Cell Reports

Cell Size-Based Decision-Making of a Viral Gene Circuit

Graphical Abstract

Highlights

- Gene expression bursts from the HIV LTR promoter increase with T cell size
- Larger T cells latently infected with HIV exclusively reactivate from latency
- Reactivation from HIV latency is cell-cycle dependent, with enhancement in G1
- Checkpoint arrestors actively modulate cell cycle to bias viral decision-making

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In Brief

Bohn-Wippert et al. investigate reactivation of T cells latently infected with HIV. They discover that only larger cells exit latency, while smaller cells remain silent. Viral expression bursts are cell size and cell-cycle dependent, presenting dynamic cell states, capable of active control, as sources of viral fate determination.
Cell Size-Based Decision-Making of a Viral Gene Circuit

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SUMMARY

Latently infected T cells able to reinitiate viral propagation throughout the body remain a major barrier to curing HIV. Distinguishing between latently infected cells and uninfected cells will advance efforts for viral eradication. HIV decision-making between latency and active replication is stochastic, and drug cocktails that increase bursts of viral gene expression enhance reactivation from latency. Here, we show that a larger host-cell size provides a natural cellular mechanism for enhancing burst size of viral expression and is necessary to destabilize the latent state and bias viral decision-making. Latently infected Jurkat and primary CD4+ T cells reactivate exclusively in larger activated cells, while smaller cells remain silent. In addition, reactivation is cell-cycle dependent and can be modulated with cell-cycle-arresting compounds. Cell size and cell-cycle dependent decision-making of viral circuits may guide stochastic design strategies and applications in synthetic biology and may provide important determinants to advance diagnostics and therapies.

INTRODUCTION

One major obstacle to curing the global HIV epidemic is the reservoir of latently infected resting CD4+ T cells (Chun et al., 1997; Finzi et al., 1997; Richman et al., 2009). Under antiretroviral therapy (ART), HIV viral load is undetectable in the plasma of infected individuals. Upon removal of ART, the viral load rapidly rebounds back to pretreatment levels of viremia due to reactivation of the latent reservoir (Davey et al., 1999). Reactivation from latency involves production and spread of virions to target-rich lymph node niches unprotected by ART (Stellbrink et al., 2002).

Researchers have worked extensively on the mechanisms and regulation of latency (Richman et al., 2009; Ruelas and Greene, 2013) and on drug treatments to both reactivate and remove cells harboring latent provirus (i.e., the shock-and-kill strategy) (Dar et al., 2014; Deeks, 2012; Spina et al., 2013). Strategies to reanimate the latent reservoir are plagued by severe challenges, including (1) incomplete reactivation of non-inducible provirus (Ho et al., 2013), (2) uncertainty regarding clearance or death of cells after latent reversal (Deng et al., 2015; Shan et al., 2012), and (3) coupling of migration and reactivation of latently infected T cells, causing additional viral spread in cell niches (Bohn-Wippert et al., 2017; Murooka et al., 2012). Recent efforts have used an alternative block-and-lock strategy toward silencing latency into a chronically inactive state (Besnard et al., 2016; Dar et al., 2014; Kessing et al., 2017). Another approach, direct removal of the latent reservoir, is challenged by our inability to identify latent cells at low expression levels. To address this, researchers have pursued identification of novel biomarkers for viral persistence (Fromentin et al., 2016; Hurst et al., 2015).

Gene expression fluctuations play an important role in determining when a virus shifts between latency and activation (Weinberger et al., 2005, 2008). Studies of gene expression bursts at levels of transcription and translation in human fibroblasts, and cell-free gene expression systems reveal a correlation between gene expression bursts and cell reaction volume (Caveney et al., 2017; Padovan-Merhar et al., 2015). Here, a burst is defined as the number of mRNA produced per transcriptional activity pulse of the promoter during episodic transcription (transcriptional burst) or the number of proteins produced per mRNA lifetime (translational burst). Both transcriptional and translational bursts contribute to total gene expression bursts (Dar et al., 2015; Kepler and Elston, 2001; Ozbudak et al., 2002). The authors show that fluorescence measured by the abundance of GFP increases with the size of a cell-free gene expression reactor, similar to increases of mRNA levels of genes in larger human fibroblasts (Figure S1) (Caveney et al., 2017; Padovan-Merhar et al., 2015). Observed increases are described by increased burst size, not by increased burst frequency (the transition rate of an inactive promoter into active transcribing state $k_{on}$, both of which can increase abundance levels (Dar et al., 2012; Kepler and Elston, 2001; Megaridis et al., 2018; Simpson et al., 2004; Singh et al., 2010). In addition, burst frequency ($k_{on}$ or $F$) has been shown to...
depend on cell cycle and decreases after DNA replication in the late G2 phase (Padovan-Merhar et al., 2015; Skinner et al., 2016). Additional studies have investigated the coupling of gene expression noise to growth rate and cell cycle of yeast (Keren et al., 2015).

Previous studies have shown that cell size influences other cell functions. A preexisting intracellular variation dependent on the volume of Escherichia coli cells has been shown to bias lambda phage developmental fate before infection (St-Pierre and Endy, 2008). Additional studies reveal that the lysis-lysogeny decision of bacteriophage λ depends on both bacterial host-cell size and MOI (Cortes et al., 2017; Zeng et al., 2010). The postinfection decision of the phage is initially made at the single-virus level with additional infections contributing to an integrated decision between lysis and lysogeny of the targeted cell (Zeng et al., 2010). The probability of entering the lysogeny state has also been shown to be inversely proportional to phage infection times (Cortes et al., 2017).

Despite the low MOI of latently infected T cells, these examples of viral-host decision-making suggest the possibility of similar determinants existing in the reactivation-latency decision of latently integrated HIV in single human T cells. Here we show that cell size and cell cycle are involved in single-cell decision-making of HIV-infected T cells. Along with consistent burst size dependence on cell size for various housekeeping promoters (Figure S1), this suggests a general role in endogenous genetic systems.

