Division of labour between Myc and G1 cyclins in cell cycle commitment and pace control

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A body of evidence has shown that the control of E2F transcription factor activity is critical for determining cell cycle entry and cell proliferation. However, an understanding of the precise determinants of this control, including the role of other cell-cycle regulatory activities, has not been clearly defined. Here, recognizing that the contributions of individual regulatory components could be masked by heterogeneity in populations of cells, we model the potential roles of individual components together with the use of an integrated system to follow E2F dynamics at the single-cell level and in real time. These analyses reveal that crossing a threshold amplitude of E2F accumulation determines cell cycle commitment. Importantly, we find that Myc is critical in modulating the amplitude, whereas cyclin D/E activities have little effect on amplitude but do contribute to the modulation of duration of E2F activation, thereby affecting the pace of cell cycle progression.
E2F transcriptional factors are a family of proteins that bind to overlapping sets of target promoters, regulating cell cycle progression and cell-fate decisions. Enforced E2F1 expression can induce quiescent cells to enter S phase, and genetic loss of all activator E2Fs (E2F1-3) completely abolishes the ability of normal fibroblasts to enter S phase. Substantial evidence supports the view that the Rb/E2F network orchestrates the precise regulation of E2F activation (Fig. 1). The canonical view is that mitogen-driven expression of D-type cyclins and activation of their partners cyclin-dependent kinase (CDK) 4/6 initialize the phosphorylation of Rb, releasing existing E2F protein from Rb sequestration. Free E2F can then transcribe Cyclin E, which together with CDK2, hyper-phosphorylates Rb, resulting in full activation of E2F. The potent oncogene, Myc, dramatically affects E2F activity, presumably through modulating G1 cyclins expression as well as cyclin-dependent kinase (CDK) activities. However, restoration of Cyclin D level, despite succeeding in restoring the kinetics of Rb phosphorylation to normal, fails to rescue slow-growth phenotypes in c-Myc-deficient cells. Moreover, it was recently showed that Myc is also required for allowing the interaction of the E2F protein with the E2F gene promoters suggesting a direct and Rb-independent regulatory role of Myc on E2F activation through interfering with E2F auto-regulation. In addition, several target genes of E2F, such as Cyclin A and Skp2, contribute to negative feedback loops and affect E2F activity through direct regulation of its transcriptional activity or protein degradation.

It has been generally accepted that the commitment into cell cycle is determined by E2F activation because of G1 cyclin/CDK complex-mediated Rb phosphorylation. However, it appears difficult to reconcile this view with the observation that major phosphorylation of Rb occurs after the restriction point other events may be more critical for the initial E2F activation.

Conventional approaches based on population analysis cannot adequately address this question, in light of extensive heterogeneity in gene expression among cells that can mask or obfuscate the contributions from different regulatory elements. Single-cell analysis provides the opportunity to follow the dynamics of signalling molecules that reflect how an individual cell enters and decodes information that result in a particular cellular outcome.

To this end, we used time-lapse fluorescence microscopy to follow E2F1 temporal dynamics in single cells. Guided by mathematical modelling, we set out to address several specific questions. In particular, do E2F dynamics determine the commitment to cell cycle entry in individual cells? If so, what aspects of E2F temporal dynamics are the major determinants of cell cycle entry? How do Myc and G1 cyclins affect different aspects of E2F temporal dynamics? How do their effects manifest themselves in the ability of a single cell to enter and pace the cell cycle? In contrast to the canonical view, our results reveal that Myc and G1 cyclins contribute to distinct aspects of the E2F temporal dynamics, despite their apparently overlapping roles. In particular, Myc primarily sets the maximum E2F level, which in turn determines commitment to cell cycle entry. G1 cyclins, however, control the timing for reaching the maximum level and thus the pace of cell cycle progression. We find that these distinctive modes of control over the E2F temporal dynamics are an intrinsic dynamic property of the core Rb/E2F network. On one hand, our results elucidate the different roles that Myc and G1 cyclins play in controlling cell cycle entry and progression. On the other hand, this ‘division of labour’ represents a novel, perhaps general, strategy to integrate different signals (Myc versus G1 cyclins) through a common ‘signal carrier’ E2F.

### Results

#### Quantification of E2F dynamics in single cells.

To measure E2F1 transcriptional dynamics in single cells, we re-engineered the reporter construct from Yao et al. to generate a brighter fluorescent signal that is largely localized to the nucleus (Fig. 2a). The improvements facilitated segmentation and signal extraction in individual cells, as nuclei are well separated from each other in the field of observation. Individual cell clones (REF52-hE2F1p::4NLS-d4Venus) expressing the reporter construct were isolated and used for experimental validation. Consistent with our expectations, the majority of the Venus fluorescence signal in independent clones was restricted to the nucleus, with a small amount of the signal localized around the nucleus (Fig. 2b).

