Phase locking and multiple oscillating attractors for the coupled mammalian clock and cell cycle

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Daily synchronous rhythms of cell division at the tissue or organism level are observed in many species and suggest that the circadian clock and cell cycle oscillators are coupled. For mammals, despite known mechanistic interactions, the effect of such coupling on cell cycle progression and, hence its biological relevance, is not understood. In particular, we do not know how the temporal organization of cell division at the single-cell level produces this daily rhythm at the tissue level. Here we use multispectral imaging of single live cells, computational methods, and mathematical modeling to address this question in proliferating mouse fibroblasts. We show that in unsynchronized cells the cell cycle and circadian clock robustly phase lock each other in a 1:1 fashion so that in an expanding cell population the two oscillators oscillate in a synchronized way with a common frequency. Dexamethasone-induced synchronization reveals additional clock states. As well as the low-period phase-locked state there are distinct coexisting states with a significantly higher period clock. Cells transition to these states after dexamethasone synchronization. The temporal coordination of cell division by phase locking to the clock at a single-cell level has significant implications because disordered circadian function is increasingly being linked to the pathogenesis of many diseases, including cancer.

Significance

Most organisms adapt their physiology and behavior to daily environmental cycles by means of endogenous circadian clocks. In mammals, the circadian timekeeping system involves a master pacemaker in the suprachiasmatic nuclei that coordinates peripheral oscillators in each cell of most organs and tissues. The core mechanism governing all these clocks is a self-sustained time-delayed transcriptional/posttranslational negative feedback loop relying on clock genes (1). This genetic oscillator conveys circadian rhythmicity to physiological outputs through the regulation of a substantial and tissue-specific set of target genes or proteins.

Several critical cell cycle components have recently been found to be clock-controlled. For instance, in mice the circadian clock regulates the cyclin-dependent kinase inhibitors p16 and p21, the G2/M kinase Wee1, as well as the checkpoint proteins CHK1 and 2, and genetic disruption of any of these links compromises cellular proliferation (2–5). Although these molecular links provide a partial mechanistic basis for the coupling between these oscillators, the consequences for the joint dynamics are far from clear, as is the extent to which the cell cycle is coordinated by the clock, and vice versa.

One-to-one phase locking of oscillators is a well-known phenomenon where two coupled oscillators have a fixed relative phase and thus oscillate with a common frequency (6). A necessary condition for two oscillators to lock in this way is for their natural frequencies, when uncoupled, to be close and for them to be coupled strongly enough. Therefore, it is reasonable to expect that functional links as above should lead to 1:1 phase locking of the clock and cell cycle when their uncoupled periods are similar. In lower organisms, evidence of circadian coupling has been published for cyanobacteria (7, 8). Likewise, phase locking of the cell cycle of budding yeast using periodic forcing of the G1 cyclin CLN2 has been demonstrated (9). Moreover, 1:1 phase locking has been shown for mechanistically detailed mathematical and automaton models of the mammalian systems (8, 10, 11).

The evidence of circadian rhythms of cell division at the tissue or organism level (12, 13) in mammals is compatible with such 1:1 phase locking at the single-cell level but is also compatible with a model where cells may or may not divide during a circadian cycle but their division is restricted by gating. Introduced in ref. 14, gating is defined there as a control whereby there are certain clock phases in which cell division is allowed to occur and other phases in which it is forbidden, thus introducing new clock-determined checkpoints. The phase-locking and gating models are distinct because for the former, unlike the latter, in ideal noise-free systems (and approximately in stochastic systems), the two oscillators cycle in step and are synchronized over the whole cycle so that knowing the phase of one system largely determines the phase of the other.

In tissues such as bone marrow, intestinal mucosa, or regenerating liver, the daily rhythm of cell division is controlled by the cell’s circadian clock. Determining how this clock organizes important processes such as cell division, apoptosis, and DNA damage repair is key to understanding the links between circadian dysfunction and malignant cell proliferation. We show that in proliferating mouse fibroblasts there is more than one way in which the clock and cell cycle synchronize their oscillations and that one of them is the biological equivalent of the phase locking first discovered by Huygens in the 17th century when he coupled two clocks together. When phase-locked two coupled oscillators have a fixed relative phase and oscillate with a common frequency.

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Phase locking of the mammalian systems has never been reported. Indeed, two recent studies reported a lack of clock regulation of the mammalian cell cycle (15, 16). However, earlier work of Nagoshi et al. (17) suggested a dynamical link distinct from 1:1 phase locking. They demonstrated a three-peak distribution of cell division events when timed by the phase of the circadian clock. We show that the cells we study do 1:1 phase-lock provided they are left unsynchronized. Moreover, by perturbing the system with a synchronizing protocol we show that the coupled system has coexisting dynamically distinct oscillating regimes with a significantly longer clock period and these include behavior similar to that seen in Nagoshi et al. (17). A simple model of the coupled system illuminates our experimental results and enables us to integrate a puzzling set of dynamical phenomena. Finally, we discuss evidence suggesting that the regulation between the clock and cell cycle is bidirectional.

Results

We study NIH 3T3 mouse fibroblast cells cultured in regular medium (DMEM) supplemented with various concentrations of FBS to modulate cell-cycle length. Combining computational methods with single-live-cell imaging of the Rev-Erbα::Venus clock gene reporter and a fluorescent cell cycle reporter based on the fluorescence ubiquitination cell cycle indicator (FUCCI) (Movie S1) enables us to reconstruct the joint phase trajectory of the clock and cell cycle in single-cell lineages. We extract the phase of the clock and also the cell kinetics through the G1 and S/G2/M cell cycle phases (Fig. 1). Therefore, for each cell at each point in time we measure the clock phase \( \phi_c(t) \) and the cell cycle phase \( \phi_c(t) \), both of which we take to be between 0 and \( 2\pi \) (Fig. 1C and D).

Unsynchronized Cells Are 1:1 Phase-Locked. In the first study, neither the clock nor the cell cycle was experimentally synchronized to observe both oscillators as undisturbed as possible. The initial density of cells was chosen so that cells did not become confluent over the course of the experiment. The distributions of the period of the clock and the cell cycle from cells maintained in 10% FBS show mean values of 21.9 ± 1.1 h and 21.3 ± 1.3 h, respectively (Fig. 2A and SI Appendix, Fig. S2A). Increasing FBS to 15% significantly decreases both mean periods to 19.4 ± 0.5 h and 18.6 ± 0.6 h, respectively (Fig. 2A and SI Appendix, Fig. S2A).

The combined phase \( \Phi(t) = (\phi_1(t), \phi_2(t)) \) of two oscillators determines a point in the square \( 0 \leq \phi_1, \phi_2 \leq 2\pi \). This square should be regarded as a torus because phases are periodic and therefore opposite sides of the square should be identified (Fig. 2C and D). Then, as time \( t \) increases the combined phase \( \Phi(t) \) traces out a continuous curve on the torus. In noise-free coupled oscillators described by deterministic equations, 1:1 phase locking is characterized by convergence of the combined phase \( \Phi(t) \) to a closed curve \( A \) (topologically a circle) called an attractor that winds around the torus in a 1:1 fashion (Fig. 2C). It follows that the phases \( \phi_1(t) \) and \( \phi_2(t) \) of the oscillators advance in a synchronized way and thus oscillate with a common frequency. This phase-locked state persists in the presence of weak stochasticity in the sense that typical trajectories wind around the torus in a thin band about the attractor \( A \). However, occasionally the trajectory will go on an excursion where it leaves this band,
joining it again approximately advanced or delayed by half a cycle. This phase-skipping phenomenon occurs when a fluctuation in the nondeterministic elements exceeds the stability domain of the attractor. Thus, it seems to mark the boundary of the stability domain of the attractor.

Synchronizing the Circadian Clock by Dexamethasone Reveals Further Stable Oscillating States. Treatment with the glucocorticoid dexamethasone is known to exert a resetting/synchronizing effect on clocks in cultured cells through induction of Per1 (18), and we hypothesized that this might modify the coupled dynamics. Therefore, we expanded our experiments using a dexamethasone pulse to synchronize the cells in the same conditions as above except that we used both 10% and 20% FBS to compare our results with those of Nagoshi et al. (17), who used a similar dexamethasone synchronization protocol with 20% FBS. The synchronization resulted in a significant change in the coupled dynamics. When using 20% FBS we found that the cell lineages were dominated by two groups. The timing and clock phase of cell divisions in the first group clustered in such a way that they reproduced the three-peak distribution of clock phases of cell divisions seen by Nagoshi et al. (17) and had median periods for the clock and cell cycle of 27 h and 17 h, respectively (Fig. 4C), roughly a 3:2 period ratio. The cells in the second group locked 1:1 (Fig. 4C).

![Fig. 2. Phase dynamics for unsynchronized cells. (A) Histograms showing distributions of periods for both the clock (blue) and cell cycle (red). Unsynchronized cells grown in 10% FBS (mean clock period, 21.9 ± 1.1 h; mean cell-cycle period, 21.3 ± 1.3 h) and 15% FBS (mean clock period, 19.4 ± 0.5 h; mean cell-cycle period, 18.6 ± 0.6 h). (B) Phase histograms for cells in 15% FBS; mean phase of division is 3.97 ± 0.14 radians. (C) Illustration of possible trajectories in 1:1 phase-locked system, showing the situation for simulated noise-free deterministic dynamics. All trajectories apart from those starting on the repeller R converge to the attracting periodic orbit A, which is a circle winding around the torus in a 1:1 fashion. Cells starting at the close points a and b will divide approximately half a clock period apart. This is what gives rise to the phase-skipping instability described in the text. Such a change in state and the consequent change in division timing can be caused by stochasticity. (D) Estimated phase diagram from experimental data for cells in 15% FBS (SI Appendix, section 2.5). The red dashed curve shows the mean trajectory and the blue levels show the density of cells passing through a region. The arrows show the mean direction that the cells flow in near that point on the torus.](image)

![Fig. 3. Circular representations of lineage tree for unsynchronized cells in 15% FBS. Each radial segment corresponds to a cell-cycle interval we observed. Each ring corresponds to a generation of cells, starting with the first observed generation as the innermost ring. When a cell divides, its segment is split in two in the next ring outward. Because we cannot know when the first observed cell-cycle interval starts (because this time is before the start of our recording), the inner boundary is blurred. The G1 phase is drawn in red, and the S/G2/M phases are shown in blue. Clock peaks are shown as yellow bars. Times have been normalized so that all cell cycles have the same length in the plot. Thus, there is no direct correspondence between the length of a cell-cycle interval and its representation in this plot. We observe that in the unsynchronized condition the clock peaks mostly coincide with the G1-S transition.](image)
Dexamethasone-synchronized cells in 10% FBS provide an interesting contrast. Although the synchronization significantly increased the clock period to a mean of 24.2 ± 0.5 h (from 21.9 ± 1.1 h for the unsynchronized cells), the mean cell-cycle period remained low at 20.1 ± 0.94 h, about an hour less than that observed for unsynchronized cells in 10% FBS (SI Appendix, Fig. S2 A and B). The population density plot of peak times of the clock marker Rev-Erbα:Venus and cell divisions show clear cyclicity but, strikingly, with these significantly different periods (SI Appendix, Fig. S2 A and D). This behavior is clearly distinct from the 1:1 phase locking seen in the unsynchronized cells in 10% FBS (SI Appendix, Fig. S2).

