Title: Regulation of the time when cells commit to terminally differentiate controls the number of differentiated cells

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SUMMARY
Terminal cell differentiation is essential for developing and maintaining tissues in all multi-cellular organisms. However, when and how the opposing processes of proliferation and terminal cell differentiation regulate permanent exit from the cell cycle and the number of differentiated cells is not well understood. Here we use adipogenesis as a model system to measure in live single cells the moment of terminal cell differentiation during cell-cycle progression. We show that terminal cell differentiation occurs through a competition during a lengthening G1 phase whereby cells either commit to terminally differentiate and close the window of proliferation, or commit to the next cell cycle and extend the window of proliferation until a later G1 phase. By regulating when cells terminally differentiate, different strengths of mitogen and differentiation stimuli control the number of terminally differentiated cells produced per progenitor cell. Together, our study suggests that tissue development, maintenance and regeneration is regulated by a competition mechanism that speeds up or delays the time when cells commit to terminally differentiate.

HIGHLIGHTS
• Progenitor cells decide to terminally differentiate by using a competition process that can last over multiple cell cycles and engages during each lengthening G1 phase
• The cyclin-CDK inhibitor p21 is induced by PPARG and extends G1 duration, and also triggers permanent cell-cycle exit once PPARG reaches the threshold for terminal differentiation
• The system architecture regulates the number of differentiated cells by speeding up or delaying the time when cells commit to terminally differentiate and close the window of proliferation
Terminal cell differentiation is essential for developing, maintaining, and regenerating tissues in all multi-cellular organisms and is the mechanism by which adipocytes (fat cells), neurons, skeletal muscle cells, and many other cell types in humans and other multicellular organisms are generated (Ruijtenberg and van den Heuvel, 2016). During terminal differentiation, precursor cells that were once proliferative permanently withdraw from the cell cycle (Buttitta and Edgar, 2007). Failure of terminally differentiated cells to exit from the cell cycle can lead to cancer and metabolic disorders (Ghaben and Scherer, 2019; Ruijtenberg and van den Heuvel, 2016). Despite the importance of properly-timed cell cycle exit during terminal differentiation, when and how this permanent cell cycle exit is achieved and how it regulates the total number of differentiated cells is poorly understood.

We are using adipogenesis as a model for terminal cell differentiation because it is an experimentally-accessible system in which the cell cycle and terminal differentiation have been shown to be linked both in vitro and in vivo (Jeffery et al., 2015; Tang et al., 2003). We were also intrigued by the finding that manipulating the cell cycle by knocking out the CIP/KIP CDK inhibitors p21 and p27 in mice causes a several-fold increase in fat cell differentiation (adipogenesis) and fat mass (Naaz et al., 2004). This result is puzzling since terminal cell differentiation and proliferation are commonly thought to be opposing processes (Ruijtenberg and van den Heuvel, 2016). Our study addresses four fundamental open questions in the field of terminal cell differentiation: First, are the processes of cell cycle exit and terminal cell differentiation independent events or not (Buttitta and Edgar, 2007; Hardwick et al., 2015; Soufi and Dalton, 2016)? Second, if the two processes are indeed connected, when precisely during a series of cell cycles do progenitor cells commit to the terminally differentiated fate? Third, what signaling mechanisms can robustly mediate and maintain the non-proliferative state of terminally differentiated cells? And the fourth question which is particularly important for controlling the size of terminally differentiated tissue: is there a defined number of cell cycles that occur before terminal differentiation (Jones et al., 2007; Tang et al., 2003), or is there perhaps instead a regulated time window during which proliferation can occur that may give organisms a way to control the total number of terminally differentiated cells generated per precursor?
The questions we are addressing arise from preceding studies that found molecular links between the cell cycle and terminal differentiation. For example, links between CDK inhibitors and differentiation have been reported in terminally differentiated cells, suggesting parallel pathways as well as tissue-specific redundancy in coordinating cell cycle exit and terminal differentiation (Buttitta et al., 2007; Parker et al., 2006; Ruijtenberg et al., 2015; Zalc et al., 2014). Also, a lengthening of G1 phase has been observed during differentiation processes which involve a slowing or temporary pausing of the cell cycle such as in human embryonic stem cell differentiation (Calder et al., 2012) and neuroendocrine cell differentiation (Krentz et al., 2017; Miyatsuka et al., 2011), as well as during terminal cell differentiation processes which involve permanent cell cycle exit such as in neurogenesis (Lange et al., 2009). However, even though molecular links have been found between terminal cell differentiation and cell cycle lengthening, there is conflicting evidence on whether and how strongly the two processes are connected. For example, studies in adipocytes and neurons suggested that G1-lengthening, cell cycle exit, and terminal differentiation happen sequentially (Lange et al., 2009; Tang et al., 2003) whereas other studies support a model that cell cycle exit and terminal differentiation occur as parallel, independently-regulated processes (Lacomme et al., 2012; Qiu et al., 2001). Still other studies suggest that the two processes are co-coordinated by dual actions of core components of the cell cycle and differentiation machinery (Hardwick et al., 2015).

One challenge in understanding the relationship between cell cycle exit and terminal cell differentiation is that there is great variability in whether and when individual progenitor cells in the same population proliferate or differentiate during the several-day long differentiation process and also how many cell divisions occur before cells terminally differentiate. To overcome this challenge, methods are needed that can measure whether and when during a multi-day differentiation timecourse an individual cell decides to irreversibly differentiate. Such methods require being able to track both cell cycle and differentiation progression simultaneously in realtime. Live-cell imaging approaches have been used in stem cells that undergo a slowing or transient exit from the cell cycle during the differentiation process (Matson et al., 2017; Pauklin and Vallier, 2013). However, live-cell imaging
studies to uncover the link between the cell cycle and terminal cell differentiation have to our knowledge not yet been made, and the underlying regulatory mechanisms are likely different when permanent exit from, rather than temporary slowing of, the cell cycle is required.

Many terminal cell differentiation processes including adipogenesis and myogenesis are regulated by a cascade of transcription factors (Blais et al., 2005; Farmer, 2006). In order to establish a temporal marker for differentiation commitment, it is essential to determine which of the factors in the transcriptional cascade controlling differentiation process exhibits bimodal and irreversible behavior and can thus be used to distinguish whether a cell still has the option to remain undifferentiated or has committed to terminally differentiate. For example, in adipogenesis, even though the early transcription factor C/EBPB is required, it is not a suitable marker of differentiation commitment. C/EBPB levels increase in all cells that are subjected to the DMI adipogenic stimulus, but the levels are not predictive of whether or not a cell will continue on to differentiate once the stimulus is removed (Bahrami-Nejad et al., 2018). Previous work using single-cell imaging showed that PPARG, the master transcriptional regulator of fat cell differentiation, exhibits bimodal, irreversible behavior and suggested that the level of PPARG can distinguish undifferentiated from differentiated cells (Ahrends et al., 2014; Bahrami-Nejad et al., 2018; Park et al., 2012) (Figures 1A-C).

Here we investigate how mitogens and differentiation stimuli synergistically control the number of terminally differentiated cells produced during the terminal differentiation process. We start by validating that a threshold level of fluorescently-tagged endogenous PPARG protein can be used in live progenitor cells to mark the precise time when preadipocytes irreversibly commit to terminally differentiate. By combining this live-cell PPARG sensor with a reporter to mark the G1 phase (Sakaue-Sawano et al., 2008), we establish a method that can simultaneously track both cell-cycle progression and the precise decision to terminally differentiate. Our live-cell measurements show that differentiation and proliferation are indeed directly linked: The S/G2/M phase of the cell cycle suppresses the gradual increase in PPARG expression that is triggered by addition of differentiation stimuli. In turn, differentiation strongly suppresses the cell cycle due to a PPARG-driven switch to high
expression and increased stability of the CDK inhibitor p21 when cells reach the differentiation commitment point. Thus, when cells commit to terminally differentiate, they immediately become post-mitotic, thereby closing the time window in which a progenitor cell can proliferate and thus limiting the number of terminally differentiated cells that can generated from a progenitor cell. Differentiation stimuli cause G1-phase to gradually lengthen, and we show that, markedly, cells undergo a competition during each lengthened G1 phase between whether a cell first reaches the commitment point for terminal differentiation, which closes the proliferative window early, or first reaches the commitment point to start the next cell cycle, thereby extending the window of proliferation and allowing for one or more cell divisions before terminal differentiation. Our study shows that this molecular competition in G1 phase allows the relative strength of interconnected mitogen and differentiation stimuli to regulate both the duration of each G1 phase, as well as the duration of the proliferative window before cells commit to terminally differentiate, which enables differential control of both the percent and total number of terminally differentiated cells produced, thus providing a molecular mechanism of how tissue size can be controlled.

RESULTS
Live-cell analysis of the precise time when preadipocytes commit to the terminally differentiated state

A major bottleneck in understanding the relationship between the cell cycle and terminal cell differentiation is the lack of a quantitative live-cell readout that marks the precise time point when a cell commits to the terminally differentiated state (Buttitta and Edgar, 2007). We start by establishing such a live-cell readout that marks the commitment point for terminal cell differentiation. We chose adipogenesis as a cell model since the cell cycle is known to regulate adipogenesis and the validity of using in vitro cell models for adipogenesis studies has been corroborated by in vivo studies (Ghaben and Scherer, 2019; Jeffery et al., 2015; Tang et al., 2003). Adipogenesis is centered on a master transcriptional regulator, PPARG, whose expression is driven by both external input signals and
internal positive feedback loops (Ahrends et al., 2014; Rosen and Spiegelman, 2014) (Figure 1A, top). In previous work, we used CRISPR-mediated genome editing to fluorescently tag endogenous PPARG as a live-cell readout of differentiation progression (Bahrami-Nejad et al., 2018). To enable automated nuclear tracking of moving cells, we also stably transfected the cells with fluorescently labeled histone H2B (Figure 1A, bottom). We now go on to determine whether fluorescently-tagged endogenous PPARG can be used to measure a threshold level of PPARG expression that marks in live cells the precise time point when an individual cell irreversibly commits to the terminally differentiated state.

To induce differentiation, we applied a commonly used adipogenic hormone cocktail (DMI, see Methods) that mimics glucocorticoids and GPCR-signals that raise cAMP. To determine whether there is a threshold for terminal differentiation, the removal of the differentiation stimulus at an intermediate timepoint is critical since it allows for a determination whether a cell can continue on to reach and maintain a distinct terminally differentiated state days later. Thus, we applied the differentiation stimulus, DMI, to preadipocytes for 48 hours and then removed it, replacing it with growth medium containing insulin for an additional 48 hours at which point the differentiation state of the cells was assessed (Figure 1B). Indeed, this protocol showed two distinct outcomes for cells at 96 hours: one group of cells that kept increasing PPARG levels after removal of the differentiation stimulus (Figure 1B, blue traces), as well as a second group of cells in which PPARG levels of cells fell back to the undifferentiated precursor state (Figure 1B, orange traces). A histogram of the cells at 96 hours showed that the distribution of PPARG levels is bimodal with a high peak representing the differentiated cells and a low peak representing the undifferentiated cells (Figure 1B, right-side histogram).