We confirmed that the reporter recapitulated E2F1 dynamics at the population level by comparing the individual trajectories and endogenous E2F1 mRNA expression over time (Supplementary Fig. 1a). Furthermore, we found that the accumulation of Venus and endogenous E2F1 proteins were also concordant, although minor differences can be seen, particularly in later time points (Supplementary Fig. 1b). Altogether, the dynamic expression of the reporter correlated with endogenous E2F1 expression throughout the time course of the experiment.

We next used this proxy system to examine the real-time dynamics of E2F in single REF52 rat fibroblasts held in G0 through serum starvation and released into the cell cycle by serum stimulation. For each time point, we quantified the level of fluorescence in individual cells, which could be accurately measured up to early M phase, when the nuclear membrane starts to fall apart (Fig. 2b, Supplementary Movies 1 and 2). The temporal E2F dynamics in individual cells were highly variable (Fig. 2c), but exhibited a consistent pattern: after an initial delay, the E2F1 signal increased from a basal level to a maximum value and then slowly decreased (Fig. 2d). Moreover, the same overall pattern was observed in single cells isolated from other clones transduced independently with our reporter gene.

We then defined a set of metrics to quantify the observed E2F dynamics (Fig. 2d). These include maximum amplitude (Amp), initial delay (t1), activation time (t2) and post-activation time (t3) for cells that undergo division only. Amp and t2 can be combined to define two additional metrics—the slope (k = Amp/t2) and the area under the curve (S = Amp × t2/2); k corresponds to the rate increase, which reflects the strength of positive feedback loop in the regulation, whereas S correlates with the total transcription work of the network directed at the E2F1 promoter (Fig. 2d). See Fig. 2d legend for additional definitions.

To evaluate these metrics, we measured E2F1 temporal dynamics in ~100 individual cells for each level of serum activation and calculated their values for each cell. Values of the four metrics (as shown for Amp, t1, t2 and t3) were highly variable among individual cells (Fig. 2e–h) because of stochastic...
gene expression. Notably, Amp exhibited bimodal distribution (ON/OFF) at an intermediate serum concentration (Fig. 2e), consistent with the bistability of the Rb/E2F switch. Moreover, decreasing serum concentration led to significant change in the distribution of Amp but moderate increase of average values of $t_1$, $t_2$ and $t_3$ (Fig. 2e–h). Pairwise correlation analyses revealed a weak correlation between Amp and $t_2$, and no significant correlations between other pairs, suggesting little dependence among these metrics (Supplementary Fig. 1c).

**Amp as the predictor of commitment and division.** We next examined the extent by which the metrics defined above could predict cell-cycle entry commitment and proliferation (Fig. 3a).

To this end, we combined measurements of single-cell E2F dynamics with measurement of 5-ethyl-2'-deoxyuridine (EdU) incorporation into newly synthesized DNA, a surrogate for determining cell cycle entry (Fig. 3b and c). We found that Amp is a reliable predictor of commitment. Specifically, plotting EdU signals against Amp values for $B_{100}$ cells revealed two distinct groups of cells: cells with high EdU incorporation had an Amp value above a threshold (Amp$_{th}$), whereas cells with no EdU labelling showed a value of Amp below it (Fig. 3d). Two Amp-related metrics—$k$ and $S$—were also informative, albeit less accurate at predicting cell cycle entry (Supplementary Fig. 2a and b). The other metrics ($t_1$ and $t_2$) were much less reliable predictors (Supplementary Fig. 2c and d).
As we showed that the Amp and k parameters correlate with the commitment into S phase, it stood to reason that they would also predict cell division. To confirm this prediction, we followed cell division over time in single cells and linked it to E2F dynamics. Data points from individual cells stimulated with different serum levels were aggregated and split into two groups depending on whether the cells had divided. A small proportion of cells (≤5%) within the undivided group displayed high Amp. These cells were likely within the t2 interval, and would have been observed to divide if we had kept monitoring them longer (Supplementary Fig. 3). In subsequent analyses, we excluded these cells as we lacked definitive information about their ultimate behaviour. The aggregate data were used to plot the distribution of cells as a function of the values of the corresponding E2F1 dynamic metrics measured in single cells. When plotted against Amp or k, we observed a clear-cut boundary between the group of cells that had divided and the one in which cells had not (Fig. 3e, Supplementary Fig. 2e). This distinction became less apparent when the same data were plotted against S and was lost for t1 or t2 (Supplementary Fig. 2f–h).