To analyze these results we found it revealing to use two different clustering algorithms to identify clusters in the timing of cell division (details in SI Appendix). We then plotted the clock phase of each cell division against its time as in Fig. 4 A and B and SI Appendix, Figs. S3–S5, highlighting the clusters identified. In both clustering approaches plotting the data in this way reveals that there is clear clustering of the cell divisions, thereby demonstrating the effect of synchronization and coupling. For the dexamethasone-synchronized cells in 20% FBS the clusters fall into two groups corresponding to the two cases identified above. The distinct identities of the two groups is further validated by how the period ratios segregate (Fig. 4C and SI Appendix, Fig. S4B). Strikingly similar results were found when the dexamethasone-synchronized 20% FBS experiment was repeated (SI Appendix, Fig. S5A).

Using a simple mathematical model (Fig. 5 A and B and SI Appendix, Fig. S6A and Methods) we are able to analyze these dynamical regimes and integrate our findings. In this model the clock phase progresses at a constant speed and the cell cycle progresses at a speed that depends upon the phase of the clock in that it is slowed down if the joint phase of the clock and cell cycle is in the region of the torus shaded in Fig. 5 A and B (see SI Appendix for equations). The position of this coupling region was largely determined by using the vectorfields (Fig. 4E and SI Appendix, Fig. S7) to obtain an approximate position for the attracting periodic solution A, and the Poincaré maps (SI Appendix, Fig. S6C) to confirm the phases of cell division and G1-exit on A. Using a ratio of periods similar to the experimental ones, this model then qualitatively reproduces the observed clustering (Fig. 4A and B and 6 and SI Appendix, Figs. S4A and B, S5A, and S6).

We compare model results for ratios close to 3:2 and 5:4 to compare with the 20% FBS and 10% FBS dexamethasone-pulsed cases, respectively. In both cases the model produces clear clusters in the 2D plot but the projections onto the axes differ. The clustering for the 3:2 ratio gives a three-peak distribution when projected horizontally onto the clock phase (Fig. 6A) but for the 5:4 ratio it gives no clear peaks because the clusters no longer line up well in the horizontal direction (SI Appendix, Fig. S6B). This agrees with the experiments but note that in the 20% FBS dexamethasone-pulsed experiments only one of the two groups in Fig. 6B is populated so only half the clusters appear and they also give a three-peak distribution. Projecting onto the time axis gives the population density plot for cell divisions. Whereas for 5:4 clear peaks are found (SI Appendix, Fig. S6D), this is not the case for 3:2 (Figs. 6C), a result of the different ways the peaks line up for different ratios. This agrees with our experiments (Fig. 4 and SI Appendix, Fig. S2) and is also consistent with the report in Nagoshi et al. (17) that cell cycle progression in their experiment was not synchronized. The fact that we reproduce the complex and apparently conflicting data coming from these different dexamethasone-pulsed experiments significantly strengthens the value of this analysis.

For mathematical models of coupled oscillators, pq phase locking is a generalization of the 1:1 locking discussed above but in this case one oscillator completes exactly p cycles whereas the other completes q. This is shown for pq = 3:2 in Fig. 5B. According to the mathematical theory (6) such locking is robust but the extent of the robustness depends on the strength of the coupling and the size of p and q (when pq is expressed in lowest-order terms) and decreases very rapidly as p and q become larger. Although pq phase locking with pq > 1 is readily seen in some low-noise physical systems we cannot expect to see it in its pure form in our stochastic system. For example, the single-cell dynamics are highly stochastic; the system kinetic parameters for daughter cells will usually vary from that of the mother and, unlike physical oscillators, the oscillator’s identity is changing at division. In addition, there will be phase skipping as described above for the 1:1 case and because the stability domain of the attractor is much smaller when p or q are greater than 1, skipping will be much more common. Similarly, for such p and q, locking is sensitive to parameter variation.
As a result of these factors there will be a relatively broad distribution of ratios and one cannot hope to observe $p/q$ locking in its pure form when $p/q > 1$. Nevertheless, the locking phenomenon will lead to a relevant observable experimental signature that we can hope to see when $p$ and $q$ are relatively small (such as 3:2), namely, a long-lived polyrhythmic behavior where the cells maintain a fluctuating fractional ratio of periods and display clustering as observed in the synchronized experiments and described below. The states observed in the dexamethasone-synchronized experiments that are not 1:1 phase-locked fit this description.

The clustering is explained in Fig. 5C for this 3:2 case and is due to the fact that after synchronization the cells split into either one or two groups depending on which of these branches of the attractor highway they are attracted to. These groups stay coherent because of the attraction to the attractor highway and because spreading of the clusters along the trajectory owing to the diffusive effect of stochasticity will be on a slower timescale.

Discussion

Our experiments have demonstrated that there are multiple coexisting robust oscillatory dynamical states of the coupled clock and cell cycle in proliferating mammalian cells and we have shown that varying the FBS level changes these states in a manner that accords with theory. In principle these states coexist in the same single cells because after the dexamethasone-synchronized cells are returned to dexamethasone-free regular medium there is in principle no difference between their current cellular context and that of the unsynchronized cells. This suggests that the coupled system is a stochastic dynamical system with multiple coexisting stable oscillating states and that the dexamethasone synchronization acts as a large perturbation that knocks the state out of the low-period 1:1 locked state into the domain of attraction of these other states. This is analogous to the bistability or multistability of equilibrium states that underlies many biological systems that switch between two or more different states. In our case the attractors are oscillating states and, although this is a well-known phenomenon in the theory of dynamical systems, so far as we are aware it has not been seen before in such biological systems.

The established circadian regulation of cell-cycle genes and proteins provides a consistent mechanistic basis to produce the coupling and phase locking described in our study. However, our results, together with previous observations by Nagoshi et al. (17), suggest that the coupling also operates in the opposite direction. In particular, in the unsynchronized cells, increasing FBS caused the period of both clock and cell cycle to decrease in unison with common periods that are significantly smaller than those for the clock in dexamethasone-synchronized experiments and also when the cells are confluent and not dividing. Although this is not currently supported by known mechanistic links,
following ref. 17, we hypothesize that key cell-cycle events including cyclin-dependent kinase network activation, cell growth, DNA replication, nuclear envelope breakdown, cytokinesis, and reduced transcription at mitosis may rhythmically and co-ordinately perturb the concentration, activity, and subcellular distribution of some clock proteins and as a result modulate the period of the clock. Presumably, in vivo and in physiological situations these intracellular signals are likely to be counteracted by extracellular circadian cues such as glucocorticoids and temperature so that the clock can impose a ~24-h periodicity to the cell cycle in proliferating tissues.

This and our other results suggests a natural hypothesis, namely, that (i) in unsynchronized cells there is bidirectional coupling enabling the clock and cell cycle to robustly entrain each other but dominated by the cell cycle, and (ii) dexamethasone synchronization perturbs the system into a state where the effect of the cell cycle on the clock is reduced so that the clock is freed to take up a period close to 24 h but to still regulate the cell cycle with its significantly lower natural (uncoupled) period and consequently produce both 1:1 phase-locked states and long-cycle with its significantly lower natural (uncoupled) period and freed to take up a period close to 24 h but to still regulate the cell cycle as dexamethasone synchronization perturbs the system into a state where the clock and the cell cycle is of primary relevance to cancer because disordered circadian function has been implicated in the pathogenesis of cancer, and a deregulated cell cycle is a hallmark of cancer cells (19, 20). Of the many processes being regulated by the circadian clock, some of the most profound are those related to specific cell-cycle events, DNA repair, and apoptosis (2, 4, 5, 21–25). The dynamics of the coupling between the cell cycle and the clock are described here are likely to be controlled through multiple and interacting pathways. Alterations in one or several of these links may therefore compromise the robustness and the adaptability of the cell cycle–circadian clock coupled oscillator system with a broad relevance for health.

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Supplementary Methods and Information for “Phase-locking and multiple oscillating attractors for the coupled mammalian clock and cell cycle”

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METHODOLOGY

1.1 Cell lines and reporters.

Circadian clock reporter cell line: NIH3T3 bearing the stably inserted Rev-erba::Venus construct were obtained from Ueli Schibler.

Cell Cycle reporter system: The Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI) reporter system, which consists in a set of two fluorescent probes fused to either Cdt1 or Geminin, two cell cycle proteins which accumulate reciprocally during the G1 and S/G2/M phases respectively, has been described in Sakaue-Sawano et al., . Based on this system, we designed a polycistronic retroviral vector that allows stable expression of the FUCCI reporter in a single vector (see below) in order to achieve a better stoichiometry of the
system. This construction includes the Cdt1 and Geminin sequences coding for G1 and S/G2/M probes respectively, fused to fluorescent reporters. They are separated by a 2A sequence to allow post-translational cleavage and followed by a puromycin resistance cassette for subsequent selection (Supplementary Fig. S1a). The 2A-like sequences consist in a 19 amino acid region harbouring a self-processing activity mediating the co-translational cleavage of viral polyproteins. This new FUCCI reporter was named “FUCCI-2A”.