The bimodality in the histogram of PPARG levels at 96 hours suggested that a threshold level of PPARG may exist that can determine differentiation outcome. However, to validate that there is indeed a defined PPARG threshold that can predetermine the fate of a cell days later, one needs live cell imaging. The fate of each cell is apparent from the PPARG levels at 96 hours (Figure 1B), but one
needs to be able to track each cell back to before the stimulus was removed and assess whether its PPARG level before the stimulus was removed could indeed predict its final fate. Because we had timecourses of PPARG expression for each cell, we could carry out this analysis. We thus could compare for each cell its PPARG levels at 48 hours - before DMI was removed - to its PPARG level at 96 hours (Figure 1C). Indeed, the level of PPARG before stimulus removal at 48 hours could predict with a less than 5% false positive rate whether a cell will keep increasing PPARG and terminally differentiate or will lower its PPARG levels and fall back to being a progenitor cell. We could thus calculate a threshold which is determined as the center between the two peaks in the histogram at the 48-hour timepoint (Figures 1B-C, black dashed line; See Methods).

In additional control experiments, we validated that high and low PPARG expression in individual cells at 96 hours is correlated with high and low expressing of different well-established markers of mature adipocytes, confirming that high PPARG is indeed a marker for the differentiated adipocyte state (Figures 1D and S1). We also confirmed that PPARG levels are predictable of final fate throughout the differentiation process, independently of when cells pass the threshold for terminal differentiation. As shown in Figure 1E, even at different timepoints throughout adipogenesis (24, 48, 72 hours), cells with higher PPARG levels have a higher probability to differentiate. Nevertheless, the probability to differentiate could not be predicted by PPARG levels at the start of the experiment (Figure 1E), suggesting that terminal differentiation fate is not predetermined.

The analysis thus far confirmed the existence of a threshold in PPARG levels that can clearly divide cells into undifferentiated and differentiated states during adipogenesis. But what drives cells to pass this threshold? Previous work showed that a positive feedback-driven bistable switch mechanism between PPARG and several co-regulators can amplify PPARG expression (Ahrends et al., 2014; Park et al., 2012; Wu et al., 1999). To determine whether such a positive feedback-driven bistable switch is responsible for the here identified PPARG threshold, we computationally aligned single-cell traces to the time when each cell crosses the PPARG threshold. Markedly, the aligned timecourses show a sharp sigmoidal increase from a slow rate of PPARG increase before the PPARG threshold to
a fast rate after that time point. This observed switch from low to high PPARG levels at the timepoint at which the PPARG threshold is reached argues that the PPARG threshold marks the precise time when the bistable PPARG switch mechanism is triggered (Figures 1F and S1).

Lastly, we found that PPARG levels were not correlated with endpoint measurements of adipocyte markers early in adipogenesis, but once the threshold was reached, PPARG levels sharply switched to being positively correlated (Figure 1G), supporting the conclusion that crossing the PPARG threshold marks a short time window of PPARG self-amplification that causes an irreversible commitment to the future terminally differentiated adipocyte state (see also Figure S1D). Interestingly, by aligning PPARG timecourses by when the threshold was reached, we are able to measure a distinct time when differentiation commitment occurs and differentiation markers switch to being strongly correlated with PPARG levels (Figure 1G). However, if we had not been able to calculate and define a PPARG threshold and we were thus unable to align the timecourses, we would only see that there is an increased relationship between PPARG expression and mature adipocyte markers; for example, see rightmost plot in Figure 1G which shows a gradual increase in correlation with PPARG with GLUT4 when timecourses are not aligned by the threshold. Thus, without being able to measure a threshold for each cell and being able to align the PPARG timecourse for each cell by this threshold, we would be unable to mark a precise timepoint for differentiation commitment, as is obvious by comparing the aligned and unaligned plots in Figures 1G and S1E.

Taken together, these different experiments validate that a threshold level can be used to mark a precise time when precursor cells commit to terminally differentiate even before the markers of mature fat cells can be measured. When adipogenic stimuli are removed, cells that passed the PPARG threshold go on to terminally differentiate two days later, while cells below the threshold return to the undifferentiated progenitor state. Thus, fluorescently-tagged endogenous PPARG can be used to directly address the questions when a cell terminally differentiates and what the connection is between the decisions to terminally differentiate and permanent exit from the cell cycle.
Simultaneous single-cell analysis shows that further entry into the cell cycle is blocked once a cell reaches in G1 the differentiation commitment point

In order to monitor cell cycle progression and the commitment to terminally differentiate simultaneously in the same cells, we made a dual-reporter cell line by transfecting a FUCCI cell cycle reporter (Sakaue-Sawano et al., 2008) into an OP9 preadipocyte cell line we had previously generated that expressed endogenous citrine-PPARG (Figure 2A)(Bahrami-Nejad et al., 2018). The cell cycle reporter is composed of a red fluorescent protein mCherry fused to a fragment of the geminin protein that includes a degron for APC/C and is degraded by both anaphase promoting complex/cyclosomes (APC/C) (here referred to as APC/C degron reporter). Specifically, the APC/C-degron reporter signal rapidly drops when cells activate the first E3 ubiquitin ligase APC/C\text{CDC20} in mitosis before cells start G1 phase, and the signal only starts to increase close to the end of G1 phase when the second APC/C\text{CDH1}, which is active during G1, is rapidly inactivated (Cappell et al., 2016). To validate that this APC/C reporter is suitable to monitor G1 length in the OP9 preadipocyte cell system, we compared its dynamics to that of a reporter of CRL4-Cdt2-mediated degradation that provides a more precise measure of the G1/S transition (Grant et al., 2018; Sakaue-Sawano et al., 2017). We confirmed that the expression of the APC/C degron reporter and degradation of the CRL4-Cdt2 reporter coincide in these cells (Figure S2). Thus, the dual-reporter system can be used to accurately measure the start of G1 and G1 length simultaneously along with the commitment to the terminally differentiated state.

In the following experiments, we purposely used sub-confluent cell plating conditions in order to maximize the number of cell divisions, to reduce the effect of cell density on cell cycle arrest, and to improve the fidelity of the automated tracking algorithm (Figure S3A-S3B).

To determine when terminal cell differentiation occurs relative to the last cell cycle, we tracked PPARG expression and APC/C reporter timecourses over four days of differentiation. Trajectories of cells in the population that terminally differentiated are marked in blue, and cells that remained undifferentiated are marked in orange (Figure 2B). The trajectories show that cells that terminally differentiate had fewer cell cycles and exited the last mitosis earlier (Figures 2B-C) compared to cells...
that did not undergo terminal differentiation. Such an inverse relationship between proliferation and terminal cell differentiation can be represented in a cumulative plot comparing the percent of cells still in S/G2/M versus the percent of cells that have crossed the PPARG threshold for terminal differentiation, as a function of time after DMI stimulation (Figure 2D). Control experiments showed no significant differences in PPARG levels between cells that underwent two versus three cell cycles which argued against the possibility that the lower differentiation observed in cycling cells was due to PPARG simply being diluted more in cells that cycle more often (Figure S3C-S3D).

When visually inspecting hundreds of single-cell traces, we found great variability in the kinetics of PPARG increases and number of cell-cycles (Figure 2E). However, no new cell-cycle entry was observed if the PPARG level in a cell increased above the threshold for terminal differentiation (marked with a yellow dot, Figure 2E), arguing that permanent cell-cycle exit is forced on cells when they reach the commitment point to terminally differentiate. We observed that PPARG levels already increased in many cells during S/G2/M phase of the cell cycle, but a large majority of differentiating cells only reached the PPARG threshold for terminal differentiation in G1 phase. Control experiments using a CDK2 activity reporter (Spencer et al., 2013) instead of the APC/C sensor confirmed that commitment to terminally differentiate happened during G1 phase, as shown by the fact that PPARG levels are only high when CDK2 activity levels are low (Figure S3E-S3F). Perhaps the clearest relationship between terminal differentiation and the last mitosis can be seen in a histogram analysis of when relative to the last mitosis each cell commits to a terminally differentiated state. While cells passed the commitment point for terminal differentiation at different times, almost all did so only after mitosis and the average time to commitment is approximately 14 hours from the last mitosis (Figure 2F). Thus, cells commit to the terminally differentiated state almost exclusively in G1. It should be noted that our live dual-reporter method in which we can measure cell cycle and differentiation progression simultaneously allows us to distinguish between cells that become 1) post-mitotic, differentiated; 2) undifferentiated, proliferating; or 3) undifferentiated, quiescent. For example, the bottom right plot in Figure 2E shows a cell that remains undifferentiated but becomes quiescent and
ceases to proliferate even when serum is refreshed at 48 hours. The percentage of cells that end up in the three different cell fates in a typical DMI-induced differentiation experiment is shown in Figure 2G.

An interesting result from this analysis was that preadipocytes undergo a variable number of cell divisions before they differentiate (Figures 2C and 2E), arguing that terminal differentiation of adipocytes does not occur after a fixed number of cell divisions before differentiation as has been previously suggested (Tang et al., 2003). Since the previous study relied on averaged, population-based measurements, the variable number of mitoses in different cells could likely not be resolved without live single-cell analysis. Not only do the number of cell cycles vary, but there is also great variability in the time after stimulation when cells start to increase PPARG levels, and also in the time cells spend in G1 before cells reach the PPARG threshold for terminal differentiation (see also Figure S4).

We conclude that terminal adipocyte differentiation occurs after a variable rather than fixed number of cell cycles, that most preadipocytes reach the commitment point for terminal differentiation after spending variable times in G1, that cells partially suppress the increase in PPARG during S/G2/M, and that cells permanently exit the cell cycle at the same time when they pass the commitment point for terminal cell differentiation.

**PPARG regulates terminal cell-cycle exit by inducing p21 and FKBPL**

The dual reporter timecourse data showed that DMI-stimulated cells that stopped proliferating earlier had consistently higher levels of PPARG (Figures 2B, 2D, and 2E), supporting that PPARG may suppress the cell cycle and also regulate permanent cell cycle exit. To test for a direct role of PPARG in suppressing proliferation, we carried out siRNA experiments in our dual reporter cells and showed that indeed depletion of PPARG resulted in an increase of the percent of proliferating cells at all timepoints throughout the differentiation process (Figure 3A). Based on our observation that differentiation commitment occurs almost exclusively only in G1-phase and out of a state with low
CDK2 activity (Figure S3E-S3F), we hypothesized that PPARG may increase the expression of one of the CDK inhibitors, which may then slow or inhibit entry into the next cell cycle.

We sought to identify putative inhibitors of proliferation by performing RNA-seq analysis on cells transfected with siRNA targeting PPARG, or control siRNAs, and collected the transcripts at different timepoints during a 144-hour DMI differentiation protocol. We examined mRNA expression profiles of canonical CDK inhibitors and identified two that were strongly regulated by PPARG expression, p18 and p21 (Figure 3B). A PPARG-mediated increase in p21 has also been reported in other cell types (Han et al., 2004). To determine whether p18 and p21 mediate cell-cycle arrest during adipogenesis, we carried out siRNA knockdown experiments and found that p21, but not p18, knockdown led to an increase in proliferation (Figure 3C). Experiments in which p21 was knocked down with siRNA further showed that p21 is required for PPARG to mediate both terminal cell differentiation as well as suppression of proliferation (Figure 3D). We tested whether the effect from PPARG on p21 expression is direct by using ChiP-Seq experiments which showed significant binding of PPARG to the promoter of p21 during adipogenesis induced by DMI stimulation (Figure 3E). To further test whether the effect of PPARG on p21 is direct, we added rosiglitazone, a small molecule that directly activates PPARG, which led to a robust increase in p21 expression (Figure 3F).