Next, we performed logistic regression to estimate the probability of cell commitment or division as a function of different metrics. Our results demonstrated that Amp serves as the most accurate predictor, with a confidence value of 95% ± 1% for commitment (96% ± 1% for division; Table 1). Importantly, we observe an ultrasensitive dependence between the probability of cell commitment/division and the Amp value, reflected in Hill coefficients of >7 for logistic regression curves (Fig. 3f). A slight increase of Amp from 2.2 to 2.8 a.u. (12% in the scale of mean

Table 1 | Correctness of commitment or division as predicted by different E2F1 dynamics metrics.

| Metric | Commitment (%) | Division (%) |
|--------|----------------|-------------|
| Amp    | 95             | 96          |
| k      | 90             | 96          |
| S      | 85             | 85          |
| t1     | 81             | 83          |
| t2     | 75             | 76          |
| Random*| 50             | 50          |

*As a control, a random guess predicts 50% correctly.
*Amp* in E2F1-activated cells) within the ultra-sensitive region can lead to a 50% relative increase in the proportion of divided cells (Fig. 3f). This observed ultrasensitive dependence further defines a threshold value *Amp* \( t_\text{th} \). If *Amp* of an individual cell reaches this threshold, it has at least a 50% probability to commit into the cell cycle. Moreover, we observed a similar requirement for *Amp* in cell-cycle commitment and division in mouse embryonic fibroblast NIH3T3 cells, in which the integrated reporter gene was driven by the mouse E2F1 promoter (Supplementary Fig. 2i–k).

**Distinct roles of G1 Cyclins and Myc revealed by modelling.** Considering the predictive power of E2F *Amp* for individual cells’ commitment to division, we wondered how it would be affected by perturbations on the Rb/E2F network. To this end, we employed an established kinetic model to perform time-course simulations, which can be compared with our experimental observations (Fig. 1, Supplementary Tables 1–4). The full model generates E2F transcriptional dynamics that are consistent with our experimental observations (Supplementary Fig. 4). The characteristic E2F pulsatile dynamics are maintained even when each parameter was varied by 100-fold around its base value. We further performed sensitivity analysis of each metric (*Amp*, \( t_2 \) and \( S \)) with respect to changes in 33 parameters in the full model. The analysis indicates that only parameters associated with Myc-dependent E2F autoregulation can lead to dramatic change in *Amp* (Supplementary Fig. 5a). Parameters associated with Myc-dependent E2F autoregulation, Rb-E2F interaction, or Cyclin and CycinE accumulation can significantly affect \( t_2 \), \( k_s \) and \( S \) (Supplementary Fig. 5b–d). In contrast, parameters associated with the E2F-negative feedback loop have little effect on either *Amp*, \( t_2 \) or \( k_s \) (Supplementary Fig. 5a–c). These results suggest that different modules within the full system influence different aspects of E2F dynamics.

We next examined the modulation of *Amp* by perturbations on three main regulators—CycD, CycinE and Myc—\( t_2 \). CycD and CycinE nodes play a critical role in controlling the function of Rb by phosphorylation, facilitating activation of E2F. They have been suggested as constituting the rate-limiting step in cell cycle entry. In contrast, Myc has been shown to be critical for the simulation analysis of each metric (*Amp*, \( t_2 \) and \( S \)) with respect to changes in 33 parameters in the full model. The analysis indicates that only parameters associated with Myc-dependent E2F autoregulation can lead to dramatic change in *Amp* (Supplementary Fig. 5a). Parameters associated with Myc-dependent E2F autoregulation, Rb-E2F interaction, or Cyclin and CycinE accumulation can significantly affect \( t_2 \), \( k_s \) and \( S \) (Supplementary Fig. 5b–d). In comparison, parameters associated with the E2F-negative feedback loop have little effect on either *Amp*, \( t_2 \) or \( k_s \) (Supplementary Fig. 5a–c). These results suggest that different modules within the full system influence different aspects of E2F dynamics.

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Figure 4 | Modelling predicts distinct modes of regulation on E2F dynamics by G1 cyclins and Myc. (a–c) Normalized Amp (to the Amp base parameter values) within 120 h under the inhibition of CDK4/6, CDK2 or Myc as function of inhibition rate. Horizontal dashed lines mark that the expected threshold value for normalized \( \text{Amp}_{\text{th}} \) is 0.5, given that the measured \( \text{Amp}_{\text{th}} \) is around half of the average Amp of cells (2.5 versus 5, Fig. 3e). (d) Log sensitivity analysis of Amp to inhibition rate. Coloured lines are E2F synthesis curves under low (black) and high (grey) cellular unphosphorylated Rb levels, respectively. The decrease in Rb phosphorylation rate leads to only a slight reduction of cyclin complexes activity. The decrease in Rb phosphorylation rate leads to only a slight reduction (ΔAmp) in steady-state E2F level. Black and grey lines are E2F synthesis curves given high (black) and low (grey) synthesis rate, respectively. (e) Normalized \( t_2 \) to its initial level as a function of inhibition rate under each inhibition case. Yellow shading highlights the region where cells are expected to commit into cell cycle. (f) Log sensitivity analysis of \( t_2 \) to inhibition rate. For \( \text{Amp}_{\text{th}} \), log sensitivity was calculated within the committed region. (g) The central motif of Myc/Rb/E2F network includes E2F auto-regulation (module for level control) and its titration by Rb (module for timing control). (h) Steady-state analysis of the central motif under the inhibition of Rb phosphorylation through reduction of cyclin complexes activity. The decrease in Rb phosphorylation rate leads to only a slight reduction (ΔAmp) in steady-state E2F level. Black and grey lines are E2F synthesis curves under low (black) and high (grey) cellular unphosphorylated Rb levels, respectively. Red line indicates the degradation curve. Blue line indicates the threshold \( \text{Amp}_{\text{th}} \). Green dots, stable steady states; red dots, unstable steady states. (k) Steady-state analysis of the central motif. Black and grey lines are E2F synthesis curves given high (black) and low (grey) synthesis rate, respectively.