1.2 Generation of FUCCI-2A transfer plasmid (pPRIPu CrUCCI)

The sequence encompassing the Geminin-T2Apeptide-mKO2-hCdt1 was synthesized by Epoch Biolabs, INC. (1306 FM1092 Rd, Ste 407 Missouri city, TX 77459-1565 http://www.EpochBiolabs.com) and cloned into a modified pBSK.

The amino acid sequences of the Geminin, mKO2, and hCdt1 are identical to those described in Sakaue-Sawano et al.,

The 2A Amino Acid sequence (T2A) is that of the Thosea asigna virus (Sequence ID: gb|AAC97195.1)

Silent mutations were introduced to facilitate insertion in our retroviral vectors.

The pPRIPu CrUCCI used in this study (Supplementary Fig. S1b) was constructed as follow: the AgeI-SspI (in lowercase above) fragment from pBSK FUCCI was inserted in the pPRIPu HAa digested with NgoMIV and HinCII. Additionally, as mAG fluorescence wavelength was not compatible with that of Venus, we replaced the mAG marker with a E2-Crimson fluorescence in our final system (sequence provided on demand). Restriction map is reported in Supplementary Fig. S1b. The integrity of the entire FUCCI-2A sequence in the latter has been confirmed by sequencing analysis.

1.3. Stable cell line generation

Replication-defective, self-inactivating retroviral constructs were used for establishing stable NIH3T3_Rev::Venus_FUCCI-2A cell line. On day 1, HEK293T were seeded in 100 mm plates (1-2.10^6 cells per plate). On day 2, cells were co-transfected with 10 µg transfer (pPRIPu CrUCCI), 5 µg packaging (pCMV-gag-pol) and 5 µg envelope (pCMV-env-VSV-G) plasmids, using a Calcium Phosphate method. After 16 hours, medium was replaced with 5 ml fresh medium (day 3). Supernatant was harvested 48h after transfection and filtered on 0.45µm PES filters to remove cell debris. NIH3T3_Rev::Venus cells seeded at 10% confluence the day before were directly infected by applying filtered supernatant + 4 µg/ml polybrene (Sigma-Aldrich) to the cells (day 4). Medium was then added to transfected HEK cells for a second round of harvesting/infection the next day, following the same protocol (day 5). Viruses were left for 3 days before splitting infected cells and adding puromycin (4µg/ml) for selection. Cells were frozen 15 days later or used in subsequent experiments.
The resulting cell line stably expressed the following markers: Reverbα::Venus as a clock marker, mKO2::Cdt1 and E2-Crimson::Geminin as G1 and S/G2/M cell cycle phases markers respectively.

1.4 Flow cytometry
To ensure that the cell cycle was not perturbed by insertion of the transgene in NIH3T3_Rev::Venus_FUCCI-2A cells, as well as to validate this modified system as an accurate cell cycle reporter, we analysed the DNA content of proliferating cells using a Fluorescence-Activated Cell Sorter (FACS). NIH3T3_Rev::Venus_FUCCI-2A cells were seeded in 100 mm plates (1.10^6 cells per plate) and left to proliferate for 48 hours before cell analysis. Cells were resuspended and incubated for 30 min in Cytofix (BD Bioscience). After centrifugation, fixed cells were resuspended in PBS containing 0.1% Triton, 20 µg/ml RNase A and 2 µg/ml Hoechst 33342 solution (Invitrogen) After a 10 min incubation, cells were harvested and analyzed using a BD LSR Fortessa (Becton Dickinson). mKO2, E2-Crimson and Hoechst were excited with a 561nm, 633 nm and 355 nm laser line, respectively. Fluorescence signals were collected at 586 nm (586/15 BP) for mKO2, at 670 nm (670/14) for E2-Crimson, and at 450 nm (450/40) for Hoechst 33342. The data were analyzed using the DIVA software (BD). Results shown in Supplementary Fig. S1c indicate that mKO2(+)E2-Crimson(-), mKO2(+)E2-Crimson(+), mKO2(-)E2-Crimson(+) cells populations had a DNA content consistent with their predicted phase of the cell cycle.

1.5 Time-lapse imaging
For recording, cells were seeded at 7-10% confluence (10^5 cells per well) in a 6 wells plate (Falcon), with white DMEM medium (high glucose) containing 1% Penicillin/Streptomycin, 10 mM HEPES and either 10%,15% or 20% FBS. Cells were left undisturbed for 48 hours. For the Dex-pulse condition, cells were incubated for 2 hours in the same medium as above, supplemented with 100 nM Dexamethasone (Dex). Cells were then returned to Dex-free regular medium just before recording. For recording, cells were placed in a Zeiss Axiovert 200M microscope (Zeiss) with a 10X Ph objective. A culture chamber, temperature and CO₂ controller (Pecon) were used to ensure constant suitable conditions for long term recording of the cells. Images were recorded every 15 minutes for 72 hours, using a Coolsnap HQ/Andor Neo sCMOS camera. Cells were briefly illuminated with a fluoarc HBO lamp (Zeiss) at reduced intensity and epifluorescence signals were recorded as follows: Venus: 1000 ms (filter cube: Ex 475/40 – Di 500 – Em 530/50), mKO2: 300 ms (filter cube: Ex 534/20 – Di 552 – Em 572/38), E2Crimson: 800 ms (filter cube: Ex 600/37 - Di 650 - LP 664).
A brightfield image for each frame was also acquired to ease cell tracking and analysis. Timelapse acquisition was controlled using a Metamorph 6.1/7.7 software.

1.6 Cell sorting
NIH3T3_Rev::Venus_FUCCI-2A cells were seeded in 100 mm plates (1.10^6 cells per plate) and left to proliferate for 48 hours before cell sorting. On the day of sorting, cells were trypsinized and resuspended in 1 ml PBS + 5% FBS + 2 mM EDTA. Based on their fluorescence, single cells were sorted as follows, using a BD FACS ARIA (Becton Dickinson): Non-marked: Early G1, mKO2 only: G1, mKO2 + E2-Crimson: Early S, E2-Crimson only: S/G2/M. mKO2 and E2-Crimson were excited with 561 nm and 633 nm laser lines, respectively. Fluorescence signals were collected at 610 nm (610/20 BP) for mKO2 and at 670 nm (670/14 BP) for E2-Crimson. A plate of non-sorted cells was used as control. The experiment was done in triplicate. After centrifugation, cells were processed for total RNA extraction with NucleoSpin RNA II kit (Macherey-Nagel) according to manufacturer’s protocol. RNA was kept at -80°C until use.

1.7 qRT-PCR
Quantitative RT-PCR. Measurements were performed with a Light Cycler 1.5 (Roche Applied Science) using SYBR green I dye detection according to the manufacturer’s recommendations. cDNA, synthesized from 2-5 µg of total RNA using random primers and Superscript II (Invitrogen), was added to a reaction mixture (Faststart DNA SYBR Green I) with appropriate primers at 0.5 µM each: Rev-erbα: forward 5’-AACCTCCAGTTTGTGTCAGG-3’ and reverse 5’-GATGACGATGATGCAGAAGAAG-3’ (Supplementary Figure S1d); Ccna2: forward 5’-AAGACTCGACGGGTTGCTC-3’ and reverse 5’-CCAGGGCATCTTACACTCT-3’ (Supplementary Figure S1e); Ccne2: forward, 5’-GCATCAGTATGAGATTAGAATTG-3’ and reverse 5’-CAGAATGCAGAACTTGAAAATGT-3’ (Supplementary Figure S1f); 36B4: forward 5’-GCTGATGGGCAAGAACACCA-3’ and reverse 5’-CCCAAAGCCTGGAAGAAGGA-3’. Relative mRNA abundance was calculated using a standard curve method. Expression levels were normalized to the levels of the constitutively expressed 36B4 ribosomal protein mRNA.

2. Image and timeseries analysis
2.1 Cell tracking. Tracking cells using LineageTracker is a semi-automated process. The input to LineageTracker is a sequence of images which were captured every 15 or 30 minutes (see Table S1: Summary of Datasets). There are different approaches to the choice
of cells to track. If the cells are sparse in the last frames, cells should be chosen from the end of the movie, making it easier to pick cells which can be tracked for a long period of time. In other movies, choosing cells from the beginning works better because the density of cells increases drastically at the end of the movie. To be able to conclusively analyse the resulting timeseries, around 20-50 lineages should be tracked per movie (we also show a list of how many lineages we tracked in Supplementary Table S2: Tracked Cell Counts and Period Lengths). Note that any cell that was visible from beginning to end of the recording was considered as a “mother cell” and was tracked, i.e. cells were not selected before tracking. As an output, we get the numerical timeseries for each of the three markers, information about when divisions occurred, and also information about the parent and children of each cell in the lineage tree. The file format and plugin we used for this have been made available at https://github.com/pkrusche/lineagetracker.jsonexport. The output of the cell tracking procedure is further illustrated in Figure 1b-d.

2.2 Numerical time series Analysis

Clock Periodicity Analysis

Our analysis of the clock period serves two goals:

1. To classify cells and lineages w.r.t. rhythmicity.
2. To determine period lengths of circadian rhythms.

We use the spectrum resampling software package developed by Costa et al.\(^7\) for a first analysis of periodicity. This software determines the stationary period length in a circadian timeseries and confidence intervals for it by means of bootstrap resampling. We obtain

- an optimal set of period lengths together with confidence bounds and variance information;
- a de-trended version of the input timeseries; and
- a nonlinear least-squares fit of a sinusoidal curve obtained using these period lengths.

We encountered a few difficulties when trying to get accurate period length estimates due to noise in our data. In Figure 1c, we see the clock trace for a cell, and some diagnostic information from the period length finding procedure:

1. We first de-trend the clock curve (shown normalized as black dots) using a strongly smoothed trend curve, which aims to remove oscillations that have a period length longer than the time interval that was observed. This trend is shown using a dotted black line. The de-trended result is shown in blue.
2. Since we only have a few clock peaks in most cells, we find these peaks before using a smoothed version of the input data (shown in yellow). We then estimate the period using the average distance \(D\) between these detected peaks. If less than two peaks are found, we discard the data (assuming that the tracked cell does not have a functioning
clock). We then run the period length estimation on the part of the time series, beginning at no more than time offset $-D/2$ before the first peak and ending at most at time offset $+D/2$ after the last peak. This region is highlighted as the area between the grey shading.

