transcripts for these genes, regardless of induction conditions.

Unlike induced genes, which are activated synchronously during a well-defined time interval by an upstream signal, the transcription of constitutive genes is achieved by independent initiation events with a constant probability over time. Notably, even transcripts from two endogenous alleles of *MDN1*, with identical promoters, were uncorrelated after accounting for the synchronizing effects of cell division (Fig. 6c). Moreover, transcripts of several classes of functionally related and unrelated constitutive genes in individual cells were uncorrelated (Figs. 4b–d and 5). These results show that individual cells are unable to coordinate the expression of constitutive genes because of inherently stochastic fluctuations in transcription initiation.

A simple model with only two free parameters is sufficient to describe mRNA variation for constitutive genes in yeast (Fig. 7a). We note that our model slightly underestimates the experimentally measured correlation coefficients. More accurate assessment of transcript half-lives would improve these predictions. It is also possible that the discrepancy arises from the fact that our model assumes transcription to be a homogenous Poisson process and does not account for gene duplication before cell division. Nevertheless, our model confirms that weak correlations between constitutive genes within a

wide range of transcript means and half-lives reflect the lower limit of extrinsic variability due to cell growth and division¹⁷ (Fig. 7b).

How, then, are cells able to carry out complex functions in a predictable and coordinated manner when the transcriptional output of constitutive genes is essentially random? It has been suggested that in higher eukaryotes, fluctuations in mRNA levels are filtered out at the protein level by long protein half-lives^{35,42}. In yeast, however, the average protein half-life is only twice as long as the average mRNA half-life^{43,44}. Therefore, protein half-lives only partially explain the low variation observed for functionally related proteins^{8,18,20,21}. There are several passive and active means to achieve predictable outcomes from a stochastic system. It is, in fact, possible to build a multi-protein complex in a predictable amount of time even if the abundance of each of its subunits varies substantially. Whereas the duration of each binding step might vary because of fluctuations in protein quantities, these fluctuations average out when they are added sequentially to produce the full complex. More generally, any biological process can be passively rectified against stochastic fluctuations, because the central limit theorem predicts that variability in the total duration of a process decreases with increasing number of intermediate steps.

There are also active models that could compensate for the lack of coordination in mRNA abundance. One possibility is that in order to yield predictable outcomes, cells impose checkpoints until all conditions for further progress are satisfied. Assembly of proteasomes, for example, is guided by various chaperones that ensure correct incorporation of each subunit in a specific order^{45,46}. Chaperones could also act to stabilize the intermediate complexes and ensure that they do not dissociate while 'waiting' for the next subunit. In this way, cells can guarantee a predictable outcome, but not the time it takes to achieve it.

Post-transcriptional gene regulation might also play an important role in optimizing the expression of each subunit for efficient assembly of complexes. Efficient regulation requires fast responses to transient variations in protein levels. Therefore, it seems reasonable to control protein abundance by tuning the latest possible step of the production process. Post-transcriptional or even post-translational regulation would provide much quicker responses compared to initiating the much longer process of transcription. RNA binding proteins have been implicated in coordinated regulation of many post-transcriptional steps in the expression of functionally related genes^{47–49}. Indeed, genes that encode subunits of stoichiometric complexes are thought to have similar transcript and protein decay rates^{43,44}.

Our perception of transcription has been influenced over the last half century by bacterial models where gene activity is regulated by its end product. Since the discovery of the lac operon in *Escherichia coli*, genes have been viewed as finely tuned thermostats that constantly sense and counter changes in the environment with a precisely coordinated response⁵⁰. Although there are examples of highly regulated gene networks in various organisms that support this view, it certainly cannot be generalized to constitutive genes. The experimental and modeling results presented here suggest that execution of gene expression programs, particularly at the level of mRNA, is not always precisely coordinated. Many constitutive genes in yeast are essentially clueless entities that produce transcripts with a constant probability over time, irrespective of the necessary concentrations of the final gene product. Whether genes can sense and regulate their end product or whether they act autonomously leads to profoundly divergent modes of transcription, and hence assembly, of essential complexes. The results presented here suggest a fundamental shift in the way we must think about coordination of biological processes within a cell. Cells have evolved very simple modes of gene expression that require much

less coordination than previously thought. Therefore, the regulation of precise stoichiometry must occur post-transcriptionally, and likely post-translationally. Determining the level of post-transcriptional control for many of these genes will show whether active processes further regulate the expression of genes encoding protein complexes or if the downstream processes are just as 'clueless' as transcription.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/nsmb/>.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

S.J.G. and D.Z. initiated the project. S.J.G. conducted the experiments and data analysis. S.J.G. and T.L. did the numerical simulations and T.L. derived the analytical solution. D.Z. and R.H.S. supervised the project. S.J.G. wrote the paper with editorial help from D.Z., T.L. and R.H.S.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cell culture. Yeast cells (w303 haploid or diploid) were grown in YPD medium at 30 °C to an optical density at 600 nm (OD_{600}) of 0.5. For galactose induction, cells were grown in yeast extract, peptone and 2% (w/v) raffinose at 30 °C to OD_{600} of 0.5. The cells were then induced by adding 20% (w/v) galactose to the cell culture to a final concentration of 2% (w/v) for 15 min.