**RESULTS**

**Model of Cell Size-Based Initiation of HIV Feedback and Reactivation from Latency**

To investigate whether HIV's shift from prolonged latency to reactivation could depend on cell size, we implemented a computational model consisting of the HIV long terminal repeat (LTR) promoter driving the expression of the HIV transactivator of transcription (Tat) (Figure 1A). Activation of LTR transcription by Tat is a potent positive feedback loop required to exit the latent state (Weinberger et al., 2005, 2008). The model consists of a 2-state episodic LTR promoter that is primarily in the inactive or non-transcribing state (k_{off} >> k_{on}) and underlies expression of Tat autoregulation, regardless of whether it is active (Figure 1A, bottom) or inactive (Figure 1A, top). At low Tat levels (Figure 1A, top), feedback is inactive and viral expression is described by the 2-state switching model with the active LTR state, switched at rate k_{on}, where viral mRNA are transcribed at rate k_{m}. This occurs...
before decay back to an inactive transcribing state at rate $k_{\text{off}}$ (Kepler and Elston, 2001; Pecced and Ycart, 1995; Simpson et al., 2004). At high Tat levels produced by lower $k_{\text{off}}$, LTR bound by Tat transcribes at an elevated transcription rate, initiates feedback, and leads to reactivation from latency. For inactive feedback, the number of mRNA produced per promoter activity pulse ($k_m/k_{\text{off}}$ in the transcribing state is defined as the transcriptional burst size (or B). Translation occurs at rate $k_p$, and the number of proteins translated per mRNA lifetime ($k_p/\gamma_m$) is defined as the translational burst size (or $b$). Total expression burst size ($B \times b$) is assumed to increase with cell size (Figure 1A, left vertical arrow), and a range of burst sizes is assessed for evidence of increased cell-size-dependent transitions between the inactive to the active feedback state (Figure 1A). This model has previously been used to understand the role of Tat feedback in HIV autonomy from host-cell state (Razooky et al., 2015).

Analysis of gene expression fluctuations, or noise, has been a valuable tool to understand the stochastic viral switch of HIV (Weinberger et al., 2005, 2008). Transcriptional bursting of the LTR promoter has been experimentally measured and modeled across the human genome, including noise modulation under diverse drug treatments (Boehm et al., 2013; Dar et al., 2012, 2014, 2016; Singh et al., 2010). Translational bursting also contributes to total gene expression noise (Dar et al., 2015; Ozbudak et al., 2002). Noise measurements from LTR expression can illustrate the dependence of total burst size ($B \times b$) on cell size and be used for modeling size-dependent behavior (Figure 1).

Total expression burst size of the inactive feedback 2-state promoter $B \times b$ is proportional to mean Tat protein levels and noise (quantified by the coefficient of variation squared ($CV^2$)) using the following equations:

$$\langle p \rangle = \frac{bBF}{\gamma_p}, \quad CV^2 = \frac{b(1 + B)}{\langle p \rangle} \quad (\text{Equation 1})$$

$$B = \frac{k_m}{k_{\text{off}}}, \quad F = \frac{k_{\text{on}}}{\gamma_m}, \quad b = \frac{k_p}{\gamma_p}, \quad B \times b = \frac{k_m k_p}{k_{\text{off}} \gamma_m} \quad (\text{Equation 2})$$

Here Tat protein is referred to as $p$; mRNA and protein are produced at rates $k_m$ and $k_p$, and degraded at rates $\gamma_m$ and $\gamma_p$, respectively; burst frequency is indicated as $F$; and other rates and burst definitions have been mentioned earlier. These equations have been previously derived under slow gene activation assumptions that $k_{\text{off}} >> k_{\text{on}}, k_{\text{off}} >> k_m, k_{\text{off}} >> \gamma_p$, and $k_m >> (\gamma_m + \gamma_p)$ (Dar et al., 2012; Kepler and Elston, 2001; Simpson et al., 2004; Singh et al., 2010). For latently infected cells, the assumption that $k_{\text{off}} >> k_{\text{on}}$ holds more than previous estimates for an active LTR promoter (Table S1).

With an assumed increasing power function dependence of total burst size ($B \times b$) on cell size, stochastic simulations were performed using a power function for all cell volumes. Single-cell expression trajectories of larger cells surpassed Tat threshold levels needed for reactivation, while smaller cells remained latent (Figures 1B and 1C). Figure 1C shows the number of cells reactivated and sorted by diameter. The result is compared to and normalized by a size distribution (6–20 $\mu$m) of the total T cell population obtained experimentally (Figure 1C, orange curve). Here $B \times b$ changes $k_{\text{off}}$ and $k_m$ simultaneously (Figure 1A, upper versus lower), while additional cases of changing $k_p$ and combinations of the three increase reactivation. The effect of Tat on the 2-state promoter switching model has been shown to effectively decrease $k_{\text{off}}$ such that in the transition of Tat to higher levels and active feedback, transcriptional bursts and total burst size are increased (Razooky et al., 2017).

In the inactive feedback model with low Tat levels, noise is inversely related to mean abundance (Equation 1; Figure 1D). Increases in transcriptional burst size ($B$) lead to a higher burst model line from which transcription can be initiated with activator drug treatments known to increase burst frequency ($F$) or the rate of switching into the active LTR state. Larger cells would surpass the threshold between promoter only and active feedback, while smaller cells would fall short.

Recently reported noise drug cocktails effectively exploit the same noise modulation strategy by using combination drug treatments to increase both $B$ and $F$ and achieve synergistic reactivation in latent cell populations (Equation 1) (Dar et al., 2014). In the current study, natural heterogeneity of latent cell size varies in total burst size and a similar effect is achieved with a single-activator treatment to increase $F$ and total $B$. Larger cell sizes are primed for stochastic reactivation before activator treatments. The model results show that cell-size-dependent total expression bursts are capable of determining between reactivated and latent states of HIV. This motivates our hypothesis that cell-size-dependent increases of burst size can determine the decision of HIV to reactivate from latency after activator drug treatment (Figure 1D). If true, cell-size-dependent decision-making of HIV in single cells will be important for understanding stochastic design and heterogeneity in other biological systems and disease (Dar and Weiss, 2018).