3. Finally, we classify cells as rhythmic or non-rhythmic. A basic requirement is that the cell has at least one clock peak within its lifetime (marked using a red rectangle). In order to extract phase information (see below), we will further increase this requirement to demanding at least two peaks, and we require the period length to be between 5 and 50 hours. Secondly, we use an rae-type value obtained from the confidence bounds $[P_{lower}, P_{upper}]$ returned from the spectrum resampling tool:

$$\text{rae} = (P_{upper} - P_{lower})/(2P_{mean})$$

If $\text{rae} < 0.25$, the confidence interval is less than twice as large as the estimated period, therefore we assume that the estimated period value is acceptable. Visually, the quality of the period estimate can be assessed by looking at the width of the peak in the period spectrum samples plot (also, several peaks may occur -- we require that the oscillatory component in the circadian range must clearly be the strongest). In Table S2: Tracked Cell Counts and Period Lengths, we show the distributions of period lengths both for the clock and for the cell cycle.

4. Using the period estimate $P$ from spectrum resampling, we fit a sinusoid of the form

$$f(t) = a \cos \left( \frac{2\pi t}{P} \right) + b \sin \left( \frac{2\pi t}{P} \right)$$

to the timeseries. We then filter out clock marker peaks which are not within a fixed distance of a peak for this sinusoid. This eliminates spurious peaks, and makes our automated clock phase estimation more reliable.

**Cell Cycle Event Extraction**

We analyse timeseries for the G1 and S/G2/M markers for each cell cycle (the time period from the start of the timeseries or a division to the next division). In each such period, we identify three turning points for each marker:

a) the point where the marker starts rising,

b) the peak,

c) the point where the marker has dropped below the basal level.

Turning points are identified using a piecewise-linear model: we attempt to find the best-fitting upward-triangle. From the turning points, we estimate the durations of cell cycle phases (G1 phase, and S/G2/M combined). Once we have traces for the FUCCI Markers as shown in our Figure 1c, we segment these traces into cell cycle intervals. A cell cycle interval
is defined as the time interval between two consecutive cell divisions (or the start/end of the
time series for the first/last intervals).
For each marker trace, we determine a baselevel. This baselevel will be used later on to
decide whether a marker’s peak is sufficiently large. Given the timeseries values \( TS = [M(t_1), M(t_2), M(t_3), ..., M(t_n)] \), we estimate the baselevel as follows:

\[
\text{baselevel} = \max([\text{quantile}(TS, 0.05), \min(TS) + 0.5 \cdot \text{std}(TS), 0]).
\]

In each cell cycle interval, and for each marker, we identify the following three timepoints:

a) \( t_a \) : The time when the marker intensity starts increasing towards the peak

b) \( t_b \) : The peak of the marker

c) \( t_c \) : The time when the intensity has dropped below the baselevel.

The timepoints are identified by finding the best fit for the following piecewise-linear model.

\[
Model(t) = \begin{cases} 
  c_1 & \text{if } t < t_a \\
  c_2 t + c_3 & \text{if } t + a \leq t < t_b \\
  c_4 t + c_5 & \text{if } t + b \leq t < t_c \\
  c_6 & \text{otherwise.}
\end{cases}
\]

We choose values for \( c_1, c_2, c_3, c_4, c_5, c_6 \) which minimise the squared error:

\[
SE = \sum_{i=1}^{n} (Model(t_i) - M(t_i))^2.
\]

In order to accept the fit, we require:

- Condition 1: The height of the peak must be above the baselevel: \( Model(t_b) > \text{baselevel} \).
- Condition 2: Model values after \( t_c \) must be below the baselevel: \( Model(t > t_c) < \text{baselevel} \) (we allow exceptions for the G1 marker in order to identify G1 arrest).

We obtain the six values of \( t_a^{G1}, t_b^{G1}, t_c^{G1}, t_a^{S/G2/M}, t_b^{S/G2/M}, t_c^{S/G2/M} \). In order to proceed, we require that \( t_b^{G1} < t_b^{S/G2/M} \), i.e. that the G1 peak must occur before the S/G2/M peak.

The time of the G1-S transition is estimated as follows.

\[
t_{G1-S} = \begin{cases} 
  t_b^{G1} & \text{if only the G1 fit was succesful,} \\
  t_b^{S/G2/M} & \text{if only the SG2M fit was successful,} \\
  \text{mean}(t_b^{G1}, t_a^{S/G2/M}) & \text{if fits for both series were successful,} \\
  \text{undefined} & \text{otherwise.}
\end{cases}
\]

This way, we can use the two markers for redundancy in cases where it is not possible to
find a good fit for the timeseries data on both the G1 and S/G2/M traces.

In Table S3: Data Quality for Analysed Cell Cycle Intervals and Clock, we show success
rates for extracting G1-S transition times using this method. In general, we appear to be able
to extract data for more than 50% of all cell cycle intervals. However, this includes intervals
at the beginning or end of the timeseries – these are not useful for our analysis since we
cannot know the exact length of the corresponding cell cycle which starts/ends outside of the
observed time interval. When restricting attention to only fully observed cell cycle intervals, the accuracy of our method increases to close to 100% in most datasets (in Table S3, the first success rate relates to fully observed cell cycles, the second one to all intervals in the dataset). Moreover, the success rate increases when we restrict our attention to cells with a functioning clock.

2.3 Circadian Phase-timing of Cell Cycle Events

Clock Phase. In order to establish whether there is a correlation between the clock and cell division timing, we calculated estimated clock phase values for each recorded time point. We consider two different methods for estimating this phase value. Since the circadian clock is a cyclic process, we consider clock phases from the interval \([0, 2\pi)\). We assign phase 0 or \(2\pi\) to the time at which the clock marker peaks. This way, we can associate a phase angle \(\varphi_{ci}(t)\) with each time-point. We use the following methods for estimating \(\varphi_{ci}(t)\) (see also Figure 1c, top part):

1. We use the fitted sinusoid obtained using the period estimate from spectrum resampling. This fit gives us a direct estimate for the phase angle. Assuming that we have obtained a fit

\[
f(t) = a \cos\left(\frac{2\pi t}{P}\right) + b \sin\left(\frac{2\pi t}{P}\right),
\]

we can obtain a phase angle \(\varphi_{ci}(0) = \arctan(b/a)\), and we subsequently set

\[
\varphi_{ci}(t) = \varphi_{ci}(0) + \frac{2\pi \cdot t}{P}.
\]

A drawback of this method is that estimates for \(\varphi_{ci}(t)\) will not be accurate if the period of the clock marker changes over time, since we assume that the period length \(P\) is constant. An advantage is that we can estimate phase angles for the entire time interval we used for determining the period length by spectrum resampling.

2. Using the peaks from our peak-finding procedure, we can interpolate the values of \(\varphi_{ci}\) from peak to peak. Assuming we have detected a peak in the Rev::VENUS marker at times \(t_1, t_2, ..., t_N\), we can estimate \(\varphi_{ci}\) as follows.

\[
\varphi_{ci}(t) = \begin{cases} 
\frac{2\pi}{P} \frac{t - t_i}{t_{i+1} - t_i} & \text{if } t \text{ is inside the interval } [t_i, t_{i+1}) \\
\text{undefined} & \text{otherwise}.
\end{cases}
\]

A drawback of this model is that we can only estimate phases in time intervals between two clock peaks (i.e. we cannot estimate the clock phase at the beginning and at the end of the time series).
Random Background Model

We would like to distinguish the distribution of the estimated phases of cell cycle events (cell division or G1-S transition) from a random background model. For this, we assume that in the case of no correlation between the events and the clock, any observed phase would be equally likely to be associated with some cell cycle event (Supplementary Fig S2b-c; grey shaded histogram and area).

For a given dataset of cells, let \( \text{Clock}(t_1, C), \ldots, \text{Clock}(t_{N(C)}, C) \) be all recorded clock marker values for all distinct cell cycles \( C \in 1 \ldots \text{Number of observed cell cycle intervals} \). We can also associate a phase angle \( \varphi(t_k, C) \) with each time \( t_k \) and cell cycle interval \( C \). We collect all these phase angles for an experiment into a set \( B = \{ \varphi(t_k, C) \text{ with } k = 1 \ldots N(C) \text{ and } C = 1 \ldots \text{Number of observed cell cycle intervals} \} \). If a class of events like cell divisions or G1-S transitions is not associated with a specific set of clock phases, the observed phases for this class of events should be a uniform random sample of all phases in set \( B \) (assuming all our phase estimates are taken at equally spaced time intervals).

Considering all division (or G1-S transition) events in a dataset, we compare the distribution of \( \{ \varphi(t, C) \mid \text{Event at } t \text{ in interval } C \} \) to the distribution of events obtained from drawing \( E \) values uniformly at random from all observed phase values in set \( B \) defined above. We perform this comparison using two different methods:

- We can compare the cumulative distributions, either visually (omitted due to space constraints), or using an appropriate statistical test. We compare cumulative distributions using the two-sample Kolmogorov-Smirnov test\(^8\), or the Kuiper test for circular distributions\(^9\). We test for:
  \[
  \begin{align*}
  H_0 &= \text{Distribution of phases cannot be distinguished from random background.} \\
  H_1 &= \text{Distribution of phases is different from background.}
  \end{align*}
  \]
  \( H_0 \) corresponds to the case where either cell divisions or G1-S transitions happen independently of the clock phase. In Table S4: Cell Cycle Event Analysis, and background vs. phase distribution test p-values, we give p-values for accepting/rejecting \( H_0 \).
- Using bootstrap sampling and kernel density estimation\(^10,\)\(^11\), we can compare the phase histograms directly (see Supplementary Fig S2b-c; yellow and red histograms).

2.4 Population-level Analysis

Plots of Population-based Event Densities over Time

To check if our tracked cells were synchronised on a population level, we created density plots and histograms for all cells in our tracked population, showing whether there are
preferred times during the experiment when cells like to divide (Supplementary Fig S2e), or have their clock marker peaks (Supplementary Fig S2d). We expect the Dexamethasone-treated cells to have synchronised clock marker peaks (since the treatment will reset the clock in all cells before the experiment). In the unsynchronised cells, we expect an irregular behaviour since most cells will not be phase-synchronised.