Control of commitment into cell cycle entry by Myc. To experimentally test the functional consequences induced by Myc inhibition, quiescent cells were stimulated with full serum in the presence of EdU and of different concentrations of either Myc-specific inhibitor 10058-F4 (which efficiently interferes with Myc/Max hetero-dimerization) or the bromodomain inhibitor (+)-JQ1 (which substantially suppresses c-Myc transcription) (Supplementary Fig. 7a). Consistent with our modelling analysis, single-cell analysis indicates that Myc inhibition dramatically prevents Amp to switch from an OFF mode to an ON mode, resulting in a sharp decrease in the proportion of both committed and divided cells (Fig. 6c, Supplementary Fig. 7b). Fitting the curves to a Hill function yielded a sharp sigmoid pattern for both division and commitment (Fig. 6c, Supplementary Fig. 7b). Moreover, we note a bimodal response at an intermediate inhibitor level (90 μM for 10058-F4 and 0.8 μM for (+)-JQ1; Fig. 6b, Supplementary Fig. 7c and d), which is around the half-inhibition threshold. At this dose, part of the cells was able to commit and they exhibited a strong E2F response (Fig. 6d). In contrast, the other cells remained in quiescence and they exhibited weak E2F responses (Fig. 6d). We also performed shRNA knockdown of endogenous c-Myc level to rule out the potential for a non-specific effect of the inhibitors (Supplementary Fig. 7e, Supplementary Table 5). A large proportion of the cells remained in the OFF mode after serum stimulation, fully supporting our findings obtained with two independent small-molecule inhibitors of Myc (Supplementary Fig. 7f). All these results are consistent with the bistable nature of E2F activation in response to upstream signals and suggest a critical role for Myc in this regulation. Importantly, they further underscore the predictable power of the parameter Amp in E2F dynamics for cell cycle commitment.

Discussion
Quantitative analysis of real-time dynamics of E2F1 transcription in single mammalian cells has allowed us to define the metrics that accurately predict cell commitment and proliferation. We find that the values of Amp and \( t_2 \) respond to two intimately linked but differentially controlled modes of regulation by the Myc/Rb/E2F network. Amp is critically dependent on Myc activity, whereas \( t_2 \) is tightly sensitive to the activity of G1 cyclin complexes.
**Amp** serves as the single most reliable predictor of cell cycle commitment in single cells. In particular, we show an ultrasensitive dependence on **Amp** for the probability of each individual cell to commit into the cell cycle. Importantly, this predictive power is probabilistic in nature. If **Amp** is at **Amp** (2.5), a cell has a 50% chance to commit. However, a slight increase in **Amp** (from 2.2 to 2.8) greatly boosts the probability (from 25 to 75%). In this case, because of the stochastic nature of single-cell behaviour, there is still a small probability by which a cell can stay in quiescence. In the temporal scale, transition across the **Amp** defines a critical time point after which a cell becomes likely to commit into division. This time point is reminiscent of the classical restriction point, but has the advantage that it can be precisely determined in single cells.

Remarkably, **Amp** has remained a reliable predictor of cell cycle commitment even under drastic perturbations of the Myc/Rb/E2F network in REF52 and NIH3T3 fibroblasts. As this network is highly conserved among different tissues and species, it will be interesting to test the generality of this principle among different cell types, including normal and cancerous cells. On the other hand, **t2** seems to account for the main variability for cell cycle duration, as its variation correlates with that of the cell cycle length. At the temporal scale, **t2** covers the late G1 phase and most (if not all) of S phase. This time window is most variable during the cell cycle, perhaps allowing cells to optimize conditions for the completion of the cell cycle in response to internal stress signals such as DNA damage response.