**Gene Expression Bursts of the HIV LTR Promoter Increase with T Cell Size**

To test whether larger cell sizes increase the HIV LTR burst size, we performed single-cell time-lapse fluorescence microscopy of Jurkat T cells infected with the LTR driving a short-lived 2-hr half-life GFP (d2GFP) (Dar et al., 2012). The greater the d2GFP fluorescence, the more viral protein and expression activity occurs. Imaging was performed on a clonal population of LTR-d2GFP for 14 hr (STAR Methods). 3,037 single-cell trajectories were quantified over time for total fluorescent intensity and binned into ten cell size ranges (Figure 2A, with mean bin diameter noted). For the 10 bins, the LTR-d2GFP Jurkats reveal that the larger the mean cell diameter, the greater the total fluorescence intensity (blue circles, Figure 2B). The d2GFP intensity per unit volume shows no change across all cell diameters (orange squares, Figure 2B). This is consistent with previous reports of increased transcriptional levels and transcriptional burst size with cell volume to maintain constant transcript concentrations (Padovan-Merhar et al., 2015). Noise was analyzed from the total fluorescent trajectories of the cells in each bin (Dar et al., 2012). Noise magnitude from finite-duration imaging (high-frequency coefficient of variation squared, or $HF-CV^2$ (Figure 2C) (Dar et al., 2012) and total burst size (Figure 2D) increase with the mean diameter of each bin (Figure 2A). Increasing noise magnitude with fluorescence indicates an increase in total burst size (Equation 1) and
was previously observed for the LTR at both mRNA and protein levels using both microscopy (HF-CV\textsuperscript{2} and CV\textsuperscript{2}) and flow cytometry (CV\textsuperscript{2}) (Dar et al., 2012, 2016). The d2GFP reporter showed a constant degradation rate with cell size under cycloheximide treatment (Figure S2), and previous reports show that mRNA degradation stays constant with cell size (Padovan-Merhar et al., 2015). Total burst size \(B_b\) determined using Equation 1 showed a non-linear increase of up to a 10-fold change across the cell diameter range (Figure 2D). To find the dependence of changes to total burst size with cell size, fold change of total burst size was fit with a power function \(R^2 = 0.99\) (Figure 2E). This was used to simulate the size-dependent increases of expression bursts from Figure 1. All diameter measurements obtained by microscopy were verified using a Moxi Z automated cell analyzer (Orflo), which exploits the Coulter principle for cell count and size (Mattern et al., 1957) (Figure S3).

![Figure 2. Burst Size of the HIV LTR Promoter Increases with T Cell Volume](image)

(A) 3,037 single-cell intensity trajectories from an LTR-d2GFP clonal cell line acquired using 14 hr of time-lapse fluorescence microscopy. Trajectories are binned into ten groups according to their average individual equivalent diameters over the experiment. Average equivalent diameter of each bin is noted.

(B) Analysis of binned cell trajectories reveals increased total fluorescent intensity (blue circles) and constant fluorescent concentration (orange squares) with increasing cell diameter. Mean and SD of each bin is plotted.

(C and D) Noise magnitude (C) increases with fluorescence and transcriptional burst size (D) increases to 5- to 10-fold in larger cells compared to smaller cells. Total burst size (D) is plotted as defined in Equation 2.

(E) Power function fit to total burst size. The fold change in total burst size of the LTR promoter increases with cell diameter with a power coefficient of 5.6 (\(R^2 = 0.99\)). Error bars in (B)–(D) represent the SDs within each bin.

High-frequency processing of noise (HF-CV\textsuperscript{2}) (Dar et al., 2012; Weinberger et al., 2008) effectively high-pass filters total noise and focuses on modulations of intrinsic noise of the gene circuit. Low-frequency extrinsic noise from global resource fluctuations is filtered. HF-CV\textsuperscript{2} and CV\textsuperscript{2} have been shown to correlate and provide precise measurements of expected noise (Dar et al., 2012, 2016; Weinberger et al., 2008).

To test whether cell-size-dependent burst size applies to additional promoters, Jurkat cells were randomly infected with the Ubiquitin C (UbC) housekeeping promoter at a low MOI (MOI = 1) expressing d2GFP (UbC-d2GFP). Polyclonal UbC-d2GFP revealed increased burst size with cell size (Figure S1). This suggests that cell-size-dependent total burst size is independent of integration site and occurs globally across the genome (Figure S1). This is consistent with increased burst size of housekeeping promoters quantified in human fibroblasts and their increases in burst size with cell size to maintain constant concentrations (Figure S1) (Padovan-Merhar et al., 2015). Finally, two Food and Drug Administration (FDA)-approved drug screens on Jurkats, one for cell size and one for noise, were compared (Figure S4) to examine the relationship between cell size modulation and noise-modulating drug treatments on the HIV LTR promoter. The screens reveal a correlation between noise enhancement and cell size, suggesting that exogenous treatments can modulate both cell size and HIV expression bursts.
Latent HIV Reactivates Exclusively in Larger T Cells while Smaller Cells Remain Silent

To measure the size of latently infected cells at their moment of reactivation, a Jurkat latency model (JLat) consisting of full-length HIV with a frameshift mutation in \( env \) and GFP replacing the \( nef \) reading frame (Jordan et al., 2003) was imaged using single-cell time-lapse fluorescence microscopy, similar to previous studies (Figure 3A) (Dar et al., 2012; Weinberger et al., 2008). GFP is an indicator for latent cells switching to a reactivated state. Generation of JLat cell lines renders them GFP\(^{-}/\text{C}0\) when untreated (Jordan et al., 2003). Three clonal JLat cell populations (6.3, 9.2, and 15.4) were activated with tumor necrosis factor alpha (TNF-\( \alpha \)) at the start of imaging (t = 0) and imaged every 10 min for 20–26 hr (Figure 3A; Video S1; STAR Methods). TNF-\( \alpha \) is a potent activator of the LTR promoter by upregulating nuclear translocation of nuclear factor \( \kappa B \) (NF-\( \kappa B \)), which binds \( \kappa B \) sites within the LTR enhancer (Duh et al., 1989) (Figure S8). JLat 9.2 has previously reactivated to \( \text{GFP}^{+}/\text{C}24\) 30% after 24-hr induction with TNF-\( \alpha \) (Bohn-Wippert et al., 2017; Jordan et al., 2003; Spina et al., 2013). After imaging, individual reactivation events were segmented by image processing and detected and quantified over time for their mean fluorescent intensity, cell area, and equivalent diameter (Figure 3A). Detected reactivation events were defined as cells having an intensity above a threshold.
determined by sample and substrate autofluorescence for at least 4 hr (Figure 3B). A single intensity and cell size value (or equivalent diameter) were quantified for each reactivation event by averaging its time-dependent trajectory for 4 hr after first detection (Figures 3B–3D and S5).