**Clock Phase vs. Cell Cycle Phase**

To study the phase coupling in our cell populations, we may first assume that the cells are in a steady state, and estimate a phase diagram for their trajectories. To do this, we will need to define a cell cycle phase similar to the way we defined a clock phase above:

\[
\varphi_{cc}(t) = \begin{cases} 
0 & \text{from birth to G1 - S transition}, \\
\pi & \text{from the G1 - S transition to cell division}.
\end{cases}
\]

Each cell that is observed at time \( t \) then has a location \((\varphi_{cl}(t), \varphi_{cc}(t))\). Both \( \varphi_{cl} \) and \( \varphi_{cc} \) are cyclic coordinates (they wrap around at \( 2\pi \)). Therefore, the movement of the cells in their \((\varphi_{cl}(t), \varphi_{cc}(t))\) coordinates can be visualised by a point moving on the surface of a torus.

Our dataset allows us at each time \( t \) to estimate the location \((\varphi_{cl}(t), \varphi_{cc}(t))\) and speed \((\dot{\varphi}_{cl}(t), \dot{\varphi}_{cc}(t))\) for all cells. Given locations and speeds of all observed cells, at each time point, we can use two dimensional kernel density estimation \(^{12}\) to estimate the average speed of movement \((\dot{\varphi}_{cl}, \dot{\varphi}_{cc})\) on a regularly-spaced grid along the surface of the torus. The result is a streamline plot. Underlaid under the streamline plot, we show estimated cell densities over the course of the experiment for each location on the torus (Figure 2d). For the unsynchronised populations (fbs_10 and fbs_15), we can see that the cells prefer to move along a common set of trajectories on the torus surface (Figure 2d).

**Analysis of the Dynamic Behaviour**

In order to better understand whether there is a transient behaviour caused by the Dexamethasone treatment at the beginning of the experiment, we have derived two methods which allow us to analyse and visualise the dynamics of how clock and cell cycle are connected.

**Timelapse of Clock and Cell Cycle Phase Progression**

A first method to visualise the dynamic coupled behaviour of clock and cell cycle is to visualise the progression of clock and cell cycle phases over time in a movie with a frame for each time point \( t \). In each frame, we plot \( \varphi_{cl}(t) \) against \( \varphi_{cc}(t) \) for all cells. The resulting movie (see Supplementary movies 2 and 3) reveals the following types of behaviour:

1. In the unsynchronized populations, we see that cells independently follow a main direction of movement along a preferred path. Some cells may skip between the main
trajectories and re-join when they meet the main path in the next cycle (Supplementary movie 2).

2. In the Dexamethasone-synchronised populations, we can see whether cells divide in groups (and at which phase). In the movies, we have coloured the cells according to the peaks in the divisions/time density histograms (Supplementary Movie 3).

**Clustering of Division Phases over Time**

Another way to visualise the phase dynamics of cell divisions is to create a 2-dimensional scatter plot of division clock phases over time. The resulting plots are shown in Supplementary Figure S3, S4 and S5.

The main idea of our clustering approaches is to:

- establish whether cell divisions occur in groups over the time of the experiments,
- relate these groups to a period ratio obtained by dividing the clock period by the cell cycle time for each cell cycle interval.

These complementary approaches reveal more information than only looking at the density of division events over time or clock phase individually, and have allowed us to better understand and visualise how the clock and the cell cycle are coupled in a non-trivial fashion.

To implement our analysis, we use the R package 'MClust'\(^{13}\). MClust is an R package for model-based clustering, classification, and density estimation based on finite normal mixture modelling. This approach is more general than the method used by Nagoshi et al.\(^{1}\), who use a similar method to show that their distribution of observed cell division phases has three peaks.

We use two approaches:

**Clustering method 1.** We plot a data point for each cell division. For the x-axis, we show the experiment time of the division, and on the y-axis, we show the estimated clock phase at this time. We cluster the resulting set of points in two dimensions using Mclust as follows.

- Each cell cycle interval for which we observe a division and which we can associate with two clock peaks adds a data point \((t_i, 24 \frac{\phi_{cl,i}}{2\pi})\) to our dataset (since Mclust uses an Euclidean distance, we scale the clock phases to lie within an interval of \([0, 24)\)).
- Since the clock phase ‘wraps around’ at \(2\pi\), we create a copy of each data point \((t_i, \phi_{cl,i})\) at \((t_i, \phi_{cl,i} + 2\pi)\) and \((t_i, \phi_{cl,i} - 2\pi)\) as illustrated by the following R code:

```r
# Assume dataframe data contains two columns, t in column 1 and phi_cl in column 2
# short range cyclic: append a fraction of the data again on the top and bottom
cyclicdata <- rbind(data, 
                   cbind(data[,1], data[,2] + 2*pi),
                   cbind(data[,1], data[,2] - 2*pi))
cyclicdata <- cyclicdata[
                     (cyclicdata[,2] >= -overlap) &
```


(cyclicdata[,2] < 2*pi+overlap),]
# Scale phases to 0-24
 cyclicdata <- cbind(cyclicdata[,1], cyclicdata[,2]/2/pi*24)

- We run Mclust to obtain a 2-D clustering / mixture distribution for all points in the dataset constructed above.

```r
cdata <- Mclust(cyclicdata, G=1:ncl, modelnames="VVV")
```

- After we have run Mclust, we merge clusters which overlap by containing copies of the same datapoints as shown above:

```r
#' compute pairwise half-torus distance (0-24 wrapping y coordinates)
halftorusdist <- function(p1, p2) {
  ply = p1[2] %/% 24
  p2y = p2[2] %/% 24
  p2d <- min(abs(ply-p2y), 24-abs(ply-p2y))
  return (sqrt((p1[1]-p2[1])^2 + p2d^2))
}

#' Pairwise distances for classification centroids
pairwiseclassdists <- function(cyclicdata, cdata) {
  dists = matrix(nrow=cdata$G, ncol=cdata$G)
  for(i in 1:cdata$G) {
    ci = c(mean(cyclicdata[cdata$classification == i, 1]),
         mean(cyclicdata[cdata$classification == i, 2]))
    for(j in i:cdata$G) {
      cj = c(mean(cyclicdata[cdata$classification == j, 1]),
         mean(cyclicdata[cdata$classification == j, 2]))
      dists[i,j] = halftorusdist(ci,cj)
    }
  }
  return(dists)
}

#' merge clusters with centroids closer than threshold
mergedclusters <- function(classification, dists, threshold) {
  newclass = classification
  for(i in 1:nrow(dists)) {
    for(j in i:nrow(dists)) {
      if(dists[i,j] < threshold) {
        newclass[newclass == j] = i
      }
    }
  }
  return(newclass)
}

dists <- pairwiseclassdists(cyclicdata, cdata)
classes <- mergedclusters(cdata$classification, dists, 2)
data$classification <- classes[1:nrow(data)]
```
• Each division produces two child cells. If one or both of these cells divide again, we can determine the cluster in which this division happens. The result of this analysis can be visualised in two different ways:
  o We can draw arrows between the division data point of each mother cell and the division data points of its two children.
  o We can aggregate the information above by connecting the clusters. We show the relative number of children dividing in each subsequent cluster by varying the thickness of the arrow (see Supplementary Figure S4 and S5, first two plots for each dataset).
• We split our lineages into groups depending on which cluster the last divisions in each lineage occur in. This is possible because cells divisions within each lineage mostly fall into the same clusters.

While Nagoshi et al.\textsuperscript{1} reported a three-peak distribution compatible with ours it is not possible from the results reported in their paper to tell whether their data contains cells of both of the two types that we find. We believe it is likely that the behaviour seen by Nagoshi et al.\textsuperscript{1} (three peaks in the phase histogram over the entire time of the experiment) is the result of the way in which the subset of peaks in the 2-D density of clock phase at cell division overlap. To illustrate this, we show phase histograms for clock phase at cell division for each cluster obtained above separately (see Figure S3, S4, S5, rightmost plot in the top row for each dataset). Moreover, in our experiments with 20% FBS (see Supplementary Figure S4b), we see that the children coming out of the first cluster of divisions are divided into two groups: one group which divides again at a mean phase of around $\pi$ (cells which divide first in the red cluster and their children dividing in the blue, and then the purple cluster), and another group of cells which divides at phases around $\pi/2$ (dark yellow/olive green cluster) and $3\pi/2$ (green cluster). When plotting the division clock phase histograms for both groups, we get two distinct behaviours, one that corresponds to 1:1 locking, and another one that corresponds to 3:2 phase-locked behaviour. In our analysis (see Figure 4b), differently from the figure shown by Nagoshi et al.\textsuperscript{1}, the three peaks of the division phases are not of equal size. This is explained by the fact that our data includes all children in each lineage that we were able to track (it is our understanding that Nagoshi et al.\textsuperscript{1} only tracked one cell per lineage). Therefore, the first peak will be the smallest, the second peak will contain approximately twice as many events (since we have two children for each division), and the third peak contains four times as many observations. Overall, we conclude that our cells reproducibly contain a subpopulation that behaves as predicted by our model for 3:2 coupling, and as in the experiment by Nagoshi et al.\textsuperscript{1}.
Clustered method 2. For each cell cycle interval, we can obtain a clock period by taking the mean time between all clock peak-to-peak intervals that overlap. Using this clock period and the length of each fully observed cell cycle interval, we can look at the distribution of clock/cell cycle period ratios. We find that in most of our datasets, the cells fall into two groups: one group of cells which couple 1:1, and another group of cells which have a higher mean clock/cell cycle period ratio.

Using Mclust to fit a 1-D Gaussian mixture model to the set of period ratios, we can separate these groups (we restrict the number of clusters to two to avoid overfitting since the ratio data is noisy):

```r
data$ratio = data$clockperiod_mean / data$cellcycle_len
m = Mclust(flattened_no_na[, c('ratio')], c(1, 2))
data$classification = mclassification
```

This method enables us to create the plots shown in the bottom row for each dataset in Figures S3, S4 and S5 (see also Table S5: Clock/Cell Cycle Period Ratios and Groups). The first/leftmost figure for each dataset (see also Figure 4a) shows a scatterplot of the clock and cell cycle periods, colored by the classification obtained using the clustering shown above, together with a 75% confidence ellipse for each group. The second plot (in the middle) shows a histogram of the periods and counts for each group. The rightmost plot shows a scatter plot of clock phases at cell division against time similar to the one created for clustering method 1 above, but with different colours: we use the classification by ratio obtained above for colouring the cells.