In the same RNA-seq data, we also found that PPARG increases the expression of FKBPL (WiSP39), a protein that was shown to stabilize p21 and increase the ability of p21 to arrest cells in response to ionizing radiation (Jascur et al., 2005)(Figure 3G). To test if p21 could be stabilized by FKBPL during the early stages of adipogenesis, we carried out cycloheximide protein degradation experiments to measure the half-life of p21 in cells transfected with siRNA targeting FKBPL. Our results showed that knockdown of FKBPL causes a small decrease in p21 half-life but did not affect the half-life of PPARG, supporting that FKBPL does regulate p21 stability during terminal cell differentiation (Figure 3G). Taken together, our results demonstrate that PPARG slows and stops proliferating cells in G1 in part by increasing p21 levels by directly increasing p21 transcription and by FKBPL-mediated slowing of p21 degradation.
Commitment to terminally differentiate triggers immediate CDK inhibitor-driven cell-cycle exit

We next focused on the question how preadipocyte cells trigger permanent exit from the cell cycle once they pass the PPARG threshold for terminal differentiation. To determine the relationship between PPARG levels and terminal cell cycle exit, we took advantage of the variable increase in PPARG following DMI stimulation and grouped cells into 10 bins according to their expression level of PPARG at 48 hours (Figure 4A). At this 48-hour timepoint, the media is changed from DMI to media with insulin and growth factors and no differentiation stimuli. The corresponding mean PPARG (left) and APC/C reporter (right) signals were plotted for each bin. We found that the group of cells that passed the PPARG threshold, but not the cells that stayed below the threshold, showed no significant APC/C reporter signal in response to fresh growth media, demonstrating that cells lose the ability to re-enter the cell-cycle entry after they cross the threshold for terminal differentiation. We confirmed that this is indeed the result of reduced cell-cycle activity by calculating the fraction of cells that underwent mitosis in response to fresh growth media (Figure 4B, red). Thus, cells that pass the PPARG threshold lose the proliferation response to freshly added growth factors, arguing that crossing the PPARG threshold marks the time when cells permanently enter a post-mitotic state.

We next investigated how p21 levels change relative to PPARG levels in individual cells. After completion of a live cell time course, we fixed and stained cells for p21 expression. We again binned cells according to PPARG levels and plotted the mean nuclear p21 fluorescence for each bin (Figure 4B, black). We found that p21 levels gradually increase along with PPARG until the PPARG threshold after which p21 plateaus and stays high, suggesting that p21 is not only lengthening G1 and mediating cell-cycle exit but also maintains the postmitotic state.

To directly test for such a maintenance role of p21, we added siRNA to knockdown p21 late in adipogenesis at the 48-hour timepoint when the adipogenic stimulus was replaced with growth factor containing media. As a control, we also started at the same time to knock down PPARG and CEBPA, a required co-activator of PPARG expression that is needed for cells to reach the threshold for

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differentiation (Bahrami-Nejad et al., 2018; Wu et al., 1999). Acute knockdowns of all three regulators after the commitment point to terminally differentiate showed that only p21 knockdown resulted in a significant increase in cell-cycle activity (Figure 4C). To quantitatively analyze this result, we grouped cells by their PPARG levels and plotted the average time course for each group during the removal of DMI and addition of growth factors. Control experiments show that if PPARG levels are above the threshold, APC/C reporter signals remain suppressed, consistent with a lack of proliferation. However, acute knockdown of p21 expression led to a loss of the ability of PPARG levels to suppress APC/C reporter signals and maintain the postmitotic state even though PPARG levels stayed above the threshold (Figure 4D). This loss of maintenance of the postmitotic state can also be seen in an analysis showing the percent of cells in the cell cycle in Figure 4E. Thus, a maintained high level of p21 is required for cells to maintain the post-mitotic state after cells passed the commitment point for terminal cell differentiation.

Notably, when we examined images of cells from Figure 4E that had p21 depleted after crossing the commitment point, we found that PPARG high cells were enriched for multinucleation events (Figure 4F). This suggests that a critical role of p21 is to permanently prevent cell division after terminal differentiation to prevent mitotic defects.

Finally, it was recently shown that the ratio of nuclear expression of cyclin D1 versus p21 can predict Rb hyperphosphorylation and re-entry into the cell cycle (Yang et al., 2017). We determined whether the role of the PPARG-induced increase in p21 expression is to shift this p21-cyclinD1 ratio towards high p21 to keep Rb dephosphorylated and ensure that cells remain unresponsive to serum stimulation. As shown in a plot of p21 versus cyclinD1 levels in a large number of single cells (Figure 4G), the ratio of p21 to cyclinD1 becomes strongly skewed towards p21 when PPARG levels go above the threshold during adipogenesis, providing an explanation of how differentiated cells can maintain a robust arrested state. Taken together, our data shows that the commitment to terminally differentiate also marks entry into a permanent post-mitotic state, and that p21 is needed to maintain the health of terminally differentiated cells by preventing cells in the postmitotic state from proliferating after
An ongoing competition during G1 between terminal differentiation and continued proliferation

Previous live-cell analysis of the cell cycle in the differentiation of stem cells, which do not terminally differentiate, has shown that cells temporally lengthen G1 as part of the differentiation process (Calder et al., 2012). The relationship between G1 lengthening and differentiation is less well understood for terminal cell differentiation and we first determined whether a gradual lengthening of G1 already occurs in the cell cycles that precedes the G1 phase from which cells terminally differentiate. Preadipocytes can undergo rapid cell cycles with a G1 phase that is on average only about 4 hours (Figure 5A). However, more than 14 hours on average is needed after the last mitosis in order for a cell to reach the PPARG threshold for terminal differentiation (Figures 2F and 5A), arguing that G1 must be lengthened before progenitor cells can commit to terminally differentiate.

To test whether adipogenic stimuli trigger a gradual lengthening of G1 over time, or only a single G1 extension when they terminally differentiate, we selected cells that underwent three mitoses following DMI stimulation and before terminal differentiation, and compared the G1 duration for the first and second observed G1 phase (G1₁ and G1₂, see scheme in Figure 5B, left). Consistent with a DMI-induced gradual lengthening of G1, the second G1 length is typically significantly longer (Figure 5B, right; Figure S6A). When G1 length is plotted as a function of the time after DMI stimulation, the length of G1 initially can be seen to increase gradually from 12 to about 18 hours and more strongly from 18 to about 30 hours (Figure S6B). Thus, adipogenic stimuli progressively lengthen their G1 period before cells reach the last mitosis and then terminally differentiate out of the last G1 phase.

This DMI-induced G1 lengthening raises the question whether the reason why cells do not differentiate more quickly after DMI stimulation is simply the result of cells having a short G1 period for the first few cell cycles so that PPARG can be mostly suppressed during S/G2/M. To test whether there is a delay before cells activate PPARG that is independent from the lengthening of G1, we made use of the high variability in cell cycle responses in the cell population. By computationally aligning the
time-courses by the time a cell completes its last mitosis, we were able to measure more precisely PPARG increases before and after the last mitosis (Figure 5C). If a cell had a last mitosis in the first 12 hours after DMI addition, PPARG did not noticeably increase both before or within 12 hours after mitosis. If a cell had its last mitosis 12-24 hours after DMI addition, PPARG showed only a small increase but only after mitosis. Markedly, this increase of PPARG after mitosis becomes stronger when a cell had its last mitosis 24-36 hours after DMI addition. Thus, there is a delay mechanism that is independent of the cell cycle that restricts the increase in PPARG not only to the G1 phase but also to a time window starting only at approximately 24-36 hours after DMI stimulation.

To more directly evaluate the delay before PPARG can increase in G1, we made the same alignments but with smaller time windows and by measuring the time each cell takes after mitosis to reach the PPARG threshold. We used the timecourse data shown in Figure 2B and binned cells into groups based on when a cell completed its last mitosis in increments of 4 hours (see scheme in Figure 5D). The data shows that the later a cell exited the last mitosis, and thus the longer the proliferating cell was exposed to the adipogenic stimuli, the less time a cell needs to spend in G1 before reaching the PPARG threshold (Figure 5E, left). Furthermore, cells that spend less time in G1 are less likely to terminally differentiate (Figure 5E, right), consistent with a repressive contribution from the ongoing S/G2/M part of the cell cycle to terminal differentiation.

Taken together, our data can be summarized in the following model for terminal cell differentiation of adipocytes (Figure 5F): Early after adipogenic stimulation preadipocytes are proliferating and have short G1 periods. Adipogenic stimuli then gradually extend G1 length and also gradually accelerate during each G1 the rate of PPARG increase to the threshold, thus setting up a race after the end of each mitosis. In one outcome of the race, a cell first reaches the commitment to terminally differentiate, which then closes the proliferative window and suppresses future cell cycles. In another outcome, cells first commit to the next cell cycle at the onset of S phase, which extends the proliferative window at least until after the next mitosis. In a third outcome, cells fail to reach either the differentiation or cell cycle commitment points. Thus, at the end of an extended G1 phase, cells end
up in one of three fates as we previously showed in Figure 2F: (1) terminally differentiated and post-mitotic, (2) undifferentiated and proliferating, or (3) undifferentiated and quiescent. Finally, this is suggestive of another important regulatory principle that cells use this competition in G1 to delay or accelerate the time of commitment to terminally differentiate and thereby shorten or lengthen a window of proliferation to allow for fewer or more cell cycles to occur before terminal cell differentiation. The next series of experiments aim to test such a model.

**Mitogens synergize with adipogenic stimuli to regulate the time when cells commit to terminally differentiate and thereby control the total number of terminally differentiated cells**

Cell-cycle entry out of G1 and the length of G1 phase are not only regulated by adipogenic stimuli but also by mitogens. We thus tested whether the time of commitment to terminally differentiate can also be accelerated or delayed by lowering mitogen stimuli or by directly manipulating the regulators of G1 phase cyclin D1 and p21. We first focused on stimulating the Ras/MEK/Erk signaling pathway. Indeed, when we stimulated cells with DMI with and without a MEK inhibitor (Figure 6A), we observed not only that less cells proliferated in the presence of the MEK inhibitor but also that the time window after stimulation during which cells proliferated was shorter. At the same time, stimulation with DMI in the presence of MEK inhibitor also increased the percentage of cells that terminally differentiated and accelerated the time at which cells committed to differentiate. Similarly, decreasing the serum concentration along with DMI stimulation not only reduced the percent of cells that proliferated and increased the percent of terminally differentiated cells, but cells also committed to terminally differentiate earlier (Figure 6B). Thus, mitogen stimuli and the Ras/MEK/Erk pathway not only regulate the length of G1 but also control the time when cells commit to terminally differentiate.

The most important conclusion here is that an important role of mitogens is to delay the time cells need to reach the threshold for terminal differentiation. Since cells become post-mitotic immediately after reaching the threshold, mitogens thereby extend a time window when differentiating cells can proliferate and thus potentially allow cells to increase the average number of cell cycles per progenitor
cell before terminal differentiation.

Because it has many targets, the EGFR-MEK-ERK signaling pathway can regulate differentiation and proliferation processes in different ways. For example, the EGFR-MEK-ERK pathway can regulate entry into the cell cycle by increasing the expression of cyclin D1 and also by reducing expression of p21 and p27. Both of these regulatory steps then activate CDK4/6 and promote entry into the cell cycle. On a first glance, based on the results in Figures 6A and 6B which support the commonly held view that proliferation and terminal differentiation are opposing processes (Ruijtenberg and van den Heuvel, 2016), one would expect that mice lacking p21 or p27 should have more proliferating progenitor cells and less adipocytes. Nevertheless, one of the most striking findings from gene knockout studies was that fat tissues of female mice with deleted CDK inhibitors p21 and p27 show a 6-fold enlargement while knockouts of p21 or p27 alone showed smaller increases (Naaz et al., 2004). We wanted to see if live single-cell analysis approaches could resolve this conundrum.