The three JLat showed consistent reactivation in larger cells with a mean diameter of ~15 μm compared to a mean population diameter of ~12 μm (Figure 3C). Cells lower than the mean population diameter showed no reactivation. To further demonstrate that reactivation of large cells occurs independently of the drug treatment used, we performed additional experiments using diverse reactivation treatments of trichostatin A (TSA); TSA+TNF-α; prostratin (Pro); Pro+TNF-α; and suberoylanilide hydroxamic acid (SAHA)+TNF-α, on JLat 9.2 and 15.4 (Figures 3D and S6; STAR Methods). Consistent with TNF-α treatment, all cells reactivated in a larger cell diameter regime above the 12 μm mean population diameter. Mean cell diameters of reactivated cells ranged from 15.5 to 17.2 μm (Figure 3D). As TNF-α treatment duration increased, reactivation events increased in cell diameter, intensity, and frequency at later detection times (Figure S5). Due to long (>20 hr) cell doubling times and minimal cell diameter, intensity, and frequency at later detection times (Figure S5), this effect cannot be attributed to dynamic changes of burst size of individual cells during imaging. For example, total reactivation events of JLat 9.2 + TNF-α between 10 and 14 hr increased by 42% compared to 14 and 18 hr (Figure S5).

Next, to assess the contribution of cytoplasm and translational bursts, we calculated the ratio of cytoplasm volume to nucleus volume for GFP+ cells (C:N ratio). Plotting C:N ratio versus total cell diameter shows values less than 1 for most reactivated cells (~80%) (Figure 3H). Along with a control showing that promoter fluctuations dominate noise measured in all cells (Figure S2), these results suggest that changes in total burst size (B × b) are dominated by changes in nucleus size and transcriptional bursts (B) are less influenced by changes in translational bursts (b).

Latently Infected Primary CD4+ T Cells Consistently Reactivate in Larger Cells

To test whether size-dependent reactivation from latency is conserved in primary human CD4+ T cells infected with the JLat vector, sorted cells were adhered and imaged for 12–15 hr under treatment with a potent transcriptional activator combination, phorbol 12-myristate 13-acetate (PMA)+ionomycin, at the start of imaging (Figure 4A). Examples of reactivating primary cells are shown (Figure 4A; Video S2; STAR Methods). A total of 805 reactivation events were detected from the infected CD4+ T cells from two donors and quantified for their 4-hr mean intensity and diameter (Figures 4B and S8). Most reactivation events had cell diameters greater than the mean diameters for the activated T cell population from each donor (red versus black distributions, Figure 4B), and vertical dashed lines for both the active and the resting primary CD4+ T cell distributions represent mean cell diameters. The increased reactivation diameters of the primary CD4+ T cells are consistent with the observed reactivation of larger Jurkat cells (Figure 3).

Unlike the JLat, reactivated primary cells did not increase in intensity with cell diameter (Figure S8). Reactivation intensity increased in the JLat only after 10 hr of imaging with TNF-α (Figures 3 and S5), which is consistent with an ~10- to 12-hr time delay needed for NF-κB activation by TNF-α (Figure S8). Increased fluorescence intensity by delayed response to PMA+ionomycin treatment was not possible to capture, because imaging of primary cells was limited to 12–15 hr. However, fluorescence-activated cell sorting (FACS) of GFP+ JLat-d2GFP-infected CD4+ T cells treated for 48 hr with diverse activators (Figure S8) showed size-dependent intensity enhancement consistent with the assumed model (Figures 1, 2, and S8).
To establish that resting and infected primary CD4+ T cells are unable to reactivate in smaller cell sizes, we performed additional microscopy experiments using PMA+ionomycin (Figure 4C). Resting T cells infected with JLatd2GFP showed decreased cell sizes and no reactivation between 4 and 18 hr of imaging, both with and without pretreatment of PMA+ionomycin. Stimulated GFP− sorted CD4+ cells from donor 1 revealed increased cell diameter and GFP+ cell counts. Cell viability of all infected cells was between 80% and 100% and assessed using propidium iodide (PI) staining.

**Reactivation of HIV Latency Depends on Cell-Cycle Phase**

Despite the idea that cell size would be naturally correlated with cell cycle, reports in fibroblasts have shown that all cell-cycle phases can exist throughout the range of cell sizes in a population (Padovan-Merhar et al., 2015). We have confirmed this in Jurkat T cells using a cell-cycle stain. TNF−α-treated JLatss reveal no correlation between cell size and cell cycle (Figure S7). In addition to cell size-dependent transcriptional bursting, studies in mammalian cells have shown that transcriptional burst frequency (F or koff) decreases with cell-cycle progression (i.e., FG1 > FG2) (Padovan-Merhar et al., 2015). Given the strict dependence of reactivation on larger cell size, we next investigated the dependence of reactivation of latency on cell cycle.

With reactivation occurring by combining increased transcriptional burst frequency (e.g., with TNF−α in Figure 3C) and burst size, latent cells predisposed with higher burst frequency at earlier cell-cycle phases are predicted to synergize to higher reactivation levels when treated with an activator (Equations 1 and 2; Figure 5A) (Dar et al., 2012, 2014; Singh et al., 2010). Conversely, treating cells with an activator at later cell-cycle phases is predicted to result in fewer threshold crossings due to lower burst frequency (i.e., FG1+TNF−α > FG2+TNF−α).

To test whether reactivation increases at earlier cell-cycle stages, we modified the computational model showing active feedback from Figure 1A for the larger cell size (higher B model line) to have two burst frequencies corresponding to G1 and G2 phases (Figures 5A and 5B). Here FG1 was defined to be double FG2 (Figure 5B) based on previously measured results (Padovan-Merhar et al., 2015), and G2 was simulated to have 2 copies of the gene (Table S1). Total burst size dependence on cell diameter was scanned similarly using the power function of Figure 2E.