We observe the following:

- In the unsynchronised populations (Figure S3), the ratios largely fall into the 1:1 group shown in red. The blue group is more scattered and contains cells which ‘skip’ between stable trajectories on our phase torus.
- In the Dexamethasone-treated cell population at 10% FBS, the clock/cell cycle period ratio of the majority-group of cells has a mean of around 1.1.
- In the Dexamethasone-treated cell populations at 20% FBS (Figures S4b and S5), there are two clearly distinct groups of cells, one with a 1:1, and another one with a 3:2 coupling ratio. Moreover, the colouring by ratio reproduces the clusters obtained by clustering method 1.

¹ The 75% confidence ellipse is estimated from a covariance matrix obtained by fitting a bivariate t-distribution to our data.
Estimation of return phase/Poincaré maps

For each observed cell cycle interval, we can plot the clock phase at its beginning against the clock phase at the end. The result is an approximation of a Poincaré map of the dynamical system of the cell: for each clock phase at which a cell may begin the cell cycle, we can see the preferred phase at the end of the cell cycle. We can make a similar plot for the cell cycle phase: for each complete circadian interval we plot the cell cycle phase at the clock marker peak $P_i$ at the beginning against the cell cycle phase at the time of the clock marker peak $P_{i+1}$ at the end of the interval.

In the case of our unsynchronized datasets (fbs_10 and fbs_15 on the left hand side of Fig. S6c), we can clearly see that the majority of cells prefers to enter/exit at similar locations in our plot, along a fixed point on the main diagonal. In the Dexamethasone-treated conditions, we see a behavior that becomes more different from the unsynchronized case as the period ratio between clock and cell cycle changes from 1:1 towards 4:5.

3. Summary of datasets

3.1 Datasets

We have recorded cells in a variety of conditions to vary the speed of the cell cycle (by means of changing the concentration of FBS), and to perturb the clock (by treating the cells with Dexamethasone to achieve population synchronisation of the clock). A summary of all datasets is shown in Table S1: Summary of Datasets, more details on the data extraction and period lengths are summarized in Table S2: Tracked Cell Counts and Period Lengths.

3.2 Tracking and Dataset Summary

When tracking cells and analysing their clock and cell cycle markers, we obtained good results for cells in 10% and 15% FBS. Clock and cell cycle periods are shown together with an estimate of the standard error of the mean ($\text{sem} = 1.96 \cdot \text{std}(\text{Periods})/\sqrt{n}$).

Note that there is good agreement within the two experiments which were repeated between the two different labs (‘fbs_10’ and ‘fbs_10_r’). Another observation is that in the unsynchronised conditions, clock and cell cycle appear to be running at very similar period lengths (Supplementary Figure S2a). In the Dexamethasone-treated cells, the mean clock period is longer (Supplementary Figure S2a).

As expected, the number of divisions (relative to the number of lineages tracked) is higher in higher concentrations of FBS.
3.3 Data Quality

We show for how many cell cycle intervals we were successfully able to extract the timing of the G1-S transition, and what the average RAE for the clock traces is in each dataset. These numbers can be considered a measure of the quality of the tracking data and our success in extracting information from the resulting time series.

We consider all cell cycle intervals for which we can determine a time of the G1-S transition. In some of these intervals, we may have only been able to match one of the G1 or S/G2/M markers, which will possibly result in a slightly less accurate estimate for the G1-S transition time. We show two success percentages in Table S3: Data Quality for Analysed Cell Cycle Intervals and Clock: in all datasets, we are able to analyse more than 90% of all fully observed cell cycle intervals successfully. When also considering partially observed intervals, this success rate drops to around 50%, since only few partially observed cell cycle intervals have enough data to apply our method (this is not problematic since we can only use fully observed cell cycles in most of our analyses; in most cases, we need to know the duration of the cell cycle interval, which can only be known if it has been observed entirely during our imaging experiment).

Clock RAE values below 0.25 indicate that the clock was functional with a stationary period on average. Only using branches of lineages having an RAE value of less than 0.25 also improves the success rate when analysing the cell cycle. We show a summary of our data quality analysis in Table S3: Data Quality for Analysed Cell Cycle Intervals and Clock.

3.4 Clock vs. Cell Cycle Phase over Time

Dataset fbs_10 and fbs_15:
See Supplementary Movie 2: Cells are unsynchronised, but follow a main trajectory. Some cells 'skip'. G1-arrested cells are shown in grey. Cells for which the clock phase was estimated using peak prediction are shown with a slightly lighter colour.

Dataset dexpulse_fbs_10
See Supplementary Movie 3: We can see that clock and cell cycle are rhythmic. We assign a colour to each group of dividing cells. Cells for which the clock phase was estimated using peak prediction are shown with a slightly lighter colour.

Dataset dexpulse_fbs_20
We did not produce a movie for this dataset, the behaviour is better visualised in the plots from our clustering analysis.
4. Modelling Clock and Cell Cycle as Coupled Oscillators

**Differential Equations for Phase.** We would like to study clock and cell cycle as a simple stochastic coupled oscillator model with unidirectional coupling. One way to model this is to consider two phase angles $\varphi_{cl}$ and $\varphi_{cc}$ of the clock and cell cycle oscillators which are coupled in one direction, from the clock to the cell cycle.

$$\varphi_{cl}' = v_1 + d\xi$$
$$\varphi_{cc}' = v_2 + f_{amp} \cdot Q(\varphi_{cl}, \varphi_{cc}) + d\xi$$

The function $Q(\varphi_{cl}, \varphi_{cc})$ is our gating/coupling function. It describes at which relative phases the clock will be slowing down the cell cycle. We define $Q$ like this, following previous work on the clock for cyanobacteria:

$$Q(\varphi_{cl}, \varphi_{cc}) = -\left| \cos \left( \frac{\varphi_{cl} - \mu_1}{2} \right) \right|^\alpha \left| \cos \left( \frac{\varphi_{cc} - \mu_2}{2} \right) \right|^\beta - N_0,$$

with

$$N_0 = \frac{\Gamma(\alpha + 1) \Gamma(\beta + 1)}{2^{\alpha + \beta} \Gamma\left(\frac{\alpha}{2} + 1\right)^2 \Gamma\left(\frac{\beta}{2} + 1\right)^2}.$$

The normalisation factor $N_0$ makes function $Q$ integrate to 1 over the range of $[0,2\pi) \times [0,2\pi)$.

We may add a white noise term $d\xi$ to simulate molecular noise.

In a deterministic setting (when $d\xi = 0$), we can consider the following scenario: we assume that the clock has a constant forcing period (of, e.g., 24h), and that the cell cycle may phase-couple to this forcing oscillator in different ways, depending on its free-running period (which, in our cell cultures will be dependent upon the amount of growth factor, or the concentration of FBS).

In supplementary Figure S6a (left panel), we can see that $\varphi_{cc}$ can lock to $\varphi_{cl}$ due to the forcing term, despite the fact that its natural speed $v_2$ is slightly faster than that of $\varphi_{cl}$ (red line). On the bottom, we show Poincaré maps for both phases (see also Figure S6c for their experimental counterparts); intuitively, these show where a trajectory entering at phase $\varphi_{cc}$ on the left (or $\varphi_{cl}$ on the bottom, respectively) of a torus plot as shown in S6a will leave on the right (or the top). Points where the resulting curve crosses the main diagonal are fixed points of the system. We see that the 1:1 coupled system has two fixed points for each phase, corresponding to the stable (red) and unstable (blue) trajectory in Figure S6a, left.

In the middle panel, we see the result for coupling with a cell cycle/clock period ratio of 5/4. The system does not lock in a stable fashion (the 5:4 locking region is very small), we see a mixture between the 1:1 and the 3:2 cases with many possible parallel mean trajectories.

The right panel in supplementary Figure S6a shows that the system can also be phase-coupled at a different period ratio – depending on the parameters, we can find coupled
solutions in which we complete three cell cycles every two clock cycles. Each of these coupling regimes would map cell divisions to different (but consistent) clock phases.

**Stochastic Simulations** When enabling the white noise term $d \xi$, we can simulate the behaviour of our coupled system in settings similar to our experiments. First, we can show that even when noise is present, unsynchronised populations will be phase-coupled (Supplementary Fig. S4b). In this simulation, we choose random starting phases $\varphi_{ct}$ and $\varphi_{cc}$ as uniformly random numbers between 0 and $2\pi$. We also fix $\alpha = \beta = 10$, and set $\mu_1 = \mu_2 = \pi$. We use $f_{amp} = 2$. We can see that even with white noise, the 1:1 coupling between clock and cell cycle remains stable.

We then looked at reproducing experiments with a 3:2 coupling ratio. This is possible (although the 3:2 coupled region is much smaller than the 1:1 region), and gives results very similar to the experiments (see Figure 6). We observe the following effects:

- We see three peaks in the distribution of all clock phases. This is the result of all peaks in the 2-dimensional density plot for clock phase at division over time 'lining up' at approximately the same phase (left histogram).

- We can also see that the histogram of divisions over time on a population level can look quite random (depending on the number of cells in our sample), since the groups of cells which divide together may overlap due to our simulated noise (right histogram).

We can also reproduce the behaviour we see in our cells with an approximate 5:4 ratio coupling (10% FBS, Dexamethasone treated). Since the 5:4 coupling region is very small in our toy model (it may be larger in a more mechanistic simulation), cells will either fall into the 1:1 and 3:2 coupled regions, depending on the amount of noise. The result is a wider peak in the histogram of clock phases at cell division, and an offset between the peaks in the division-phase density over time (Supplementary Figure S6b, on the right) – therefore, the peaks in this 2-dimensional plot do not line up perfectly anymore, which blurs the distribution of clock phases at cell division (while still allowing a population-level synchronisation of the cell cycle to be clearly visible, see Supplementary Figure S6b, in the middle).