Since the EGFR-MEK-ERK pathway increases cyclin D1 expression, we first tested the effect of reducing cyclin D1 expression on the total number of differentiated cells produced. Consistent with our model that an increase in G1 duration allows more cells to build up PPARG levels and differentiate, we found that cyclin D1 knockdown led to an increase in G1 duration, lowered cell cycle activity, and resulted in an increase in the percent of differentiated cells (Figure 6C). However, unexpectedly, fewer total terminally differentiated cells were produced, raising the question whether the percent and number of terminally differentiated cells can be regulated in opposite ways by regulating when cells commit to terminally differentiate.

We considered whether our finding that cyclin D knockdown cells have fewer total differentiated cells may explain the puzzling in vivo result that p21-knockout mice displayed increased fat mass (Naaz et al., 2004). We first confirmed that cells with knocked-down p21 in vitro spend overall less time in G1 phase, consistent with p21 functioning as an inhibitor of proliferation that lengthens G1 (Figure 6D, right). Cells with knocked-down p21 also delayed the commitment to terminally differentiate and had a longer window of proliferation - similar to the effect of more mitogen stimulation.
Furthermore, we found the same increase in the percent of proliferating cells and also decrease in the percent of differentiated cells, which can again be explained by short G1 periods giving less opportunity for PPARG levels to increase during each G1 (Figure 6D, middle). However, despite the lower percentage of terminally differentiated cells, the total number of differentiated adipocytes significantly increased in the p21 knockdown condition (Figure 6D, right). The increase in the total number of terminally differentiated cells can be explained by the longer window of proliferation and corresponding increase in the average number of cell divisions before cells reach the PPARG threshold for terminal differentiation. Conversely, overexpressing p21 using a DHFR induction system yielded the opposite effect: the percent of proliferating cells decreased and there was a corresponding small increase in the percent of differentiated cells and a lower total number of differentiated cells (Figure 6E). Thus, the puzzling finding of high fat mass in mice lacking p21 or p27 type CDK inhibitors can be explained by this in vitro analysis of cells lacking p21, namely that delayed commitment to terminally differentiate results not only in a smaller percentage differentiating but also in progenitor cells undergoing more cell cycles and thereby ultimately increasing the total number of produced adipocytes.

Since p21 can affect both G1 and G2 phases, we carried out experiments to test whether regulation of G1 duration or G2 duration controls terminal cell differentiation of adipocytes. We found that the adipogenic DMI stimuli primarily lengthens G1 rather than S/G2/M (Fig S6C), arguing that PPARG-mediated p21 expression primarily acts by lengthening G1 rather than G2-phase. Furthermore, we selectively lengthened S/G2/M by knocking down CDC25B or CDC25C, which promote CDK2/1 activation in G2, and did not observe a noticeable effect on differentiation outcome (Fig S6D-S6E). Together, these findings are consistent with the interpretation that cyclin D1 and p21 act in opposite directions to both regulate the duration of G1 phase, as well as to control the duration of a “proliferative window” that starts with addition of the adipogenic stimulation and ends after one or more cell cycles if and when cells commit to terminally differentiate.
Different optimal ranges of mitogenic and differentiation stimuli control the percent and total number of terminally differentiated cells

Mammals maintain a large pool of preadipocytes near the vasculature in fat tissue (Tang et al., 2008; Tchoukalova et al., 2004), yet adipocytes are replaced only at a low rate (Spalding et al., 2008), arguing that adipogenesis is a relatively slow process with only a small percentage of preadipocytes differentiating at a given time. This slow rate of differentiation led to the earlier hypothesis that a main role of the strength of adipogenic stimuli is to control the percent of progenitor cells that start the terminal differentiation program (Ahrends et al., 2014; Park et al., 2012). At the same time, the data in Figure 6 suggests that mitogen stimuli indirectly regulate the number of terminally differentiated cells by regulating the number of cell divisions before cells commit to terminally differentiate. As shown in the scheme in Figure 7A, when the commitment to terminally differentiate is delayed, extra divisions can lead to more differentiated cells. For example, when the commitment to terminally differentiate is sped up, a progenitor cell may have 2 cell divisions, a final outcome of 75% differentiated cells, and 3 total fat cells produced. Another progenitor cell with a delayed commitment may have 3 cell divisions, the final cell population may only be 50% differentiated cells but still a total of 8 adipocytes can be produced.

If a tissue needs more differentiated cells, why not just keep increasing the proliferative window before differentiation? In vitro and likely also in vivo, cells can end up after a differentiation stimulus in an undifferentiated proliferating or quiescent progenitor state due to inhibitory signals from sources such as the differentiation stimulus itself, increased cell contact or stress. Once cells are quiescent for longer time periods, they are expected to become less capable to differentiate compared to cells that recently proliferated since proliferation has been shown to mediate epigenetic reprogramming needed for cell differentiation. For the DMI stimulation protocol we used, approximately 35% of progenitor cells become quiescent without undergoing terminal differentiation (Figure 2F). Thus, the relative strength of mitogenic and differentiation signals that maximize the percent of cells that differentiate is expected to be different from the relative strength of mitogenic and differentiation signals that maximizes the
total number of differentiated cells produced. Figure 7B describes a proposed model in which mitogenic and differentiation stimuli can differentially control percent of differentiated cells (how many cells reach the differentiation commitment threshold) versus total number of differentiated cells (how many divisions can occur during a regulated window of proliferation that starts with addition of the differentiation stimulus and closes when cells reach the commitment point for terminal differentiation and become post-mitotic.

To test this hypothesis that the strength of mitogen and adipogenic stimuli is critical for determining the total number of terminally differentiated cells that can be produced, we performed experiments in which we systematically applied different relative strengths of adipogenic and mitogenic stimuli for 48 hours and then removed the stimulus such that we could determine at 96 hours (i) the total number of cells, (ii) the percentage of terminally differentiated cells, and (iii) the total number of terminally differentiated cells. When focusing on the total number of cells at the end of the experiment, higher mitogen stimuli invariably increased the total number of cells produced independently of the strength of the adipogenic stimulus (Figure 7C). In contrast, since adipogenic stimuli suppress proliferation, the total number of cells decreased for increasing adipogenic stimuli. When focusing on the percent of cells that end up terminally differentiated, higher adipogenic stimuli invariably increased the percent of cells that end up terminally differentiated independent of the strength of the mitogen stimulus. Since proliferation inhibits differentiation, the percent of differentiated cells is nevertheless reduced with increasing mitogen stimuli which is particularly clear for the higher range of DMI stimuli.

As a consequence of the dual regulation, combined mitogen and adipogenic stimuli result in an overall triangular effect on the total number of differentiated cells, with higher mitogen and higher adipogenic stimuli synergistically increasing the total differentiated cells produced from the same number of progenitor cells. At the highest levels of mitogen and adipogenic stimuli, maximal adipogenic stimuli start to reduce the total number of differentiated cells, suggesting that there can be optimal ranges of mitogen and adipogenic stimuli that maximize the number of terminally differentiated
cells. Experiments in which we increased both the DMI adipogenic stimulus as well as the FBS concentration to even higher levels (Figure S7) confirmed the results we had seen in Figure 7C.

We conclude that terminal cell differentiation is based on a race between mitogen and adipogenic stimuli that drive a competition where cells in G1 either reach the commitment point for terminal differentiation, which can close the proliferative window early, or reach the commitment point to start the next cell cycle and extend the window of proliferation by allowing more cell divisions. Thus, a main consequence of this competition in G1 is a regulated time period of cell division between when adipogenic stimulation is added and before cells commit to terminally differentiate.

**DISCUSSION**

Using adipogenesis as a model system for terminal cell differentiation, we carried out live single-cell analysis to determine the molecular mechanisms and timing between cell-cycle exit and terminal cell differentiation. Our study highlights several advantages of studying terminal differentiation in adipocytes as we were able to develop a live molecular marker to precisely determine when cells make the transition to the terminally differentiated state. We show that the decision to terminally differentiate in cells is made out of a lengthening G1 phase after cells exit mitosis. We further showed that the duration of the extended G1 phase varies between cells, can be regulated by changing cyclin D1 and p21 expression, and increases gradually, in part by an increase in p21 as cells undergo one or more cell cycles following adipogenic stimulation. In addition, we found that expression of PPARG is repressed during S/G2/M phases and starts to significantly increase only during each extended G1 after mitosis. This rate of PPARG increase after mitosis varies between cells but becomes on average faster the later a mitosis occurs following adipogenic stimulation.

We further showed that PPARG has to reach a critical threshold level before the end of an extended G1 period in order for cells to irreversibly commit to differentiate and close the window of proliferation. However, if a cell is able to reach the end of the extended G1 phase before it reaches the PPARG threshold, the cell will enter the next cell cycle, extend the window of proliferation, and
suppress both the increase in PPARG and differentiation until the end of the next mitosis. Thus, our data argues that terminal differentiation is based on a competition that engages over one or more cell cycles and which both regulates the length of G1 and the duration of a window of proliferation.

We showed that the competition between proliferation and terminal differentiation can be driven by a PPARG-mediated increase in expression of the CDK inhibitor p21 that occurs both by PPARG-mediated direct transcriptional induction and by a FKBPL-mediated increase in p21 half-life. The fact that p21 has a short-half-life of about an hour at both the protein and mRNA levels (Figure 3H and Yang et al., 2017) raises a question common to all terminal differentiation processes: how can a permanent post-mitotic state be maintained? In adipogenesis, PPARG can function as a continuously active driver of p21 expression since once PPARG levels reach the threshold for terminal differentiation commitment, positive feedbacks with PPARG become so strong that PPARG levels will continue to increase and stay high independently of the input stimulus. These high, positive-feedback driven PPARG levels will both permanently prevent cells from returning to the undifferentiated state as well as will permanently drive p21 expression high enough to maintain the postmitotic state. We show that acutely suppressing p21 after cells have passed the threshold for terminal differentiation re-activates the cell cycle but results in mitotic defects, arguing that p21 mediates the precise timing between differentiation and terminal cell-cycle exit and that this timing is critical for cell health.

Notably, in our experiments we plated cells at low density conditions such that p21 was the dominant CDK inhibitor. At higher cell densities, the homolog CDK inhibitor p27 becomes the main regulator of CDK activity and likely has a synergistic role along with p21 in regulating terminal cell differentiation. Such a synergistic role of both CDK inhibitors is consistent with knockout data in mice which showed a 6-fold increase in fat mass when p27 and p21 where knocked out together as compared to smaller increases with only individual knockouts (Naaz et al., 2004).