Stochastic simulations show that reactivation of cells in both cell-cycle phases increases with diameter. G1 cells reach about double the reactivation compared to G2 cells (Figure 5C).

To experimentally test the model prediction of increased reactivation at earlier cell cycles, we compared the cell-cycle distribution of reactivated JLat cells to their whole population distribution (both GFP+ and GFP− cells) (Figure 5D). JLat 9.2 and 15.4

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**Figure 4. Reactivation of Latently Infected Primary CD4+ T Cells Occurs in Larger Cells**

(A) 12- to 15-hr time-lapse microscopy of OFF sorted primary cells infected with a JLatd2GFP vector (upper). Cells are treated with PMA+ionomycin at t = 0 hr, and single-cell reactivation events are quantified (Video S2). Two reactivating cells are tracked and indicated with arrows in 1-hr snapshots (scale bar, 10 μm).

(B) Distributions of reactivated primary CD4+ T cells from two donors (upper and lower) shift to a larger size regime compared to their respective total population size distribution (black curve with average dashed line). The average size of resting CD4+ T cells is also indicated (gray curve and gray dashed line). No reactivation was detected in smaller sizes of the activated CD4+ cells.

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(C) Single-cell microscopy of resting and activated primary CD4+ T cells infected with JLatd2GFP and treated with either PMA+ionomycin at t = 0 hr or pretreated for 24 hr reveals cell-size-dependent reactivation between the start and the end of imaging. (Left) Resting primary cells do not reactivate between 4 and 18 hr. In contrast, stimulated primary CD4+ T cells show an increase of reactivation events (GFP+) over time. (Right) Cell-size dependence of CD4+ T cells in activated cells (red) and resting cells (gray).
were stimulated with TNF-α or TNF-α+SAHA for only 12 hr to exclude any measurement of GFP+ cells produced from doubling events after treatment, which could skew the cell-cycle distribution (Figure S6). DNA content using a phycoerythrin (PE)-conjugated cell-cycle stain and GFP expression was measured using flow cytometry (Figure S9). For JLat 9.2, treatment with TNF-α or TNF-α+SAHA indicates that reactivation occurs to some extent in all cell-cycle phases (red, Figure 5D). Similar results were observed for JLat 15.4.

Cell-cycle phase-dependent reactivation was calculated by the ratio between GFP+-reactivated cells and all cells in the JLat population after normalization of each distribution by its integrated cell count (Figure 5D). Ratios of normalized GFP+ to all cells were examined across the PE-Cy-5 range (Figure 5E). A cell-cycle phase ratio value of 1 would mean that the number of cells expected to reactivate at a specific cell-cycle phase is consistent with an a priori assumption that reactivation occurs with equal probability across all cell-cycle phases of the latent population and would reject the hypothesis that G1 cells with higher burst frequency have increased reactivation (Figure 5A). For TNF-α treatment, the ratio at G1 results in a 3-fold increase from expected reactivation and a 2-fold increase for G2 (Figure 5E). S phase shows a 60% decrease in the expected amount of reactivation. Reduced reactivation in the S phase is confirmed by TNF-α+SAHA treatment (Figures 5D and 5E). This is consistent with a reported role for S phase in stabilizing latency and providing a barrier for active replication of the virus (Foli et al., 2007; Wang et al., 2011). In contrast to TNF-α-treated cells (black, Figure 5E), the amplified ratio in G1 is suppressed with TNF-α+SAHA treatment while the S and G2 phases of the ratio curve follow similar trends (black and purple, Figure 5E). This is consistent with a reported G1 cell-cycle arrest by SAHA in different cell lines (Bernhart et al., 2017). It also motivates the active control and stochastic design of viral decision-making by modulating burst frequency for specific cell-cycle states (Dar et al., 2014; Dar and Weiss, 2018; Megaridis et al., 2018).

Cell-Cycle Modulators Alter Synergistic Reactivation from Latency

To assess whether cell-cycle-dependent reactivation can be actively modulated, we performed treatments for 24 hr with an array of known cell-cycle-arresting compounds on JLats 9.2 and 15.4. Treatments included rapamycin, resveratrol or cytarabine, and 17β-estradiol known to arrest cell cycle in G1, S, and G2, respectively (Figure 6A) (Ford et al., 2015; Jenkins et al., 2001; Joe et al., 2002; Sampath et al., 2006; Zhao et al., 2009).
After cell-cycle arrest treatment for 12 hr, TNF-α was added to the cell-cycle arrest for an additional 12 hr. Cell-cycle states of reactivated GFP+ cells after cell-cycle arrest were compared to the distributions of JLat treated with only TNF-α (Figures 5D and 6B). Drug-treated cells showed high cell viability and no reactivation when treated with cell-cycle-arresting treatments alone (Figure S10). Cell-cycle phase distributions for the reactivated cells with treatments are shown in Figures 6B–6E. Exposure to rapamycin resulted in reactivation in the G1 phase, which was calculated by the percentage of GFP+ cells detected by flow cytometry gating (Figure S9). GFP+ cells accumulated in the G1 phase compared to G2, and their distribution is plotted in red (Figure 6B). Treatment with resveratrol resulted in an increase of activated cells in S phase and a decrease of cells in G1 and G2 phases (Figure 6C). Although cytarabine also arrests T cells at S phase (Ford et al., 2015), treatment significantly
blocked transition of cells from G1 to S phase and reactivation predominantly occurred in G1 (Figure 6D). Treatment with 17β-estradiol resulted in a significant increase of 2.3× and 1.5× in G2 reactivation for JLat 9.2 and 15.4, respectively, compared to TNF-α treatment alone (Figures 6B and 6E). This effect was combined with a reduction of activated cells in G1 and S phases. However, these drugs alone may also change HIV expression through alternative mechanisms (Hawley et al., 2013; Martin et al., 2017; Szotek et al., 2013). These results demonstrate the possibility of active cell-cycle control for latent reactivation at specific cell-cycle phases. This presents an additional cell-state strategy to bias viral decision-making.