Although the role of the Rb/E2F pathway in regulating the cell cycle has been well documented, the precise contribution of how its core constituents mediate temporal steps in its execution has not been fully established. Our single-cell analysis of E2F activation dynamics has generated new mechanistic insights into this issue. First, we find that Myc is critical for cell cycle entry. Myc has long been recognized as a potent proto-oncogene, and its effect on cell proliferation was largely attributed to its regulation of G1 cyclins expression and CDK activities. However, our results show that Myc’s contribution is primarily due to its direct modulation of the E2F **Amp**. Our findings therefore challenge the

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**Figure 5 | Control of timing in cell-cycle progression but not commitment decision by G1 Cyclin/CDK complexes.** (a) Scatter plot based on commitment as determined by **Amp** under inhibition of CDKs (PD032991 for CDK4/6 and CVT-313 for CDK2). Committed cells were measured by EdU labelling after 48 h incubation, and divided cells were counted 48 h after addition of 10% BGS. (b) Histogram based on cell division as determined by **Amp** under inhibition of CDKs (48 h observation). (c) Proportion of committed/divided cells under different inhibitions of CDKs during a 48- and 92-h (the latter only for the combined inhibition) observation window. The exact number of total cells followed and that of cells committed/divided are indicated on top of each error bar. (d) Four representative E2F1 dynamics trajectories under the same conditions as a. Each colour bar on top indicates the length of **t2** of its corresponding trajectory. (e) Distribution of **t2** of individual cells in mock, CDK4/6-, CDK2- and combinational inhibited samples. Mean ± s.d. is shown on top right of each panel, 54 (Vehicle), 106 (15 nM PD032991), 102 (100 nM PD032991), 109 (1 μM PD032991), 94 (1 μM CVT313), 105 (5 μM CVT313) and 101 (5 μM PD032991 and 5 μM CVT313) cells were analysed for each condition, respectively. (f) Plot of the first cell cycle length (T) under the condition as in e. Every dot represents a single cell. Black cross indicates the median of T among the cells divided within 48 h. *P* = 0.01855, Wilcoxon one-side test. For all testing results, *P* < 0.005 unless marked.
general view that G1 cyclins are predominantly responsible for controlling the commitment to cell cycle entry. Instead, our results show that the primary role of G1 cyclins is to tune the timing and duration of the cell cycle. This conclusion is consistent with observations in knockout mouse models where G1 cyclins and CDKs are largely dispensable for cell cycle entry, although they affect efficacy or timing of commitment49–53. Taken together, our results reveal that the Myc/Rb/E2F network coordinates two independent control modes of cell cycle progression: the decision of ‘whether’ by Myc and that of ‘when’ by G1 cyclins. These two control modes may also be targeted to regulate cell cycle exit. For instance, reduction of Myc levels and activity would lead to a quick exit from the cell cycle, as is required for contact inhibition or terminal differentiation14,54. Furthermore, potent inhibition of G1 cyclins would likely trap cells in an infinitely long and futile cell cycle, resulting in cellular senescence.

Yao et al.4 have established the relationship between bistable E2F activation and the crossing of the restriction point using flow-cytometry measurements. In contrast, use of fluorescence microscopy enabled us to measure E2F temporal dynamics in single cells, and to correlate this information with single-cell decisions. Our approach revealed a clear distinction in the roles that Myc and G1 cyclins have on the control of E2F activation. Our results reveal an unappreciated and critical requirement for Myc-dependent E2F autoregulation that controls the cell-cycle commitment decision of fibroblasts released from G0 after serum stimulation.

Using a CDK2 activity reporter, Spencer et al.55 recently reported that the proliferation-to-quiescence decision in continuously growing cells is controlled by a bifurcation in CDK2 activity after mitotic exit. They also showed that the bifurcation is regulated by the CDK inhibitor p21 and mitogen signals. It is interesting to note that the stability of Myc is tightly regulated by mitogen signals through MAPK and Akt pathways56,57. Moreover, p21 can function as a repressor of Myc transcription or directly interfere with Myc transcriptional activity by blocking Myc/Max complex formation58,59. Given our finding that Myc is the driver of cell cycle commitment, Myc activity may also influence the control of proliferation-to-quiescence decision by p21 and mitogen signals. Therefore, it will be interesting to combine the E2F and CDK2 reporter systems together to better understand the temporal and mutual (if any) relationship in the dynamics of these two factors and to investigate how Myc, CDK2, p21 and the mitogen signals combine to regulate the normal cell cycle.