It is commonly accepted that increasing the differentiation stimulus should increase the number of differentiated cells. In a first type of mechanism to increase the number of differentiated cells, previous studies (Ahrends et al., 2014; Park et al., 2012) and our analysis in Figure 7 show that
increasing adipogenic stimuli triggers an increasing percentage of progenitor cells to terminally differentiate. In a second type of mechanism, cells also regulate when after they are stimulated they commit to terminally differentiate. By regulating such a window of proliferation, the relative strength of mitogen and adipogenic stimuli allows for 1, 2, or 3 cell cycles to occur before cells reach the threshold, thus generating 2, 4 or 8 differentiated cells per progenitor cell, respectively (Figure 7A). Thus, our study argues that a functional consequence of a system that regulates the time when progenitors commit to terminally differentiate is that organisms can increase the number of adipocytes produced either by increasing adipogenic stimuli to speed up the process and increase the percent of cells that commitment to terminally differentiate or by increasing mitogen stimuli to delay the commitment to terminally differentiate and produce more differentiated cells. The large in vivo effect of p21 and p27 inhibitor knockouts on fat mass (Naaz et al., 2004), and the role of p21 shown here to regulate the time when cells commit to terminally differentiate, provides support for therapeutic strategies aimed at regulating the time when cells commit to terminally differentiate to control tissue maintenance and regeneration. Under normal conditions, the role of p21 in adipogenesis may particularly show itself during conditions of stress, DNA damage, and aging that are associated with increased p21 levels.
METHODS

**Generation of PPAR and APC/C dual-reporter cell line.**
OP9 cells with endogenously tagged citrine-PPARG2 and stably infected H2B-mTurqoise was generated as previously (Bahrami-Nejad et al., 2018). Lentivirus was generated for the APC/C reporter from the vector pCSII-EF-Geminin(1-110)-mCherry. A third-generation lentiviral packaging system was used and consisted of the following packaging vectors: pMDlg, pCMV-VSVG, and pRSV-Rev. The APC/C reporter was then stably infected into H2B-mTurqoise/citrine-PPARG2 cells to generate the dual-reporter cell lines. Selection of dual-reporter cells was done with FACS for mCherry-positive cells.

**Generation of a PPAR, APC/C, and CDK2 triple reporter cell line.**
Lentivirus was generated for the CDK2 sensor from the vector pCSII-EF-DHB-mTurquoise (gift from the lab of Tobias Meyer) in the same manner described above and used to infect the dual reporter PPARG/geminin cells. Selection of triple-reporter cells was done with FACS for cells that were positive for both mCherry and mTurquoise.

**Generation of a PPARG and CRL4-Cdt2 dual reporter cell line.**
The CRL4-Cdt2 construct was developed by Atsushi Miyawaki’s lab (Sakaue-Sawano et al., 2017) and was obtained from the lab of Tobias Meyer. We changed the fluorescent tag to iRFP670 and generated lentivirus in the same manner described above. Selection of triple-reporter cells stably expressing iRFP670-CRL4-Cdt2 was done with FACS for cells that were positive for both mCherry and iRFP670.

**Cell culture and differentiation.**
Wildtype and reporter OP9 cell lines were cultured according to previously published protocols (Ahrends et al., 2014; Bahrami-Nejad et al., 2018; Wolins et al., 2006). Briefly, the cells were cultured in growth media consisting of MEM-α media (ThermoFisher Scientific) containing 100 units/mL Penicillin, 100mg/mL Streptomycin, and 292 mg/mL L-glutamate supplemented with 20% FBS. To induce differentiation, two methods were used. In the first method, a standard DMI cocktail was used: cells were treated with 125 µM IBMX (Sigma-Aldrich), 1 µM dexamethasone (Sigma-Aldrich), and 1.75 nM insulin (Sigma-Aldrich) for 48h, followed by 1.75 nM insulin for 48h. In the second method, cells were treated with 1 µM of Rosiglitazone (Cayman, USA) for 48 hours, followed by 1.75 nM insulin for another 48 hours. For fixed cell experiments, the differentiation stimuli were added to the growth media described above with one modification: 10% FBS was used (instead of 20% FBS) during differentiation.
conditions. The one exception is in the reduced serum experiments in Figure 4D, in which 2% FBS was used in the growth media during differentiation. For all live cell experiments, the differentiation stimuli were added to Fluorobrite DMEM media (ThermoFisher Scientific) containing 100 units/mL Penicillin, 100mg/mL Streptomycin, and 292 mg/mL L-glutamate supplemented with 10% FBS. For Figure 4D, EGF (Sigma-Aldrich E9644) was used at a final concentration of 1 µg/mL, and a MEK inhibitor PD0325091 was used at a final concentration of 100 nM.

**siRNA-mediated gene silencing.**

siRNA targeting *Pparg*, *Cebpa*, *p21*, *CyclinD1*, *Fkbpl* and the AllStars Negative Control siRNA were purchased from QIAGEN. For siRNA knockdown in the live-cell imaging experiments in dual-reporter cells (Figure 4, Figures 5a, 5d, and 5e), OP9 cells were transfected by reverse-transfection using µL Lipofectamine RNAiMax (Invitrogen). Briefly, our reverse-transfection protocol per well is as follows: mixed 20 µL of Optimem, 0.5 µL of a 10 µM siRNA stock solution, and 0.3 µL of RNAiMax. Let solution incubate at room temperature for 10 minutes and then add 80 µL of culture media containing the desired number of cells per well. Then the entire (~100µL) volume is plated into one well of a 96-well plate. The siRNA/RNAiMax mixture was left on the cells for 24 hours before being aspirated away and replaced with fresh culture media containing DMI to begin the differentiation protocol.

For the live-cell imaging experiments in dual-reporter cells transfected at the 48-hour timepoint (Figure 6), the following protocol per well was used: siRNA mixture was prepared using 0.6 µL Lipofectamine RNAiMAX, 0.5 µL of a 10 µM siRNA stock solution, and 20 µL of Optimem. Incubate the mixture for 10 minutes then add 180 µL of Fluorobrite media consisting of 1.75 nM insulin. The entire solution (~200µL total volume) was then added to cells at the 48-hour time point and left on until the end of the experiment.

**Overexpression of p21**

A retroviral vector containing DHFR-Chy-p21 (Spencer et al., 2013) (gift from the lab of Tobias Meyer) was used to generate viral particles to stably infect DHFR-Chy-p21 into a modified dual-reporter cell line. This cell line was also stably infected with H2B-iRFP670 and a version of the APC/C reporter fused to mCerulean3. Positive clones were selected for by FACS in cell culture media containing 10 µM TMP. Cells were sorted into culture media with no TMP and grown in the absence of TMP. All overexpression experiments were done by adding 10 µM TMP into the culture media or differentiation media. In control experiments, 10 µM DMSO was added instead of TMP.
**Immunofluorescence (IF) staining**

All cultured cells were fixed with 4% PFA in PBS for 30 min at room temperature, followed by five washes with PBS using an automated plate washer (Biotek). Cells were then permeabilized with 0.1% Triton X-100 in PBS for 15 minutes at 4°C, followed by blocking for 1 hour in 5% bovine serum albumin (BSA, Sigma Aldrich) in PBS. The cells were incubated with primary antibodies in 2% BSA in PBS overnight at 4°C: mouse anti-PPARγ (Santa Cruz Biotech, sc-7273, 1:1,000), rabbit anti-CEBPα (Santa Cruz Biotech, sc-61, 1:1,000), mouse anti-p21 (Santa Cruz Biotech, sc-6246, 1:100), cyclinD1 (Abcam, ab137145, 1:1,000), adiponectin (Abcam, ab22554, 1:1,000), Glut4 (Santa Cruz Biotech, sc-1608, 1:500), FABP4 (R&D Systems, AF1443, 1:1,000). After washing, cells were incubated with Hoechst (1:20,000) and secondary antibodies in 2% BSA / PBS for 1 hour. Secondary antibodies included AlexaFluor-conjugated anti-rabbit, anti-mouse, and anti-goat antibodies (Thermo Fisher Scientific). All secondary antibodies were used at a 1:1,000 dilution. Where indicated, lipids were co-stained by adding HCS LipidTOX Deep Red Neutral Lipid Stain 637/655 (1:1,000, ThermoFisher Scientific H34477) to secondary antibody solution. Cells were washed five times with PBS in an automated plate washer prior to imaging. For fixed-cell timecourse experiments, approximately 7,000 wildtype or dual-reporter OP9 cells were used to calculate mean values at each timepoint for each technical replicate.

**RNAseq**

siRNA targeting Pparg (# L-040712-00-0005) and Negative Control siRNA (# D-001810-10-05) were purchased from Dharmacon and transfected into OP9 cells using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer’s protocol. siRNA was used at a concentration of 25 nM, and the RNAiMAX/siRNA mixture was applied for 48 hours prior to the induction of differentiation. For gene expression analysis of OP9 cell samples, the cells were differentiated for 144 hours using a previously described protocol (Ahrends et al., 2014). RNA from three independent biological experiments were collected at different time points before and after induction of differentiation including (d0-d6) the extraction was completed using RNeasy Mini Kit (QIAGEN, Cat. 74104). RNA quality of all samples (n=7 time points and n=3 experiments from independent passages) was evaluated by both Nanodrop for (A260/280 >2) and Bioanalyzer 2100 High Sensitivity RNA Analysis chips (Agilent, Cat. 5067-1513) which displayed intact RNA integrity (RIN >9). mRNA samples were concentrated to ≤ 5 µl by MinElute column (QIAGEN, Cat. 74204). For generation of RNA-seq libraries, polyadenylated mRNA was isolated from 300 ng of total RNA by incubation with oligo-DT attached magnetic beads and followed by strand-specific library preparation using the TruSeq Stranded mRNA Library Preparation kit (Ilumina, Cat. 20020595). Briefly, isolated polyadenylated mRNA was fragmented using divalent
cations under elevated temperature and 1st and 2nd strands DNA were synthesized using SuperScript II Reverse Transcriptase (provided with Illumina kit). A-tailing and adapter ligation was performed according to the manufacturer’s protocol; the resulting dsDNA was enriched in a PCR reaction based on predetermined CT values and cleaned using AMPure XP beads (provided with Illumina kit). Concentrations of enriched dsDNA fragments with specific adapters were determined and base pair average size as well as library integrity were analyzed using the Bioanalyzer DNA High Sensitivity chips (Agilent, Cat. 5067-4626). Samples were pooled and sequenced on the Illumina NextSeq 500/550 High Output platform (Illumina, FC-404-2002) up to 18 samples per lane with 1% PhiX spike as a control.

The read quality of the raw FASTQ files was checked with FastQC (Andrews and Babraham Bioinformatics, 2010) (v0.11.7). Next, reads were pseudo-aligned to the mouse reference transcriptome (Mus_musculus.GRCm38.cdna) using Kallisto (Bray et al., 2016) (v0.44.0) with the quantification algorithm enabled, the number of bootstraps set to 100, and run in paired-end mode. The Kallisto output files were read into R using Sleuth, and the transcripts per million (TPM), a measurement of the proportion of transcripts in the RNA pool, was used for downstream differential expression analysis (Pimentel et al., 2017).

**Measuring protein decay rates using cycloheximide**

Protein decay rates were quantified as previously described (Bahrami-Nejad et al., 2018). Briefly, 10,000 OP9 cells were seeded in 96-well plates) one plate for each timepoint. Cells were induced to differentiate with DMI for 24 hours. Cyclohexamide was added to the media at a final concentration of 30 μM. Cells were fixed and stained at different times after addition of cyclohexamide, and immunofluorescence was used to quantify protein concentration. Half-lives were obtained by fitting first order exponential decay curves to the data.

**Fluorescent imaging**

Imaging was conducted using an ImageXpress MicroXL (Molecular Devices, USA) with a 10X Plan Apo 0.45 NA objective. Live fluorescent imaging was conducted at 37°C with 5% CO2. A camera bin of 2x2 was used for all imaging condition. Cells were plated in optically clear 96-well plates: plastic-bottom Costar plates (#3904) for fixed imaging or Ibidi µ-Plate (#89626) for live imaging. Living cells were imaged in FluoroBrite DMEM media (Invitrogen) with 10% FBS, 1% Penicillin/Streptomycin and insulin to reduce background fluorescence. Images were taken every 12 min in different fluorescent channels: CFP, YFP and/or RFP. Total light exposure time was kept less than 700 ms for each time.
point. Four, non-overlapping sites in each well were imaged. Cell culture media were changed at least every 48h.