**References**


Supplementary Tables

Table S1: Summary of Datasets

<table>
<thead>
<tr>
<th>Dataset name</th>
<th>Lab</th>
<th>Condition</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>fbs_10</td>
<td>Nice</td>
<td>unsynchronised, 10% FBS</td>
<td>72h, 15mins/frame</td>
</tr>
<tr>
<td>fbs_10_r</td>
<td>Rotterdam</td>
<td>unsynchronised, 10% FBS</td>
<td>72h, 15mins/frame</td>
</tr>
<tr>
<td>fbs_15</td>
<td>Nice</td>
<td>unsynchronised, 15% FBS</td>
<td>72h, 15mins/frame</td>
</tr>
<tr>
<td>dexpulse_fbs_10</td>
<td>Rotterdam</td>
<td>Dexamethasone treated, 10% FBS</td>
<td>84h, 30mins/frame</td>
</tr>
<tr>
<td>dexpulse_fbs_20</td>
<td>Rotterdam</td>
<td>Dexamethasone treated, 20% FBS</td>
<td>72h, 30mins/frame</td>
</tr>
<tr>
<td>dexpulse_fbs_20_2</td>
<td>Rotterdam</td>
<td>Dexamethasone treated, 20% FBS</td>
<td>72h, 15mins/frame</td>
</tr>
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</table>

Table S2: Tracked Cell Counts and Period Lengths

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<tr>
<th>Dataset name</th>
<th>Lineages</th>
<th>Divisions</th>
<th>Cell Cycles</th>
<th>Clock Period</th>
<th>Cell Cycle Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>fbs_10</td>
<td>76</td>
<td>173</td>
<td>422</td>
<td>21.9 +/- 1.1 h</td>
<td>21.3 +/- 1.3 h</td>
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<tr>
<td>fbs_15</td>
<td>69</td>
<td>477</td>
<td>1022</td>
<td>19.4 +/- 0.5 h</td>
<td>18.6 +/- 0.6 h</td>
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<tr>
<td>fbs_10_r</td>
<td>31</td>
<td>129</td>
<td>283</td>
<td>21.6 +/- 0.9 h</td>
<td>22.3 +/- 2.2 h</td>
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<td>dexpulse_fbs_10</td>
<td>73</td>
<td>380</td>
<td>660</td>
<td>24.2 +/- 0.5 h</td>
<td>20.1 +/- 0.9 h</td>
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<tr>
<td>dexpulse_fbs_20</td>
<td>57</td>
<td>316</td>
<td>677</td>
<td>23.1 +/- 0.9 h</td>
<td>19.1 +/- 0.7 h</td>
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<td>dexpulse_fbs_20_2</td>
<td>38</td>
<td>234</td>
<td>487</td>
<td>23.9 +/- 1.4 h</td>
<td>21.3 +/- 1.1 h</td>
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<tr>
<td>Dataset name</td>
<td>Success</td>
<td>G1/SG2M only</td>
<td>G1 Arrest/No Fit</td>
<td>Mean RAE</td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>-------------</td>
<td>--------------</td>
<td>------------------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>fbs_10</td>
<td>261 (97% / 62%)</td>
<td>21 / 25</td>
<td>96 / 161</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>fbs_10 (rae &lt; 0.25)</td>
<td>150 (96% / 66%)</td>
<td>12 / 12</td>
<td>50 / 76</td>
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<tr>
<td>fbs_10_r</td>
<td>134 (96% / 47%)</td>
<td>2 / 17</td>
<td>17 / 149</td>
<td>0.24</td>
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<tr>
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<td>100 (96% / 53%)</td>
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<td>9 / 88</td>
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<td>fbs_15</td>
<td>583 (100% / 57%)</td>
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<td>107 / 439</td>
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<tr>
<td>fbs_15 (rae &lt; 0.25)</td>
<td>452 (99% / 64%)</td>
<td>41 / 31</td>
<td>69 / 258</td>
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<tr>
<td>dexpulse_fbs_10</td>
<td>409 (98% / 62%)</td>
<td>1 / 89</td>
<td>135 / 251</td>
<td>0.17</td>
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<tr>
<td>dexpulse_fbs_10 (rae &lt; 0.25)</td>
<td>392 (99% / 68%)</td>
<td>1 / 85</td>
<td>125 / 185</td>
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<tr>
<td>dexpulse_fbs_20</td>
<td>368 (94% / 54%)</td>
<td>0 / 64</td>
<td>74 / 309</td>
<td>0.13</td>
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<tr>
<td>dexpulse_fbs_20 (rae &lt; 0.25)</td>
<td>315 (94% / 66%)</td>
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<td>47 / 164</td>
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<tr>
<td>dexpulse_fbs_20_2</td>
<td>186 (67% / 38%)</td>
<td>6 / 28</td>
<td>27 / 301</td>
<td>0.25</td>
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<tr>
<td>dexpulse_fbs_20_2 (rae &lt; 0.25)</td>
<td>111 (65% / 44%)</td>
<td>4 / 9</td>
<td>12 / 144</td>
<td>0.16</td>
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Table S4: Cell Cycle Event Analysis, and background vs. phase distribution test p-values

<table>
<thead>
<tr>
<th>Dataset Name</th>
<th>G1 Length</th>
<th>S/G2/M Length</th>
<th>Mean Division Phase</th>
<th>Mean G1-S Phase</th>
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<tr>
<td>fbs_10</td>
<td>10.5 ± 1.1 h</td>
<td>15.6 ± 1.7 h</td>
<td>3.83 ± 0.25</td>
<td>0.04 ± 0.24</td>
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<td>fbs_10_r</td>
<td>10.1 ± 1.3 h</td>
<td>14.6 ± 2.1 h</td>
<td>3.91 ± 0.27</td>
<td>0.02 ± 0.27</td>
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<td>(rae &lt; 0.25)</td>
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<td></td>
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<tr>
<td>fbs_10_r</td>
<td>9.5 ± 1.0 h</td>
<td>13.4 ± 1.3 h</td>
<td>3.78 ± 0.35</td>
<td>5.81 ± 0.33</td>
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<td>(rae &lt; 0.25)</td>
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<td>fbs_15</td>
<td>8.6 ± 0.4 h</td>
<td>10.2 ± 0.4 h</td>
<td>3.97 ± 0.14</td>
<td>0.11 ± 0.15</td>
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<td>(rae &lt; 0.25)</td>
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<tr>
<td>fbs_15</td>
<td>8.7 ± 0.4 h</td>
<td>9.7 ± 0.3 h</td>
<td>3.99 ± 0.14</td>
<td>0.12 ± 0.16</td>
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<tr>
<td>dexpulse_fbs_10</td>
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<td>11.4 ± 0.7 h</td>
<td>3.50 ± 0.44</td>
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<td>dexpulse_fbs_20</td>
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<tr>
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<td>6.4 ± 0.5 h</td>
<td>11.9 ± 0.5 h</td>
<td>3.68 ± 0.24</td>
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<td>3.90 ± 0.64</td>
<td>0.03 ± 0.47</td>
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<td>Grp.</td>
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<td>31</td>
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SUPPLEMENTARY FIGURES LEGEND

Figure S1: the FUCCI-2A system

a, Scheme of the FUCCI-2A construct. CMV pro: CMV promoter; RU5: 5’ long terminal repeats (LTR) from MMLV; (gag) - U3: contains CIS viral elements (e.g. Psi); E2-Crimson::Gem (blue): E2-Crimson::Geminin fusion. It is separated from the mKO2::hCdt1 (red) upon translation, through cleavage of the 2A peptide; IRES-puro: puromycin resistance allowing for selection of dual FUCCI positive cells, under control of an IRES. b, Restriction map of the FUCCI-2A transfer plasmid (pPRIPu CrUCCI). c, Cell Cycle analysis in proliferating NIH3T3_Reverbα::Venus-FUCCI-2A cells. Hoechst staining profile and cell fluorescence have been superimposed. P5 area represents cells with a 2C DNA content (G1 or Early S). In P9 area, DNA content progressively increases from 2C to 4C (S phase). In P10 area, cells have a 4C DNA content (G2). As expected, cells that only express mKO2 fluorescence (red) are in G1 and cells that only express E2-Crimson (blue) are in S/G2. Cells that express both markers have a DNA content consistent with “Early S” phase. d, e, and f, Box plot showing Reverba (d), CcnE2 (e) and CcnA2 (f) mRNA levels found in cells in different cell cycle phases. Cells were sorted into early G1, G1, early S and S/G2/M based on cell cycle markers. qRT-PCR revealed that Reverba and cyclins expression was as expected (i.e. Reverba peaking in early G1/G1, CyclinA2 is absent in G1 and CyclinE2 is expressed in G1 and early S).

Figure S2: Timeseries Analysis Results

a, Clock (blue) and Cell Cycle (red) Period lengths analysis. From left to right: 10% FBS unsynchronised, 15% FBS unsynchronised, 10% Dexamethasone-treated, 20% Dexamethasone-treated, and repeat experiment for 20% Dexamethasone treatment below on the right. b, Phase histograms for G1-S transition, from left to right: 10% FBS unsynchronised, 15% FBS unsynchronised, 10% Dexamethasone-treated. c, Phase histograms for cell divisions, from left to right: 10% FBS unsynchronised, 15% FBS unsynchronised, 10% Dexamethasone-treated. d, Population level analysis of clock peaks (blue/top), cell divisions (red/middle), and G1-S transitions (yellow/bottom) over the course of the experiment. From left to right: 10% FBS unsynchronised, 15% FBS unsynchronised, 10% Dexamethasone-treated. e, Split-population analysis for Clock/Cell Cycle Period ratios of 1:1 and 3:2. On the left: The first/purple histograms show the clock phases at cell division for the 1:1 coupled subpopulation. The second/green histograms show the 3:2 coupled subpopulation. On the right: population-level clock peak and cell division histograms. We show data from two separate experiments in 20% FBS with Dexamethasone treatment.
(dexpulse_fbs_20 and dexpulse_fbs_20_2), and the result of combining the datasets on the bottom.