Last, the measurement of single-cell E2F dynamics provides a quantitative and sophisticated framework for understanding the control logic of cell cycle entry. Clear delineation of the precise function of each effector in the Myc/Rb/E2F network implicated in cell cycle control has implications for developing effective strategies for cancer therapy.
Methods

E2F1 transcriptional reporter system. A DNA fragment encoding d4Venus (destabilized Venus with ~4 h half-life) was cut from pdVenus-N1 and fused with four repeats of SV40 nuclear localization sequence (NLS) at its N-terminus to generate the 4NLS-d4Venus-expressing cassette.20 This cassette was then subcloned after the human E2F1 promoter (~784 to ~32) into a pcQXIP vector (Clontech) to construct the pcQXIP-hE2F1p-4NLS-d4Venus reporter plasmid.21 The derivative construct was transfected into an eukaryotic packaging cell line, Plat-E1. Forty-eight hours after transfection, the culture medium containing retrovirus particles was filtered and applied to REF52 cells. A clonal pool genetically integrated with the reporter system was selected after cells were cultured in medium containing puromycin (2 μg/ml) for approximately 10 days. Single clones (REF52-hE2F1p:4NLS-d4Venus) were picked up from the clonal pool by dilution in μ-diluted pcQXIP-hE2F1p-4NLS-d4Venus reporter system leads to two significant improvements appropriate for single-cell analysis. On one hand, the incorporation of four NLSs in the protein drives its translation into the nucleus, thereby increasing the intensity of the fluorescent signal. On the other hand, it facilitates segmentation and signal extraction in individual cells, as nuclei in different cells are well separated. Based on a similar strategy, a mouse E2F1 promoter (~1,165 to +123) was amplified from mouse embryonic fibroblast NIH3T3 cells to derive the NIH3T3-mE2F1p:4NLS-d4Venus reporter cells.

Cell culture. REF52 (an immortal line of postcrisis Fischer rat embryo cells) and NIH3T3 mouse fibroblasts (CRL-1658, ATCC) were routinely grown in Minimum Essential Medium Alpha Medium (Gibco/Invitrogen) supplemented with 10% bovine growth medium (BGS, Hyclone/Thermo Scientific). For the preparation of medium, calf serum (C-0539, Sigma-Aldrich) was diluted in Minimum Medium Alpha Medium (Gibco/Invitrogen). For infection, cells were plated in 10 cm tissue culture dishes and cultured in minimum essential medium (tissue culture treated, ibidi) channel slides by adding 1 ml volume of the cell medium for 36 h. For perturbation experiments, PD0332991 (CDK4/6 inhibitor, Selleckchem), UO126 (MEK inhibitor, Selleckchem), 10058-F4 (c-Myc inhibitor, Enzo Life Science), CVT-313 (CDK2 inhibitor, Enzo Life Science), 10058-F4 (c-Myc inhibitor, Sigma Aldrich) and (−) JQ1 (bromodomain and extra terminal domain inhibitor, Cayman Chemical) were added into cells immediately after cells were released from serum starvation (by adding 10% BGS) in either single or combined way. For 92 h perturbation experiment with both PD0332991 and CVT-313, cells were growing with replaced fresh medium with fresh inhibitors after the initial 48 h.

Live cell imaging. For time-lapse microscopy, quiescent cells growing in μ-Slide I slides were released from starvation by shifting to serum containing medium and placed under Leica DMI 6000 B inverted fluorescence microscope (Leica). Images were taken using Leica N PLAN L 20 × 0.4 objective lens with phase contrast or aSemrock Brightline YFP filter set (502/542 nm, excitation/emission) and Hamamatsu ORCA AG digital camera (Hamamatsu) with uniform parameter setting: binning = 4, offset = 0, gain = 255 and exposure time = 0.01 s (phase channel) or 0.15 s (YFP channel). The microscope was enclosed with an environmental chamber with 37°C temperature, atmosphere (5% CO2) and humidity. Images were acquired every 30 or 60 min for 24–48 h. Time-series image acquisition was controlled by SimplePCI6 Software (Hamamatsu).

EdU staining and imaging. EdU staining was performed after E2F dynamics measurement in ibidi μ-Slide I slides by using Click-it EdU Imaging Kits (Invitrogen) with Alexa Fluor 594 azide according the manufacturer’s instructions. The only modification of the protocol was to wash cells with PBS with 0.2% Tween three times after all the staining steps. Images were taken using Leica N PLAN L 20 × 0.4 objective lens with phase contrast or a Semrock Brightline Texas Red filter set (562/624 nm, excitation/emission) and Hamamatsu ORCA AG digital camera with uniform parameter setting: binning = 4, offset = 0, gain = 255 and exposure time = 0.01 s (phase channel) or 0.8 s (Red channel).

Western blot analysis. Antibodies against E2F1 (1:1,000, Cell Signaling), phosphorylated RB Ser780 (C-15, 1:1,000, Santa Cruz), phosphorylated RB Thr821/826 (C-15, 1:1,000, Santa Cruz), phosphorylated Rb Ser780 (C-15, 1:1,000, Santa Cruz), phosphorylated Rb Thr821/826 (C-15, 1:1,000, Santa Cruz), c-Myc (sc-16669-R, 1:1,000, Santa Cruz), GFP (XP Rabbit mAb, 1:1,000, Cell Signaling), CDK2 (78B2 Rabbit mAb, 1:1,000; Santa Cruz) and Actin (C-2, 1:1,000; Santa Cruz) were selected for Western blot analysis. Filters were blocked up from the blocking solution by dilution in μ-diluted pcQXIP-hE2F1p-4NLS-d4Venus reporter system.