**Imaging data processing**

Data processing of fluorescent images was conducted in MATLAB R2016a (MathWorks). Unless stated otherwise, fluorescent imaging data were obtained by automated image segmentation, tracking and measurement using the MACKtrack package for MATLAB. Quantification of PPAR- and CEBPA-positive cells in fixed samples was based on quantification of mean fluorescence signal over nuclei. Cells were scored as PPAR- and CEBPA-positive if the marker expression level was above a preset cut-off determined by the bimodal expression at the end of the experiment.

For live imaging data of OP9 cells, the CFP channel capturing H2B-mTurqoise fluorescence was used for nuclear segmentation and cell tracking. Obtained single-cell traces were filtered to removed incomplete or mistracked traces according to the following criteria: cells absent within 6 hours of the endpoint, cell traces that started more than 4 hours after the first timepoint, cells that had large increase or decrease in PPAR intensity normalized to the previous timepoint, cells where H2B drops did not match drops in the APC/C reporter. If cells were binned according to their PPAR expression, cells were binned based on their mean nuclear PPAR expression at the described timepoints.

The percent of cells in the S/G2/M phases at each time point is calculated by counting the cells that expressed the APC/C reporter during the 96-hour differentiation period divided by the total number of cells. The percent of PPAR high cells was assessed by counting cells that above the PPAR threshold at that time point and dividing by the total number of cells at that time point.

**Estimating a differentiation commitment point (i.e. PPAR threshold)**

PPAR values at the end of a differentiation experiment typically exhibit a bimodal distribution. In order to estimate a commitment point, PPAR values at the last frame of the experiment was fit to a 2 component gaussian mixture model. Cells were then classified as either differentiated or undifferentiated based on whether they more closely associated with the high or low component of the mixture model, respectively. The commitment point was then assessed as the value of PPAR at the 48-hour time point, before the stimuli was removed, that predicted the final differentiation classification with a false positive rate of less than 5%. In experiments where multiple conditions are present, the gaussian mixture model was only fitted to the negative control and the commitment point was selected based on the negative control model and applied to all other conditions in the same experiment.
Statistics

Unless specified otherwise, data are expressed as mean +/- standard error of the mean (S.E.M). Live traces are expressed as median +/- interquartile range (25th-75th percentiles). For histograms with a y-axis labeled “Fraction of Cells,” each histogram (not each plot) is normalized to the total number of cells in the population of that histogram such that all bars in the histogram add to 1. Representative results are representative of at least two independent experiments.

Data availability

All relevant data from this manuscript are available upon request.
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**AUTHOR CONTRIBUTIONS**

M.L.Z. and M.N.T. conceived experiments. M.L.Z., K.K., A.R., and Z.B. performed experiments and analyzed data. M.L.Z. and B.T. wrote the image analysis scripts. M.L.Z. and M.N.T wrote the paper with input from all authors.

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FIGURE LEGENDS

Figure 1. Measurement of the precise time when cells irreversibly commit to differentiate based on live single-cell analysis of endogenous expression levels of PPARG.

(A) Images of cells expressing endogenous citrine-PPARG that were differentiated using the standard 96-hour DMI protocol. Scale bar, 50 µm.

(B) Results of a typical experiment in which thousands of single cells were stimulated to differentiate. Thirty single-cell traces are shown as examples. Representative of 4 independent experiments.

(C) Scatter plot using data from (B) showing PPARG levels for each cell at 48 hours, just before the DMI stimulus was removed, and at 96 hours. Dashed black line represents the PPARG threshold (see Methods). Cells were defined as differentiated (blue) or undifferentiated (orange) by whether their PPARG levels were in the high or low intensity distributions, respectively.

(D) Citrine-PPARG cells were differentiated using the DMI protocol, and immunocytochemistry was performed at 96 hours for adipocyte markers (for each scatter plot: n > 4000 cells, representative of 2 independent experiments).

(E) The timecourses from (B) were split into equal-width bins by their PPARG values at 0, 24, 48, and 72 hours. The fraction of differentiated cells represents the number cells that crossed the PPARG threshold at the end of the experiment divided by the number of cells in the bin.

(F) Differentiating cells from (B) were computationally aligned so that the zero timepoint represents the time when the cell crossed the PPARG threshold. Plot shows 5 representative single cell traces, the median (solid black line), and the 5th-95th percentile (shaded region).

(G) PPARG timecourses from the cells that differentiated after 96 hours in (D) were computationally aligned as in (F) and plotted (blue curves). At each aligned time point, the Pearson correlation coefficient between the aligned PPARG values and the endpoint immunofluorescence values for adipocyte markers was calculated (red curves). As a comparison, PPARG values for the Glut4 panel were aligned to a randomized PPARG threshold crossing point. The randomized crossing point was generated by scrambling the vector of measured threshold points for each cell so that each threshold point is matched with different cell.

(B-F) The dotted line represents the calculated PPARG threshold for that experiment.

Figure 2. Development of a system to measure timecourses of cell cycle phases and terminal cell differentiation dynamics simultaneously in thousands of individual cells.

(A) Dual reporter cells were made by stably expressing H2B-mTurquoise(CFP) and APC/C-reportermCherry(RFP). Data from a representative single cell entering the cell cycle is shown. Anaphase is shown both by a split in the H2B-mTurquoise signal (top images) and by a sharp drop in APC/C reporter signal (bottom timecourse). Scale bar, 20 µm. White outlines mark the position of nuclei in cells after anaphase. Figure S2 confirms that the APC/C reporter is suitable to monitor G1 length in the OP9 preadipocyte cell system by showing very similar dynamics to that of a reporter of CRL4-Cdt2-mediated degradation (Grant et al., 2018; Sakaue-Sawano et al., 2017).

(B) The dual reporter cells allow simultaneous measurement in thousands of single cells of differentiation state using PPARG levels (left) and cell cycle state using the APC/C sensor (right). The timepoints at which mitosis occurred were determined by using the split in H2B signal (black open circles). Representative of 4 independent experiments.

(C) Comparison of the number of observed mitotic events that occurred in cells that were differentiated
versus cells that remained undifferentiate at the end of the 96-hour experiment shown in (B).
(D) Plot showing how the fraction of cells in S/G2/M (red) or with PPARG levels higher than the threshold (blue) varies during a 96-hour differentiation timecourse induced by DMI.
(E) Examples of PPARG (blue) and APC/C reporter (red) timecourses obtained in the same single cell. Cells were stimulated to differentiate with DMI using the standard adipogenic protocol. The yellow dot in each plot marks the time at which that cell reached the PPARG threshold and irreversibly committed to the differentiated state. Bottom row shows examples of 3 undifferentiated/proliferating cells and 1 undifferentiated/quiescent cell that no longer proliferates even after a serum refresh at 48 hours.
(F) Histogram of the difference between the time when the PPARG threshold is crossed and when mitosis last occurred for each cell in the experiment shown in (B). The PPARG threshold is reached on average ~14 hours after the last mitosis is completed. Median value is 11 hours. Negative values indicate cells that reached the PPARG threshold before the last mitosis was completed.
(G) Percent of differentiated/post-mitotic, undifferentiated/proliferating, and undifferentiated/quiescent cells generated in three independent DMI-induced differentiation experiments.

**Figure 3. PPARG controls cell cycle activity in part by upregulating p21 expression.**

(A) Dual-reporter cells were transfected with PPARG or control siRNA and then stimulated to differentiate. The percent of cells in S/G2/M phases at each time point is calculated by counting the cells that expressed the APC/C reporter during the 96-hour differentiation period divided by the total number of cells. The percent of PPARG high cells was assessed by counting the cells with PPARG levels above the threshold divided by the total number of cells at the respective timepoint. Cells were induced to differentiate with the standard DMI protocol. Approximately 5000 cells were analyzed per experiment. Representative of 3 independent experiments.

(B) Wildtype OP9 cells were transfected with PPARG or nontargeting siRNA and then stimulated to differentiate with DMI. RNA samples were collected every 24 hours for 144 hours. Data is plotted as transcripts per million (TPM), and mean ± 1 SD is shown for three replicates.

(C) Dual-reporter cells were transfected with p21, p18, or nontargeting siRNAs and stimulated to differentiate with DMI. The number of cell divisions per cell is reported in the normalized histograms. Representative of 2 independent experiments.

(D) Wildtype OP9 cells were stimulated to differentiate by addition of 1 µM rosiglitazone. p21 levels were measured by fixing and staining cells at subsequent times. Approximately 5000 cells were analyzed per experiment. Data is plotted as mean (line) along with the values for each of the three experiments (points).

(E) Wildtype OP9 cells were stimulated with 1 µM rosiglitazone for 48 hours. Chromatin immunoprecipitation (ChIP) of PPARG was performed followed by qPCR. Three sites on the p21 promoter are shown. The promoters of insulin and Arbp/36b4 served as negative controls, and known PPARG target genes Fabp4/aP2 and Pdk4 (pyruvate dehydrogenase kinase, isoenzyme 4) were used as positive controls. Data are normalized to a nontarget genomic site and IgG enrichment. Two biological experiments were used. Two-way ANOVA with Bonferroni's multiple comparisons test was applied for statistical analysis. Values represent means±SEM. p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

(F) Wildtype OP9 cells were transfected with p21 or Control siRNA and stimulated to differentiate by addition of 1 µM rosiglitazone. Differentiation and cell cycle progression were assessed in the same manner as in Figure 3A.
(G) FKBPL expression under nontargeting vs PPARG knockdown were obtained from the RNA-seq data in (B). Data is reported as TPM, mean ± 1 SD.

(H) Wildtype OP9 cells were transfected with FKBPL or nontargeting siRNAs and stimulated to differentiate with DMI. Stability of p21 and PPARG were assessed by adding 30 µM cycloheximide to the media 24 hours after DMI addition and then fixing and staining for protein levels at different subsequent times. Approximately 5000 cells were analyzed per experiment. Data is plotted as mean ± 1 SD of three replicates.

**Figure 4. Cells crossing the PPARG threshold for differentiation become unresponsive to mitogenic stimulation and are locked into a post-mitotic state by p21.**

(A) Citrine-PPARG and APC/C reporter levels were measured in dual-reporter cells in response to media replacement at 48 hours. Cells were separated into ten bins based on their PPARG levels at 48 hours. Plotted lines show mean values for each bin. Inset shows the APC/C reporter signal between 48–96 hours. Representative of 2 independent experiments.

(B) PPARG values before serum refresh were binned from 100 to 2500 a.u. in 100 a.u. increments. The plot shows for each bin the fraction of cells with a minimum of one division (measured by a split in nuclear H2B signal, red) and the final p21 level (black) and in response to serum refresh. The histogram shows the number of cells in each bin. The dotted black line shows the PPARG threshold for this experiment. Data from experiment in (A).

(C) PPARG, CEBPA, p21, and nontargeting siRNA were transfected into the dual-reporter cells at 48 hours after DMI addition. siRNA knockdown efficiency is shown in Figure S5. Representative of 3 independent experiments.

(D) A similar analysis as described in (A) was performed on the nontargeting and p21 knockdown conditions from (C).