To test the possibility of reactivation with cell-cycle arrest, treatments combined with TNF-α were performed (Figures 6F and 6G). Cell cycle of JLat 9.2 and 15.4 was arrested with 24 hr of treatment, and TNF-α stimulation of cells was carried out either in combination with cell-cycle-arresting treatment for 24 hr or 12 hr after arrest was initiated. The percentage of reactivated GFP+ cells with combined treatment was analyzed and compared to the percentage of cells reactivated with TNF-α alone (Figures 6F and 6G). 24-hr cell-cycle arrest and treatment with 12-hr activator showed synergy and increased reactivation in G1 compared to TNF-α treatment (red dashed line, Figure 6F). G2 phase showed reduced synergy with reactivation comparable to TNF-α. For 24 hr of combined treatment, G1 displayed synergy, while G2 and S phases showed decreased reactivation levels, or antagonism to TNF-α. This is consistent with an expected decrease in burst frequency with cell-cycle progression compared to the increased frequency by TNF-α treatment alone (Figure 6G).

Cell-cycle-dependent synergies for enhanced latent reactivation were further investigated using a previously reported FDA-approved noise enhancing compound library (Dar et al., 2014). A portion of the reported library is known to arrest cell cycle (Figure 6H; Table S2). Previous measurements show that JLat 8.6 treated with TNF-α in combination with 18 cell-cycle-arresting noise enhancers results in cell-cycle-dependent synergy, consistent with the results observed here (Figure 6H) (Dar et al., 2014). The results demonstrate that synergistic reactivation of latent cells is minimal in S and G2 but significantly increases for G1 and G1/S phases (p < 0.05). This trend is consistent with $F_{G1+TNF-\alpha} > F_{G2+TNF-\alpha}$ as seen in Figures 6F and 6G.

**DISCUSSION**

Studies of stochastic gene expression have investigated the role of noise in diverse gene circuits, networks, and phenotypes (Balázs et al., 2011; Raj and van Oudenaarden, 2008). Extending the dependence of lambda phage developmental fate on host-cell volume before infection (St-Pierre and Endy, 2008), we show that similar host-cell-dominating effects apply to infected human cells and demonstrate cell size-dependent decision-making of latently integrated HIV. Increases of expression bursts depend on cell size, and intracellular global resources couple into known intrinsic noise mechanisms of the HIV viral circuit (Dar et al., 2012; Weinberger et al., 2005, 2008). A combination of cell size and hardwired deterministic and stochastic Tat circuitry determines stability of the latent state (Dar et al., 2014; Razooky et al., 2015; Weinberger et al., 2005, 2008). We show that smaller latently infected host cells are deterministically silent, while larger host-cell sizes exhibit larger expression bursts. Larger bursts lead to threshold crossings of viral products, feedback activation, and stochastic decision-making. While the dependence of burst size on cell size may eventually reach an upper limit before the power function reaches infinity, this did not occur within the range of cell sizes observed.

From an integrated gene circuit and stochastic decision-making perspective, this study presents an example of a cell size-based fate decision, consistent with reports of increasing gene expression burst size with cell reaction volume (Caveney et al., 2017; Padovan-Merhar et al., 2015). These observations may guide strategies for viral therapies by influencing both cell size and cell cycle. Redistributing latent cells toward smaller sizes and later cell-cycle phases (for smaller B and F) (Figures 1D and 5A) would provide a potential strategy for therapies aiming to stabilize latency (e.g., block-and-lock strategy) (Kessing et al., 2017). Conversely, modulating latent cell populations toward larger cell size and earlier G1 phase (for larger B and F) would benefit strategies aiming to reactivate cells from latency (e.g., shock and kill) (Archin et al., 2012).

The existence of cell size-based decision-making of an integrated viral circuit may contribute to current biomarker and therapeutic efforts in disease. It may also advance efforts to engineer noise by elucidating how cell state influences gene expression fluctuation intrinsic to gene circuits and networks for cellular decisions. Engineering noise toward forward stochastic design would benefit synthetic biology and bioengineering applications (Dar and Weiss, 2018). The a priori knowledge relating cell size and viral decision-making provides insight into the mechanisms of viral-host control and a basis for sorting and characterizing cell sub-populations likely to harbor latent HIV.

Collectively, these findings present HIV as a viral gene circuit with stochastic decision-making based on mammalian host-cell size. Large cells with increased burst size are required for reactivation of latent cells. Furthermore, cell growth presents a naturally timed noise generator capable of providing asynchronous stochastic decisions in a defined cell size window for each cell. In a study, Wang and colleagues report the importance of cell growth, size, and division state memory in quiescence-exit heterogeneity (Wang et al., 2017). This study of tissue repair and tissue engineering presents an additional example of growth-dependent stochastic switching. Cell size-based gene expression programs may be of interest for multicellular systems and synthetic biology applications.

Quiescence exit and reactivation from latency suggest the existence of a broader class of cell size-based decisions in diverse cell types and biological contexts (Balázs et al., 2011). These may include development and patterning in tissue growth (Meyer and Roeder, 2014), pluripotent gene expression and stem cell differentiation (Singer et al., 2014; Skinner et al., 2016), intratumor heterogeneity (Brock et al., 2009; Gupta et al., 2011), and circadian clock-based stochastic gene expression programs (Chabot et al., 2007). Furthermore, the consistent relationship measured for housekeeping promoters in Jurkats (Figure S1) and fibroblasts (Padovan-Merhar et al., 2015) suggests that size-dependent gene regulation and decision-making may be fairly common and exploited throughout the genome in diverse
pathways, viral-host interactions, and organisms. Evidence of cell size-dependent increases of global genome-wide bursts suggests that in addition to Tat feedback of HIV, signaling mechanisms are likely involved in the decision observed, which are not accounted for in the current model.