Quantitative reverse transcription–PCR analysis. RNA extracts were prepared from REF52-hE2F1p::4NLS-d4Venus reporter cells using the RNeasy Protect Mini Kit (Qiagen) according to the manufacturer’s instructions. E2F1 RNA expression was interrogated by real-time PCR using the Power SYBR Green RNA-to-Ct 1-step Kit (Applied Biosystems). Gene-specific primers used: 5′-TTG ACCCTCTGGATTTG-3′ and 5′-CCCTTGGTGCTCAGATGT-3′ for rat E2F1; 5′-GTCGACATCCGACTGTGTT-3′ and 5′-CTCTCAAGTGCTGTT- GAA-3′ for rat β-actin.

RNA interference. shRNAs for targeting CDK2 or c-Myc were generated by cloning shRNA sequences (Supplementary Table 5) into Tet-LEK-2A-mCherry vector.34 Lentiviral packaging reactions were performed in the 293T cell line in the presence of packaging plasmids psPAX and pMD2.G using Lipofectamine Transfection Reagent (Invitrogen). Viral supernatants were collected 48 h after transfection, filtered through a 0.45 μm acrylic membrane filter (Corning). For infection, cells were plated in 10 cm tissue culture dishes and allowed to achieve 20–30% confluence before adding viral supernatant in the presence of 7μg/ml polybrene for 12 h (EMD Millipore). Cells were collected and sorted by flow cytometry for 5% of the population with highest mCherry expression. Sorted cells were grown up and induced to express shRNA at different concentration of doxycycline (Sigma-Aldrich) for 48 h. After induction, cells were lysed and knockdown efficiency was determined by western blot analysis. Meanwhile, sorted cells with confirmed knockdown effect were subjected to the measurement of E2F dynamics in the presence of doxycycline at different doses.

Image analysis. The time-lapse microscopy resulted in two series of raw images in the cells in 30 or 60 min increments for 24–48 h. One fifth for the phase channel and the YFP channel. E2F signals were extracted from these images using ImageJ (NIH) software. The first time-point of each series of images was loaded side-by-side into the software. Using the ROI Manager Tool, a circular selective marker with a fixed area was placed around each cell nucleus in the phase channel image. The location of each marker was then copied to the YFP channel image and the integrated grey value of the selected pixels was measured. In the case of cell division, the selective marker was applied to one of the daughter cells. This value was normalized to the background by subtracting the integrated grey value of the same area of pixels in an empty part of the image. This process of measuring normalized grey value of the nuclei was repeated for each time point by adjusting the location of the selective marker to account for the movement of cells, thus giving a time-series measurement of the fluorescence reporter on the E2F sequence. Throughout this process, data from cells that left the field of view at any time were discarded; and the time of cell division was also noted. The similar approach was applied to quantify the fluorescence signal from EdU staining.

Analysis of E2F dynamics trajectories. Time-series E2F dynamics trajectories were analysed in Matlab (MathWorks) to determine various E2F parameters, for example, time delay, amplitude and so on. We developed a smoothing algorithm based on the recognition to remove the large fluorescence spike that occurs during mitosis, possibly as a result of either the increased concentration of endogenous E2F or the compactness of cell mass shrinkage or the change from a ‘globular’ to a ‘flat-lobed’ cell shape that immediately precedes division. The processed trajectories without spikes were then smoothed by using a three-window Gaussian averaging algorithm. Each smoothed and processed trajectory was fit to the following two-phase regression model (equation 2, Supplementary Fig. 8) to automatically derive optimal values for t1 and t2 (ref. 65):

\[
y(t) = \begin{cases} 
y_0, & 0 \leq t < t_1; 
(y_{\text{max}} - y_0)(t-1) / t_1, & t_1 \leq t \leq t_2; 
y_{\text{max}}(t_{2} - y_0)^{2}, & t > t_2;
\end{cases}
\]

(2)

Thus, the problem equals to search for \( t_1, t_2 \) that gives \( \min \sum_{t} \left| y(t) - y(t) \right|^2 \). Relevant parameters represent:

- t1: initial delay
- t2: activation time
- y0: E2F basal level (the average of fluorescence values of the initial four time points)
- \( y_{\text{max}} \): E2F peak level (maximum fluorescence value of each trajectory)
- \( y(t) \): E2F signal in dynamic trajectory at the moment t.