(E) Left, A similar analysis as in (B) was performed on the nontargeting and p21 knockout conditions from (D). Right, the same data normalized to the first PPARG bin.

(F) Images of control and p21-knockdown cells from (C) obtained 48 hours after siRNA transfection (at 96 hours). Red arrows indicate representative multi-nucleated cells. Scale bar, 50 µm.

(G) OP9 cells were induced to differentiate with rosiglitazone. Cyclin D1, p21 and PPARG levels were assessed by immunocytochemistry. The PPARG threshold for the whole experiment (dotted black line) was calculated at the end of the 96-hour differentiation protocol. Representative of 2 independent experiments.

**Figure 5. Adipogenic stimuli initiate a competition between proliferation and differentiation during a gradually extending G1 phase.**

(A) Comparison of the time to commit to the next cell cycle (marked by upregulation of the APC/C reporter) versus time to commit to differentiation in cells that underwent 2-3 mitoses before differentiating. Right, Schematic showing that the end of the second-to-last mitosis is used as the starting reference time for each cell. Left, histograms comparing the two times measured in the same cell (data from Figure 2B, n > 4000 cells, representative of 4 replicates).

(B) Left, Schematic showing which G1-periods were compared. Right, Histograms of the durations of the 1st and 2nd G1-periods in cells from (A) that have undergone at least 3 divisions. See also Figure S6.
(C) Differentiated cells from (A) were separated into three groups based on when they last exited mitosis. The traces were aligned by the last mitosis frame. The median PPARG levels were plotted for each group (dark line). Shaded region represents the 95th confidence interval.

(D) Timecourses from (A) were categorized into 9 bins based on time of last mitosis. The APC/C reporter peak for each bin is plotted to illustrate when the last mitosis occurred relative to addition of the adipogenic (DMI) stimulus.

(E) Left, Plot showing the average time of the last mitosis versus the average time it took for cells in that bin to increase PPARG levels to the differentiation commitment point. Right, Plot of binned cells from (E) showing the average time spent in G1 versus the percent of cells in that bin that were PPARG high.

(F) Schematic of the three stages of G1-extension, in response to an adipogenic (DMI) stimulus. During the intermediate phase of G1-extension, stochastic competition between proliferation and differentiation causes cells to probabilistically exit into a differentiated or proliferating state. This phase defines how many differentiated cells are generated on average from a precursor cell.

Figure 6. Mitogens synergize with adipogenic stimuli to regulate the time when cells commit to terminally differentiate and thereby control the total number of terminally differentiated cells

(A) Dual-reporter cells were differentiated in the presence of a MEK inhibitor (PD0325091). Representative of 2 independent experiments.

(B) Dual-reporter cells were differentiated in normal (10%) and reduced (2%) serum concentrations. Representative of 2 independent experiments.

(C) Dual-reporter cells were transfected with cyclin D1 or nontargeting (control) siRNAs. Representative of 3 independent experiments.

(D) Dual-reporter cells were transfected with p21 or nontargeting (control) siRNAs. Representative of 3 independent experiments.

(E) Dual-reporter cells stably expressing a DHFR-p21-mCherry fusion protein were differentiated in the presence of 10 µM TMP (to increase expression of p21) or DMSO (control). Representative of 2 independent experiments.

(A-E) Cells were induced to differentiate with the standard DMI protocol. Differentiation and cell cycle progression were assessed in the same manner as in Figure 3A. Histograms show the total time spent in G1 phase for each cell trace across all cell cycles for the respective experimental condition.

Figure 7. Different optimal ranges of combined mitogenic and differentiation stimuli control the percent and total number of terminally differentiated cells

(A) Schematic of how the percent versus total number of differentiated cells can differ.

(B) Schematic of how increased proliferation before the PPARG threshold could control the number of adipocytes.

(C) The maximum number of differentiated cells occurs through a competition between mitogenic and adipogenic stimuli. See also Figure S7.
SUPPLEMENTARY MATERIAL

• **Supplementary Video 1**: Dual-reporter OP9 cells induced to undergo adipogenesis by addition of the commonly-used DMI adipogenic stimulus. Daughter 1 (blue trace) and daughter 2 (red trace) only represent the product of the second mitosis (~58 hr) in the time trace. The outline represents the outline of nuclear segmentation based on the H2B channel. APC/C reporter images and trace (gray) and H2B signal are shown only for daughter 1.

• **Supplementary Video 2**: Dual-reporter OP9 cells transfected also with a CDK2 live-cell sensor induced to undergo adipogenesis by addition of the commonly-used DMI adipogenic stimulus.

• 7 Supplementary Figures
SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Additional validation of citrine-PPARG as a marker of differentiation commitment.
(A) The PPARG derivative and integral values are poorer predictors of differentiation. Traces from Figure 1B were smoothed using a Butterworth filter and a five-point stencil was applied to the smoothed traces to estimate the PPARG derivative at each timepoint. The PPARG derivative traces were then analyzed in a similar manner as in Figure 1E. The bins range from -300 to 300 in intervals of 50.

(B) The PPARG integral at each time point was estimated using the trapz() function in MATLAB (MathWorks). The integral values were then analyzed in a similar manner as described in Figure 1E. The bins range from 0 to 5x10^5 in intervals of 104. Note that both the derivative and integral are poor predictors for the final differentiated state. In the case of the derivative, the range between the lowest predicted probability and the highest predicted probability is smaller than found in Figure 1E. The integral values, although a good predictor for a single time point, suffers from the lack of consistency across timepoints, and a single integral value cannot be used to separate undifferentiated and differentiated cells for all timepoints.

(C) The analysis in Figure 1E was done for more time points that span the duration of the experiments and presented as a heatmap where rows represent a timepoint and the columns represents the PPARG bins as described in Figure 1E. The dotted line represents the estimated PPARG threshold for the experiment. The expanded analysis shows that the PPARG threshold remains stable throughout the experiment.

(D) The switch from PPARG low to PPARG high occurs over a relatively short time window. Blue traces represent unaligned population medians of the PPARG abundance (top) and PPARG derivative (bottom). The red trace represents the PPARG abundance (top) and PPARG derivative (bottom) after computationally aligning all traces by the time when the PPARG threshold is crossed and is represented by the zero timepoint. The dashed lines highlight the time window around the peak in the PPARG derivative of the aligned traces and suggests that the switch between PPARG low and PPARG high states occurs over a short window of about 4 hours. All shaded regions represent the interquartile range (25th-75th percentiles).

(E) Unaligned correlations of endpoint markers of adipogenesis to PPARG over a typical 4-day DMI differentiation experiment. PPARG timecourses from the cells that differentiated after 96 hours in Figure 1D were averaged. At each time point, the Pearson correlation coefficient between the unaligned PPARG values, and the endpoint immunofluorescence values for adipocyte markers was calculated.

Figure S2. The APC/C reporter behaves similarly to the Crl4-Cdt2-based sensor in marking the start of S-phase in OP9 cells.
(A) Dual reporter cells infected with a Crl4-Cdt2 sensor tagged with iRFP670. Cells were stimulated to differentiate using the DMI cocktail, and timecourses from individual cells are plotted to compare the dynamics of the APC/C reporter and Crl4-Cdt2 sensor.

(B) Comparison of median levels of the APC/C reporter and Crl4-Cdt2 sensors with t=0 marking the onset of S phase. Shaded regions represent the interquartile range (25th to 75th percentiles).

(C) Comparison of the median levels of APC/C reporter and Crl4-Cdt2 sensors at the onset of S-phases across multiple days of imaging. Shaded regions represent the interquartile range.
(A-C) In summary, the difference between the two probes in measuring the G1/S transition is only on the order of 1-2 hours at most, which would not change any of our conclusions in the context of our 4-day long experiments.

**Figure S3. Additional results supporting Figure 2.**

**(A)** Characterization of the plating conditions. In this study, we used subconfluent plating conditions (5K per well) instead of plating at confluent (15K per well) conditions that we normally use. Subconfluent plating increases the number of cell division events observed during adipogenesis and allows for easier cell tracking. The dual reporter cells were differentiated using the standard DMI cocktail. Left, The plot represents the fraction of cells that are considered past the PPARG threshold at each time point for both cell density conditions. Right, A comparison of the fraction of dual reporter cells in S/G2/M phases of the cell cycle, as assessed by the APC/C reporter, for both plating conditions.

(B) The number of mitotic events for both plating conditions are reported in the histogram as the fraction of cells observed with a given number of mitosis events. The 15,000 cells per well plating condition represents a standard differentiation protocol and yields high rates of differentiation and relatively low cell cycle activity. However, plating cells at a density of 5000 cells per well leads to a lower degree of differentiation and a higher degree of cell cycle activity. The live cell experiments in this manuscript are plated at a density of 5000 cells per well.

(C) Dilution through cell division does not significantly affect PPARG dynamics in differentiated cells. PPARG dynamics in differentiated cells were compared between cells that divided two (blue) or three (red) times in the span of the experiment, as indicated by the APC/C reporter (right). Additionally, the selected cells all completed the last mitosis at similar times. PPARG (left) and APC/C reporter (right) traces were computationally aligned to the last mitosis time. Bold traces represent median values and the shaded region represents the 95th confidence interval of the median.

(D) The trade-off between continued proliferation and differentiation exists even in cells that have been selected for undergoing exactly two divisions during the timespan of a 96-hour live-cell experiment.

(E) A CDK2 sensor (orange trace) was added to the PPARG and APC/C dual reporter cells to create triple reporter cells. Triple reporter cells were differentiated using the standard DMI protocol, and a representative trace is shown. The yellow dot represents the time when the cell reached the PPARG threshold and irreversibly committed to the differentiated state. Representative of 2 independent experiments.

(F) Scatter plot showing the CDK2 activity versus PPARG level in each single cell at every time point. The red dashed line represents the PPARG threshold.

**Figure S4. Significant cell-to-cell variability is apparent even accounting for factors such as cell cycle phase when DMI was added, number of previous cell cycles, and refreshing the stimulus/serum.**

(A) Plot shows cells that differentiated in the experiment separated by what phase of the cell cycle the cells were in when DMI was added. Cells in which the adipogenic stimulus is added in G1 have fewer average additional divisions compared to cells where adipogenic stimuli are applied in S/G2/M.

(B) Analysis of the PPARG increase in cells with 0, 1 or 2 divisions before terminal cell differentiation.

(C) When the time of second mitosis is late, cells have on average slower increases in PPARG compared to cells where the second mitosis is earlier.
(D) Control experiment showing that replacement of DMI medium every 12 hours does not significantly change differentiation outcome.

Figure S5. Validation of the siRNA knockdown efficiency when cells were transfected at 48 hours after induction of adipogenesis.
(A) Cells were transfected with siRNA 48 hours after addition of the adipogenic DMI stimulus and knockdown efficiency was assessed 48 hours later (at the end of the 96-hour long time-lapse experiment). To validate the siRNA knockdown efficiency of p21 and CEBPA, cells were fixed with paraformaldehyde and immunostained for p21 or CEBPA levels.
(B) PPARG knockdown was assessed using the live cell citrine-PPARG signal.

Figure S6. Adipogenic stimuli initiate a competition between proliferation and differentiation during a gradually extending G1 phase.
(A) Histogram of the difference between G1$_1$ and G1$_2$ for each cell from Figure 5B.
(B) Plot of G1 duration versus how long a cell had been exposed to the adipogenic (DMI) stimulus at the start of G1 for each cell from (A). Red line marks average G1 duration of all cells.
(C) Analysis of timecourses in Figure 2B showing duration of G1 versus S/G2/M for differentiated and undifferentiated cells. Representative of 3 independent experiments.
(D-E) Analysis of timecourses of OP9 cells transfected with siRNA targeting cdc25c and cdc25c and induced to differentiate using a standard DMI protocol.