PP7 strain creation. An array of 24 RNA hairpins from bacteriophage PP7, the kanamycin-resistance gene for selection, and the CYC terminator were inserted in the 3' untranslated region of one of the *MDN1* alleles in diploid w303 yeast cells by homologous recombination.

Δ HTZ1 strain creation. The 405-bp open reading frame of the *HTZ1* gene was replaced with a kanamycin-resistance gene in haploid w303a yeast cells by homologous recombination.

In situ probes. Five or six oligodeoxynucleotide probes for each gene were designed, synthesized and labeled as described previously²². Each probe was 50–53 nt long and contained five amino-modified nucleotides (amino-allyl T). The free amines were chemically coupled to cyanine 3 or cyanine 3.5 fluorescent dyes after synthesis. The sequences for probes used to detect the mRNA of genes in this study are provided in **Supplementary Methods**.

Fluorescence in situ hybridization. Multiplexed FISH was used according to the procedure outlined previously²⁴. Cells were fixed by adding 32% (v/v) paraformaldehyde to the culture to a final concentration of 4% (v/v) for 45 min at room temperature. After the fixative was washed away, the cell wall was digested with lyticase (Sigma). The cells were then attached to poly-L-lysine (Sigma)-coated coverslips and stored in 70% (v/v) ethanol at –20 °C. Stored coverslips were rehydrated and inverted onto 20 μ l of hybridization solution containing a mixture of probes for two genes, one labeled with cyanine 3 and the other with cyanine 3.5. The cells were hybridized overnight at 37 °C and washed. The nuclei were stained with DAPI and the coverslips were then mounted with ProLong Gold antifade reagent (Invitrogen).

Image acquisition. Images were acquired on an Olympus BX61 epifluorescence microscope with a UPlanApo 100 \times , 1.35 numerical aperture oil-immersion objective (Olympus). An X-Cite 120 PC (EXFO) light source was used for illumination, with filter sets 31000 (DAPI), 41001 (autofluorescence), SP-102v1 (Cy3) and SP-103v1 (Cy3.5) (Chroma Technology). Vertical stacks of 30 images with a z step size of 0.2 μ m were acquired using a CoolSNAP HQ camera (Photometrics)

with a 6.4 μ m pixel size CCD. IPLab (BD Biosciences) software platform was used for instrument control as well as image acquisition.

Data analysis. Three-dimensional image stacks were reduced to two-dimensional images by maximum intensity projection along the z axis. A previous implementation of the Gaussian mask algorithm in IDL (ITT Visual Information Solutions) was used to compute the location and intensity of diffraction-limited fluorescence signals from individual mRNAs. Cellular boundaries were defined by a hand-drawn mask, and nuclei were segmented by thresholding the DAPI signal in IPLab. Outputs from the Gaussian mask and segmentation algorithms were combined with custom made software in IDL to generate+ single-cell expression profiles containing the abundance, locations and signal intensities of mRNAs for each gene in a cell. The single-cell mRNA distributions were then used to calculate the correlation coefficient ($r_{x,y}$) between gene X and Y:

$$r_{x,y} = \frac{\sum_i (x_i - \mu_x)(y_i - \mu_y)}{\sigma_x \sigma_y}$$

where x_i and y_i are the mRNA abundances of genes X and Y, respectively, in cell i . μ and σ represent the means and s.d., respectively, of mRNA distributions of genes X and Y.

Mathematical modeling. A Markovian model for gene expression based on a random birth-and-death process has been described previously³⁴. The model has been used to calculate steady-state mRNA distributions in mammalian cells as well as yeast^{24,35}. We modified this model to account for binomial partitioning of mRNAs at cell division and obtained mRNA abundances for multiple genes in the same cell.

We obtained an exact analytical solution for the time-dependent mRNA distributions in a cell by solving the master equation (see **Supplementary Methods**). The mRNA abundance at any given time follows a Poisson distribution with a mean that varies over the cell cycle⁵¹ (**Fig. 7c,d**). We then obtained a time-averaged distribution of mRNA abundance for each gene to describe the experimentally measured mRNA distributions (**Fig. 7a**). The time-averaged mRNA distributions were used to calculate the mean, variance, covariance and correlations for mRNAs of genes with various sets of k_i and k_d parameters (**Fig. 7b**). Simulated time traces for mRNA abundance and pairwise correlations between different genes in a cell were obtained from Monte Carlo simulations done in Matlab 7.0.1 (The Mathworks).

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