Modelling and simulation analysis. A modified version of a previously developed ODE model of Myc/Rb/E2F network was applied for simulation analysis.41 Compared with models applied in previous analysis, we neglected the hypothetical feed-forward loop from Myc to E2F and microRNA-mediated negative feedback loops, rendering the model more concise but completely maintain essential regulatory characteristics. However, we include a representative factor R, which represents regulators in E2F-related negative feedback loops, such as Cyclin A and Skp2 (Fig. 1)59,20,60. The introduction of R does not influence steady-state analysis, but shapes the curves with slight decrease after A\( \text{mp} \) reaching the maximum level, which is consistent with experimental observation. Given the fact that Myc and Cyclin D levels usually rise 6–8 h after serum addition during cell cycle entry67, we reasoned that the initial delay (\( t_1 \)) is mainly due to the delayed activation of these upstream factors. Therefore, we performed...
simulation with an initial constraint that Myc and Cyclin D are forced to be zero until the simulation proceeds to 8-h time point at the temporal scale. Simulations based on these assumptions were able to generate E2F dynamics trajectories including a significant length of $t_1$ (Supplementary Fig. 4). On the other hand, we noted the fact that a time lag exists between Cyclin E activation and that of Cyclin A$^\text{D1069}$. Moreover, population dynamics of Skp2 show a peak after that of E2F1 (ref. 20). Therefore, we reasoned that the activation of negative feedback loop through R is a delayed effect after E2F1 increases. We introduced a time lag between these two events by setting a threshold for E2F1 (0.4 μM), above which E2F starts to transcribe R. The introduction of this delayed negative feedback loop was able to generate a slight decrease in E2F1 dynamics after it reaches the peak level, consistent with our experimental observations (Fig. 2C,d).

To mimic the perturbation of Cyclin D/E or Myc node, we multiplied the degradation term with a coefficient ($k_{nah}$, inhibition rate) as shown in the following equation (3):

$$\frac{d[\text{MYC}]}{dt} = k_{\text{HSP}} \cdot \frac{S}{k_s + S} - k_{\text{nah}} \cdot d[\text{MYC}]$$

Through varying the inhibition rate $k_{nah}$ within a wide range (1- to 100-fold), we performed simulations and derived a series of E2F temporal trajectories. The normalized peak values of E2F (corresponding to $t_2$) was calculated from each trajectory by using the similar method as described above and plotted as Fig. 4e-g.

Log sensitivity analysis. Log sensitivity of the four metrics to each parameter was calculated by the following equation (4):

$$\text{Sensitivity}_{\text{log metric}}(\text{metric}) = \frac{\partial \text{log metric}}{\partial \text{log parameter}}.$$  

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Acknowledgements

We thank J. Wong, G. Yao and M. He for the plasmid with human endogenous E2F1 promoter, REF52 cells and the shRNA vector, respectively; S.A. Johnson, Y. Gao and B.R. Carlson for their help in time-lapse microscopy; S. Payne, Y. Tanouchi and A. Pai for their help in experiments; Des A.J. Hartemink, X. Wang, J. Harer and N.L. Allbritton for their suggestions on experimental design; B. Li, C. Tan, T.J. Lee, G. Yao and I. Shats for comments and discussion. This research was supported by the grant from North Carolina Biotech Center Multidisciplinary Research Grant Grogram (2012-MRG-1102; L.Y. and B.M.P.), David and Lucile Packard Fellowship (L.Y.), DuPont Young Professorship (L.Y.) and NSF Career Award (L.Y.).

Author contributions

P.D., B.M.-P. and L.Y. designed research and developed the concept of the paper; P.D. performed experiments with contributions from F.T. and B.M.-P.; P.D. and J.K.S. performed simulation analyses with contributions from L.Y.; P.D. and M.V.M. analysed and interpreted data; P.D., B.M.-P. and L.Y. wrote the manuscript with contributions from J.R.N. and M.V.M.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Dong, P. et al. Division of labour between Myc and G1 cyclins in cell cycle commitment and pace control. Nat. Commun. 5:4750 doi: 10.1038/ncomms5750 (2014).

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Author Correction: Division of labour between Myc and G1 cyclins in cell cycle commitment and pace control

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Correction to: Nature Communications https://doi.org/10.1038/ncomms5750; published online 01 September 2014

This Article contains errors in Supplementary Table 3. The sixth equation in the table should read:

\[ \frac{d[RB]}{dt} = k_{RB} + k_{RBDP} \cdot \frac{[RP]}{K_{RP} + [RP]} - k_{RE} \cdot [RB] \cdot [E2Fp] - k_{RBP1} \cdot \frac{[CD]}{K_{CD} + [RB]} - k_{RBP2} \cdot \frac{[CE]}{K_{CE} + [RB]} - d_{RB} \cdot [RB] \]

The simulation results in the Article were based on the correct formula and thus the results are not affected by this correction. The errors have not been fixed in the original Article.

Published online: 13 November 2018

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