Figure S7. Titration of DMI versus FBS going up to higher maximal concentrations.
**Figure 1.** Measurement of the precise time when cells irreversibly commit to differentiate based on live single-cell analysis of endogenous expression levels of PPARG.

(A) Images of cells expressing endogenous citrine-PPARG that were differentiated using the standard 96-hour DMI protocol. Scale bar, 50 μm.

(B) Results of a typical experiment in which thousands of single cells were stimulated to differentiate. Thirty single-cell traces are shown as examples. Representative of 4 independent experiments.

(C) Scatter plot using data from (b) showing PPARG levels for each cell at 48 hours, just before the DMI stimulus was removed, and at 96 hours. Dashed black line represents the PPARG threshold (see Methods). Cells were defined as differentiated (blue) or undifferentiated (orange) by whether their PPARG levels were in the high or low intensity distributions, respectively.

(D) Citrine-PPARG cells were differentiated using the DMI protocol, and immunocytochemistry was performed at 96 hours for adipocyte markers (for each scatter plot: n > 4000 cells, representative of 2 independent experiments).

(E) The timecourses from (B) were split into equal-width bins by their PPARG values at 0, 24, 48, and 72 hours. The fraction of differentiated cells represents the number cells that crossed the PPARG threshold at the end of the experiment divided by the number of cells in the bin.

(F) Differentiating cells from (B) were computationally aligned so that the zero timepoint represents the time when the cell crossed the PPARG threshold. We plotted 5 representative single cell traces, the median (solid black line), and the 5th-95th percentile (shaded region).

(G) PPARG timecourses from the cells that differentiated after 96 hours in (D) were computationally aligned as in (F) and plotted (blue curves). At each aligned time point, the Pearson correlation coefficient between the aligned PPARG values and the endpoint immunofluorescence values for adipocyte markers was calculated (red curves). As a comparison, PPARG values for the Glut4 panel were aligned to a randomized PPARG threshold crossing point. The randomized crossing point was generated by scrambling the vector of measured threshold points for each cell so that each threshold point is matched with different cell.

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(B) The dual reporter cells allow simultaneous measurement in thousands of single cells of differentiation state using PPARG levels (left) and cell cycle state using the APC/C sensor (right). The timepoints at which mitosis occurred were determined by using the split in H2B signal (black open circles). Cells were stimulated to differentiate with DMI using the standard adipogenic protocol. Representative of 4 independent experiments.

(C) Comparison of the number of observed mitotic events that occurred in cells that were differentiated versus cells that remained undifferentiated at the end of the 96-hour experiment shown in (B).

(D) Plot showing how the fraction of cells in S/G2/M (red) or with PPARG levels higher than the threshold (blue) varies during a 96-hour differentiation timecourse induced by DMI.

(E) Examples of PPARG (blue) and APC/C reporter (red) timecourses obtained in the same single cell. The yellow dot in each plot marks the time at which that cell reached the PPARG threshold and irreversibly committed to the differentiated state. Bottom row shows examples of 3 undifferentiated/proliferating cells and 1 undifferentiated/quiescent cell that no longer proliferates even after a serum refresh at 48 hours.

(F) Histogram of the difference between the time when the PPARG threshold is crossed and when mitosis last occurred for each cell in the experiment shown in (B). The PPARG threshold is reached on average ~14 hours after the last mitosis is completed. Median value is 11 hours. Negative values indicate cells that reached the PPARG threshold before the last mitosis was completed.

(G) Percent of differentiated/post-mitotic, undifferentiated/proliferating, and undifferentiated/quiescent cells generated in three independent DMI-induced differentiation experiments.
Figure 3. PPARγ controls cell cycle activity in part by upregulating p21 expression.

(A) Dual-reporter cells were transfected with PPARγ or control siRNA and then stimulated to differentiate. The percent of cells in S/G2/M phases at each time point is calculated by counting the cells that expressed the APC/C reporter during the 96-hour differentiation period divided by the total number of cells. The percent of PPARγ high cells was assessed by counting the cells with PPARγ levels above the threshold divided by the total number of cells at the respective timepoint. Cells were induced to differentiate with the standard DMI protocol. Approximately 5000 cells were analyzed per experiment. Representative of 3 independent experiments.

(B) Wildtype OP9 cells were transfected with PPARγ or nontargeting siRNA and then stimulated to differentiate with DMI. RNA samples were collected every 24 hours for 144 hours. Data is plotted as transcripts per million (TPM), and mean ± 1 SD is shown for three replicates.

(C) Dual-reporter cells were transfected with p21, p18, or nontargeting siRNAs and stimulated to differentiate with DMI. The number of cell divisions per cell is reported in the normalized histograms. Representative of 2 independent experiments.

(D) Wildtype OP9 cells were stimulated to differentiate by addition of 1 µM rosiglitazone. p21 levels were measured by fixing and staining cells at subsequent times. Approximately 5000 cells were analyzed per experiment. Data is plotted as mean (line) along with the values for each of the three experiments (points).

(E) Wildtype OP9 cells were stimulated with 1 µM rosiglitazone for 48 hours. Chromatin immunoprecipitation (ChIP) of PPARγ was performed followed by qPCR. Three sites on the p21 promoter are shown. The promoters of insulin and Arbp/36b4 served as negative controls, and known PPARγ target genes Fabp4/aP2 and Pdk4 (pyruvate dehydrogenase kinase, isoenzyme 4) were used as positive controls. Data are normalized to a nontarget genomic site and IgG enrichment. Two biological experiments were used. Two-way ANOVA with Bonferroni's multiple comparisons test was applied for statistical analysis. Values represent means ± SEM. p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

(F) Wildtype OP9 cells were transfected with p21 or Control siRNA and stimulated to differentiate by addition of 1 µM rosiglitazone. Differentiation and cell cycle progression were assessed in the same manner as in Figure 3A.

(G) FKBPL expression under nontargeting vs PPARγ knockdown were obtained from the RNA-seq data in (B). Data is reported as TPM, mean ± 1 SD.

(H) Wildtype OP9 cells were transfected with FKBPL or nontargeting siRNAs and stimulated to differentiate with DMI. Stability of p21 and PPARγ were assessed by adding 30 µM cycloheximide to the media 24 hours after DMI addition and then fixing and staining for protein levels at different subsequent times. Approximately 5000 cells were analyzed per experiment. Data is plotted as mean ± 1 SD of three replicates.
Figure 4. Cells crossing the PPARG threshold for differentiation become unresponsive to mitogenic stimulation and are locked into a post-mitotic state by p21.

(A) Citrine-PPARG and APC/C reporter levels were measured in dual-reporter cells in response to media replacement at 48 hours. Cells were separated into ten bins based on their PPARG levels at 48 hours. Plotted lines show mean values for each bin. Inset shows the APC/C reporter signal between 48-96 hours. Representative of 2 independent experiments.

(B) PPARG values before serum refresh were binned from 100 to 2500 a.u. in 100 a.u. increments. The plot shows for each bin the fraction of cells with a minimum of one division (measured by a split in nuclear H2B signal, red) and the final p21 level (black) and in response to serum refresh. The histogram shows the number of cells in each bin. The dotted black line shows the PPARG threshold for this experiment. Data from experiment in (A).

(C) PPARG, CEBPA, p21, and nontargeting siRNA were transfected into the dual-reporter cells at 48 hours after DMI addition. siRNA knockdown efficiency is shown in Figure S6. Representative of 3 independent experiments.

(D) A similar analysis as described in (A) was performed on the nontargeting and p21 knockdown conditions from (C).

(E) Left, A similar analysis as in (B) was performed on the nontargeting and p21 knockout conditions from (D). Right, the same data normalized to the first PPARG bin.

(F) Images of control and p21-knockdown cells from (C) obtained 48 hours after siRNA transfection (at 96 hours). Red arrows indicate representative multi-nucleated cells. Scale bar, 50 μm.

(G) OP9 cells were induced to differentiate with rosiglitazone. Cyclin D1, p21 and PPARG levels were assessed by immunocytochemistry. The PPARG threshold for the whole experiment (dotted black line) was calculated at the end of the 96-hour differentiation protocol. Representative of 2 independent experiments.
Figure 5. Adipogenic stimuli initiate a competition between proliferation and differentiation during a gradually extending G1 phase. (A) Comparison of the time to commit to the next cell cycle (marked by upregulation of the APC/C reporter signal) versus time to commit to differentiation in cells that underwent 2-3 mitoses before differentiating. Right, Schematic showing that the end of the second-to-last mitosis is used as the starting reference time for each cell. Left, histograms comparing the two times measured in the same cell (data from Figure 2B, n > 4000 cells, representative of 4 replicates). (B) Left, Schematic showing which G1-periods were compared. Right, Histograms of the durations of the 1st and 2nd G1-periods in cells from (A) that have undergone at least 3 divisions. See also Figure S6. (C) Differentiated cells from (A) were separated into three groups based on when they last exited mitosis. The traces were aligned by the last mitosis frame. The median PPARG levels were plotted for each group (dark line). Shaded region represents the 95th confidence interval. (D) Timecourses from (A) were categorized into 9 bins based on time of last mitosis. The APC/C reporter peak for each bin is plotted to illustrate when the last mitosis occurred relative to addition of the adipogenic (DMI) stimulus. (E) Left, Plot showing the average time of the last mitosis versus the average time it took for cells in that bin to increase PPARG levels to the differentiation commitment point. Right, Plot of binned cells from (E) showing the average time spent in G1 versus the percent of cells in that bin that were PPARG high. (F) Schematic of the three stages of G1-extension, in response to an adipogenic (DMI) stimulus. During the intermediate phase of G1-extension, stochastic competition between proliferation and differentiation causes cells to probabilistically exit into a differentiated or proliferating state. This phase defines how many differentiated cells are generated on average from a precursor cell.
Figure 6. Mitogens synergize with adipogenic stimuli to regulate the time when cells commit to terminally differentiate and thereby control the total number of terminally differentiated cells.

(A) Dual-reporter cells were differentiated in the presence of a MEK inhibitor (PD0325901). Representative of 2 independent experiments.

(B) Dual-reporter cells were differentiated in normal (10%) and reduced (2%) serum concentrations. Representative of 2 independent experiments.

(C) Dual-reporter cells were transfected with cyclin D1 or nontargeting (control) siRNAs. Representative of 3 independent experiments.

(D) Dual-reporter cells were transfected with p21 or nontargeting (control) siRNAs. Representative of 3 independent experiments.

(E) Dual-reporter cells stably expressing a DHFR-p21-mCherry fusion protein were differentiated in the presence of 10 μM TMP (to increase expression of p21) or DMSO (control). Representative of 2 independent experiments.

(A-E) Cells were induced to differentiate with the standard DMI protocol. Differentiation and cell cycle progression were assessed in the same manner as in Figure 3A. Histograms show the total time spent in G1 phase for each cell trace across all cell cycles for the respective experimental condition.
Figure 7. Different optimal ranges of combined mitogenic and differentiation stimuli control the percent and total number of terminally differentiated cells

(A) Schematic of how the percent versus total number of differentiated cells can differ.

(B) Schematic of how increased proliferation before the PPARG threshold could control the number of adipocytes.

(C) The maximum number of differentiated cells occurs through a competition between mitogenic and adipogenic stimuli. See also Figure S7.