Conditions: fbs_10 = Unsynchronized cells with 10% FBS; fbs_15 = Unsynchronized cells with 15% FBS; dexpulse_fbs_10 = Dex-synchronized cells with 10% FBS, dexpulse_fbs_20 = Dex-synchronized cells with 20% FBS

Figure S3: Cell Division Clustering Analysis for Unsynchronised Cell Populations
We show the results from our two types of clustering for the fbs_10 (subfigure a.) and fbs_15 (subfigure b.) datasets. The top row shows the clusters obtained using 2-D clustering on the clock phase at cell division, and the experiment time. On the left, we show the clusters, on the right, we show the histograms for the clock phase at cell division that correspond to each cluster. Below, we show the clusters obtained when grouping cell cycle intervals by their clock/cell cycle period ratio. We show a scatterplot of clock and cell cycle periods on the left, the resulting period histograms and counts for both groups in the middle, and the resulting clusters on a scatterplot of clock phase at cell division over time on the right. In both cases, the ratio isolates a dominant subgroup of cells which have a 1:1 coupling ratio, and a separate, smaller group of cells which corresponds to stochastic ‘skipping’ behaviour (i.e. cells that move between stable trajectories on the torus due to molecular noise).

Conditions: fbs_10 = Unsynchronized cells with 10% FBS; fbs_15 = Unsynchronized cells with 15% FBS

Figure S4: Cell Division Clustering Analysis for Dexamethasone-treated Cell Populations
We show the results from our two types of clustering for the dexpulse_fbs_10 (subfigure a.) and dexpulse_fbs_20 (subfigure b.) datasets. The top row shows the clusters obtained using 2-D clustering on the clock phase at cell division, and the experiment time. On the left, we show the clusters. On the right, we show the histograms for the clock phase at cell division that correspond to each cluster. Below, we show the clusters obtained when grouping cell cycle intervals by their clock/cell cycle period ratio. We show a scatterplot of clock and cell cycle periods on the left, the resulting period histograms and counts for both groups in the middle, and the resulting clusters on a scatterplot of clock phase at cell division over time on the right.

For 10% FBS, we can see that there may be a small subpopulation that couples at a ratio of > 1.1, and a dominant subpopulation that couples at a ratio of 1:1. The clock phase at cell
division/experiment time clustering shows that clusters of cell divisions exist, however, the transitions between these clusters within the same lineage are not very stable.

For 20% FBS, we clearly see two subpopulations of lineages. On the top, these can be identified as 1) lineages with their first division in the red cluster, and subsequent divisions in the blue and purple clusters (arrows going right, and right), and 2) lineages with their first division in the red cluster and subsequent divisions in the yellow and green clusters (arrows going down and then up). Subgroups of a very similar composition are identified when clustering by clock/cell cycle period ratio.

Conditions: dexpulse_fbs_10 = Dexamethasone-synchronised cells with 10% FBS; dexpulse_fbs_20 = Dexamethasone-synchronised cells with 20% FBS

**Figure S5: Cell Division Clustering Analysis for Dexamethasone-treated Cell Populations, repeat experiment**

We show the results of a second experiment with Dexamethasone-synchronised cells in 20% FBS. Our findings largely agree with the results shown in Figure S4 b (however, note that the number of tracked cells is significantly lower compared to dataset dexpulse_fbs_20).

**Figure S6: Modelling Clock and Cell Cycle as Coupled Oscillators**

a, Solutions for the (deterministic) phase differential equations. Left panel: ratio = 1:1, middle panel: ratio = 5:4, right panel: 3:2;

b, Stochastic simulation results for 5:4 coupling;

c, Estimated Poincaré maps from four datasets which were used to inform our modelling. 1:1 coupled subpopulations produce a concentrations of return points around \( (\frac{\pi}{2}, \frac{\pi}{2}) \), which is where our deterministic model has its stable fixed point.

**Figure S7: Estimated vectorfields**

Estimated vectorfields/phase diagrams from experimental data for cells in the conditions indicated above each plot. The red dashed curve shows the mean trajectory and the blue levels show the density of cells passing through a region. The arrows show the mean direction that the cells flow in near that point on the torus.
Legends for the supporting videos.

**Supplementary Movie 1**: Dividing cells cultured in 15% FBS. We overlay the brightfield image with the three channels for the nuclear markers (red: Cdt1:mKO2 / G1 phase, blue: Geminin:E2Crimson / S-G2-M phases, and green: Reverba:Venus / Clock). We can see many cells start transparent or green (corresponding to low/high levels of the clock marker), then turn red during the G1 phase. Many cells will in fact turn yellow (i.e. a mixture of red and green), indicating that the clock marker is at a high level during the G1-S transition. Around the G1-S transition, the red marker drops to near zero, and the blue marker starts rising, reaching its maximum level (making blue or cyan nuclei) just before cell division.

**Supplementary Movie 2**: Temporal progression of clock and cell cycle phases for unstimulated cells in 15% FBS. We show the clock phase on the horizontal axis, illustrated by a green and black bar on the top which shows the relative clock marker level (high on the sides, low in the middle). The vertical axis shows the progression of the cell cycle. The coloured bar on the right-hand side illustrates the relative levels of the cell cycle markers (black to red in G1, and red to blue in S-G2-M). We also mark G1-S transition and cell division as horizontal yellow lines. Cells are drawn as blue dots (turning grey once they become confluent) which move from the bottom to the top as they progress though the cell cycle, and from the left to the right according to their clock phase (in this diagram measured as the normalized time between two clock peaks). In the background, we show an estimated vector field which indicates the mean direction cells are taking at each point in this phase space. On the sides, we show density estimates for the fraction of cells in in each phase. We see that most cells follow a main stream through the middle of the image, crossing the G1-S transition and cell division lines at a distinct mean clock phase each. Moreover, we observe that some cells skip: they leave the main stream of cells because they progress through the cell cycle phase at a slower speed, and re-join the other cells once they arrive at the main trajectory again. Note that we connect sibling cells by a dashed line when possible.

**Supplementary Movie 3**: Temporal progression of clock and cell cycle phases for Dex-stimulated cells in 10% FBS. This movie is similar to Supplementary Movie 2, however, we now colour the cells according to their division time. The density histogram on the top shows that cells most cluster around similar clock phases due to the Dex-synchronisation. Also, there is no main stream of cells anymore like in SM2. Rather, cells go through cell division in groups at a wider range of clock phases than in the unstimulated case. Towards the end of the movie, most cells become confluent.
Supplementary Figure S1 (Rand)
Supplementary-Figure-S2 (Rand)
Dataset fbs_10: 10% FBS, unsynchronised

Clustering by clock phase at cell division and time

Clustering by clock/cell cycle period ratios

Dataset fbs_15: 15% FBS, unsynchronised

Clustering by clock phase at cell division and time

Clustering by clock/cell cycle period ratios
Supplementary-Figure-S4 (Rand)
a Dataset dexpulse_fbs_20_2: 20% FBS, Dex-stimulated

Clock Phase at Division

0 0 2π 0 0 2π 0 0 2π

Experiment Time [h]

0 0 2π 0 0 2π 0 0 2π

1 2 3 4 5 6

Clock Phase at Division

0 0 2π 0 0 2π 0 0 2π

Count

0 0 2π 0 0 2π 0 0 2π

b Dataset fbs_15: 15% FBS, unsynch.

c Dataset dexpulse_fbs_10: 10% FBS, Dex-stimulated

Cell Cycles Observed

Clock Period [h]

0 0 2π 0 0 2π 0 0 2π

0 0 2π 0 0 2π 0 0 2π

r=1.0, n=50

Experiment Time [h]

0 0 2π 0 0 2π 0 0 2π

0 0 2π 0 0 2π 0 0 2π

Supplementary-Figure-S5 (Rand)
Supplementary-Figure-6 (Rand)
Dataset fbs_10: 10% FBS, unsynchronised

Dataset fbs_15: 15% FBS, unsynchronised

Dataset dexpulse_fbs_10: 10% FBS, Dex-stimulated

Dataset dexpulse_fbs_20: 20% FBS, Dex-stimulated

Supplementary-Figure-7 (Rand)
Supporting Information
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Movie S1. Dividing cells cultured in 15% FBS. We overlay the brightfield image with the three channels for the nuclear markers (red, Cdt1:mKO2/G1 phase; blue, Geminin:E2Crimson/5–G2–M phases; and green, Reverba:Venus/Clock). We can see many cells start transparent or green (corresponding to low/high levels of the clock marker) then turn red during the G1 phase. Many cells will in fact turn yellow (i.e., a mixture of red and green), indicating that the clock marker is at a high level during the G1–S transition. Around the G1–S transition, the red marker drops to near zero, and the blue marker starts rising, reaching its maximum level (making blue or cyan nuclei) just before cell division.

Movie S1

Movie S2. Temporal progression of clock and cell-cycle phases for unstimulated cells in 15% FBS. We show the clock phase on the horizontal axis, illustrated by a green and black bar on the top that shows the relative clock marker level (high on the sides, low in the middle). The vertical axis shows the progression of the cell cycle. The colored bar on the right-hand side illustrates the relative levels of the cell cycle markers (black to red in G1, and red to blue in S–G2–M). We also mark the G1–S transition and cell division as horizontal yellow lines. Cells are drawn as blue dots (turning gray once they become confluent) that move from the bottom to the top as they progress through the cell cycle, and from the left to the right according to their clock phase (in this diagram measured as the normalized time between two clock peaks). In the background, we show an estimated vector field that indicates the mean direction cells are taking at each point in this phase space. On the sides, we show density estimates for the fraction of cells in each phase. We see that most cells follow a main stream through the middle of the image, crossing the G1–S transition and cell-division lines at a distinct mean clock phase each. Moreover, we observe that some cells skip: They leave the main stream of cells because they progress through the cell cycle phase at a slower speed and rejoin the other cells once they arrive at the main trajectory again. Note that we connect sibling cells by a dashed line when possible.

Movie S2
Temporal progression of clock and cell-cycle phases for dexamethasone-stimulated cells in 10% FBS. This movie is similar to Movie S2; however, we now color the cells according to their division time. The density histogram on the top shows that cells most cluster around similar clock phases owing to the dexamethasone synchronization. Also, there is no main stream of cells anymore like in SM2. Rather, cells go through cell division in groups at a wider range of clock phases than in the unstimulated case. Toward the end of the movie, most cells become confluent.

Movie S3

Other Supporting Information Files

SI Appendix (PDF)