Strategies using host-cell size and cell cycle for monitoring and controlling latency may support drug treatments for HIV-infected individuals. The combined modulation of cell size and cell cycle provides a space for stochastic design of drug therapies (Dar et al., 2014; Dar and Weiss, 2018). Reports demonstrate difficulty in eradicating the latent reservoir using drug treatments in HIV-infected individuals on ART due to the proliferation and expression of reactivated latent cells (Wiegand et al., 2017). Cell-cycle states display a large range of overlapping cell sizes and mRNA levels, allowing the independent modulation of cell size and cell-cycle phase (Dolatabadi et al., 2017; Padovan-Merhar et al., 2015). Checkpoint regulators may provide novel targets for controlling latent stability in larger cells. HIV is already known to stall the cell from entering mitosis by controlling G2 arrest with its viral protein vpr (Andersen et al., 2008), when viral promoter activity is highest (Goh et al., 1998). The a priori knowledge that reactivation events occur with higher probability in larger cell sizes, while remaining silent in smaller cells, provides a paradigm for diagnostics for HIV-infected individuals, latency-reversal treatments, and therapeutic strategies toward a cure.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes 10 figures, two tables, and two videos and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.12.009.

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AUTHOR CONTRIBUTIONS

R.D.D. conceived the experimental, analytical, and computational work. K.B.-W., E.N.T., Y.L., M.R.M., and R.D.D. designed and performed the fluorescence microscopy experiments, flow cytometry measurements, and drug treatments. K.B.-W. performed the primary cell experiments. E.N.T., Y.L., M.-Y.H., and R.D.D. carried out the computational work. K.B.-W., E.N.T., Y.L., and R.D.D. analyzed and interpreted the experimental data. K.B.-W., E.N.T., Y.L., M.R.M., and R.D.D. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| DAPI Nuclear Stain  | Sigma-Aldrich | D9542      |
| NUCLEAR-ID™ Red DNA Stain | ENZO Life Science Inc. | Cat# EWZ-51008-100 |
| Anti-human CD69 monoclonal antibody (PE), clone FN50 | Thermo Fisher Scientific | Cat# 12-0699-73; RRID: AB_465735 |
| Anti-human CD4 monoclonal antibody (SIM.4) | NIH AIDS Reagent Program | Cat# 724 |
| Goat anti-mouse IgG-PE | Santa Cruz Biotechnology | Cat# sc-3738 |
| **Biological Samples** |        |            |
| Single Donor Human Whole Blood | Innovative Research | Cat# IPLA-WB1 |
| Single Donor Human Whole Blood | BioreclamationIVT | Biofluids (Blood-derived), Human whole blood |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Tumor Necrosis Factor alpha (TNF) | R&D Systems | Cat# 210-TA-005 |
| Propidium iodide (PI) | Thermo Fisher Scientific | Cat# P3566 |
| Phorbol 12-myristate 13-acetate (PMA) | Cayman Chemical | Cat# 10008014 |
| Human Interleukin 2 (IL-2) | Miltenyi Biotec Inc | Cat# 130-097-742 |
| Flavopiridol | Cayman Chemical | Cat# 10009197 |
| Prostratin (Pro) | Cayman Chemical | Cat# 10272 |
| Cycloheximide | Cayman Chemical | Cat# 14126 |
| Trichostatin A (TSA) | Cayman Chemical | Cat# 89730 |
| Suberoylanilide hydroxamic acid (SAHA) | Cayman Chemical | Cat# 10574 |
| Ionomycin | Cayman Chemical | Cat# 10004974 |
| Rapamycin | Cayman Chemical | Cat# 53128-88-9 |
| trans-Resveratrol | Cayman Chemical | Cat# 70675 |
| Cytarabine (S-phase arrest) | Cayman Chemical | Cat# 16069 |
| **Critical Commercial Assays** |        |            |
| Permeabilization Buffer (Cell Signaling Buffer Set A) | Miltenyi Biotec Inc. | Cat# 130-100-827 |
| 3.9% Fixation Buffer Inside Fix (Cell Signaling Buffer Set A) | Miltenyi Biotec Inc. | Cat# 130-100-827 |
| Cell-Tak Cell and Tissue Adhesive | Corning Life Science | Cat# 354241 |
| Dynabeads Human T-Activator CD3/CD28 | Thermo Fisher Scientific | Cat# 11161D |
| RosetteSep Human CD4+ T cell Enrichment Cocktail | StemCell Technologies, Inc. | Cat# 15022 |
| FuGENE6 Transfection Reagent | Promega | Cat# E2691 |
| **Experimental Models: Cell Lines** |        |            |
| JLat 6.3 | NIH AIDS Reagents Program, (Jordan et al., 2003) | Cat# 9846 |
| JLat 9.2 | NIH AIDS Reagents Program, (Jordan et al., 2003) | Cat# 9848 |
| JLat 15.4 | NIH AIDS Reagents Program, (Jordan et al., 2003) | Cat# 9850 |
| HEK293T | ATCC | ATCC CRL-3216 |
| Naive Jurkats, clone E6-1 | ATCC | ATCC TIB-152 |
| **Recombinant DNA** |        |            |
| JLat-d2GFP (NL4.3. DeltaEnv-d2GFP) | Weinberger Lab (Gladstone Institutes), (Hansen et al., 2018; Razooky et al., 2015) | N/A |
| UbC-d2GFP | Weinberger Lab (Gladstone Institutes), (Dar et al., 2012) | N/A |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Roy Dar (roydar@illinois.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Culture and Cell Lines
Jurkat cells and primary CD4+ T cells were grown in RPMI 1640 media supplemented with L-glutamine, 10% fetal bovine serum (FBS), and 1% Penicillin/Streptomycin (Corning Cellgro). Primary CD4+ T cells were also supplemented with 25 mM HEPES (Thermo Scientific). Cells were incubated with 5% CO2 at 37°C. Naive Jurkats and JLat isoclones 6.3, 9.2, and 15.4 were obtained from ATCC and the NIH AIDS Reagent Program. LTR-d2GFP isoclone 20 in Figure 2 was previously published in a noise drug screen (Dar et al., 2014) and kindly provided by the Weinberger Laboratory at the Gladstone Institute at UCSF. Infection of naive Jurkats for the production of LTR-d2GFP isoclones has been previously described (Dar et al., 2012; Singh et al., 2010). For HIV latency studies, JLat isoclones 6.3, 9.2, and 15.4 consisting of full-length HIV with GFP replacing the nef reading frame and a deletion of env were selected from a previously generated library (Jordan et al., 2003). Studies with primary CD4+ T cells included the production of JLatd2GFP lentivirus containing a replication incompetent full-length HIV-1 genome with a deletion of the viral env protein and replacement of the nef reading frame by a destabilized d2GFP (Weinberger Laboratory at the Gladstone Institute at UCSF). Furthermore, T cell lines created for Figure S1 consisting of the Ubc promoter with a d2GFP (UbC-d2GFP) and a LTR promoter with a d2GFP-IRES-Tat cassette (LTR-d2GFP-IRES-Tat or Ld2GIT) (Dar et al., 2012). The JLat-d2GFP, Ld2GIT, and UbC-d2GFP plasmids were obtained from the Weinberger Laboratory at the Gladstone Institutes at UCSF.

Isolation of Primary CD4+ cells
Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation of buffy coats from HIV-seronegative donors (Innovative Research, MI, USA and BioreclamationIVT, NY, USA). PBMCs were immediately processed to isolate CD4+ T cells using RosetteSep Human CD4+ T Cell Enrichment Kit (Stem Cell Technologies) and negative selection. Cultivation and stimulation of isolated CD4+ T cells was performed as previously described using a modified version of the Greene model without the use of saquinavir (Bohn-Wippert et al., 2017; Lassen et al., 2012). Subpopulations of resting and dividing CD4+ T cells were used after infection for experiments in Figure 4. For activation of resting CD4+ T cells, CD3/CD28 activation beads (ThermoFisher Scientific) were used for 72h.

Transfection of Primary Cells
For creating primary CD4+ T cells containing the JLat-d2GFP vector, HEK293T cells, were obtained from ATCC, were transfected with viral supernatant containing JLatd2GFP, and the viral supernatant was harvested 48h post-transfection. Infection of stimulated primary CD4+ T cells with lentiviral supernatant was performed using spinoculation and sorting was carried out as previously described (Bohn-Wippert et al., 2017; Lassen et al., 2012). Transfection of resting primary CD4+ T cells, used a slightly modified Greene model (Bohn-Wippert et al., 2017) by isolating the cells using the RosetteSep human CD4+ T cell enrichment cocktail (Stemcell Technology Inc., Vancouver, Canada). Cells were then cultured in RPMI 1640 containing 10% FCS and 1% Penicillin/Streptomycin. 1 day after isolation, 2.5 x 10^6 resting primary CD4+ T cells were spinoculated with 1:70 concentrated viral supernatant containing JLatd2GFP at 1200 x g for 2h at room temperature. Infected cells were plated at 1x10^6 cells/ml using RPMI 1640 supplemented with 10% FCS, 1% Pencillin/Streptomycin, 25 mM HEPES, and cultured without saquinavir treatment. Single-cell fluorescence microscopy experiments

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| LTR-d2GFP isoclone 20 | Weinberger Lab (Gladstone Institutes), (Dar et al., 2014) | N/A |
| LTR-d2GFP polyclonal | Weinberger Lab (Gladstone Institutes), (Dar et al., 2012, 2014) | N/A |
| LTR-d2GFP-IRES-Tat | Weinberger Lab (Gladstone Institutes), This paper | N/A |

Software and Algorithms
Nikon Elements Software
Nikon Instruments Inc. https://www.nikoninstruments.com/Products/Software/NIS-Elements-Advanced-Research

MATLAB
MathWorks https://www.mathworks.com/
of resting primary CD4+ T cells were performed either 1 d after infection for samples treated with PMA+ionomycin at t = 0 of imaging or 2 days after infection for samples pretreated for 24 hour with PMA+ionomycin.

METHODS DETAILS

Stochastic simulations and modeling
Stochastic simulations of the model in Figures 1 and 5 are implemented using the Gillespie algorithm (Gillespie, 1977). Parameters used are recorded in Table S1. A total of 1,200 units of time are simulated for each single cell and the recording interval of Tat copy number is once every 1 time unit. For modulation of fold change in B × b (Figures 1 and 5), 2/3 of the change is attributed to decrease of koff, and 1/3 is attributed to increase of km. To model G2 phase in cell cycle (Figure 5), the number of molecule representing the gene is increased from 1 to 2 in the simulation; for G1 phase, the rate parameter km is doubled.

Quantification of cell size versus expression burst size
To calculate mean fluorescence < FL >, high-frequency noise magnitude (HF-CV), and transcriptional burst size for the integrated LTR promoter as a function of cell size (or diameter), imaged cells in Figure 2A were binned by their individual mean size. Next, their corresponding intensity trajectories were used for each bin size to calculate mean single-cell fluorescence, population general trends (time-dependent average of each size cluster), noise magnitude, and burst sizes using previously described noise analysis (Dar et al., 2012; Kepler and Elston, 2001; Simpson et al., 2004; Singh et al., 2010) (Equation 1 of main text).

Sorting of infected primary CD4+ T cells using flow cytometry
Three days after infection with JLatd2GFP virus and T cell stimulation, infected primary CD4+ T cells were sorted using a primary anti-human CD4 monoclonal (SIM.4) antibody (NIH AIDS Reagent Program, MD, USA) and a secondary goat anti-mouse IgG-PE antibody (Santa Cruz Biotechnology, TX, USA). Live cell staining of cells was carried out for 30 min at 4 °C for the primary antibody followed by an additional step of 20 min at 4 °C for the secondary antibody. Washed cells were sorted for GFP+ and GFP- populations.

Reagents for reactivation and cell-cycle assays
Populations of Jurkat LTR-d2GFP isoclone and JLat clones 6.3, 9.2, and 15.4 were stimulated with TNF at a final concentration of 10 ng/ml or with TNF in combination with 3 μM prostratin. Histone deacetylase inhibitor TSA (Sigma-Aldrich) was applied at a final concentration of 400 nM. GFP- sorted primary CD4+ T cells were stimulated with a combination of 200 ng/ml PMA and 1 μM ionomycin. suberoylanilide hydroxamic acid (SAHA) was used at a final concentration of 25 μM (Kumar et al., 2016). For cell-cycle arrest studies, JLat 15.4 and 9.2 were treated with 400 nM Rapamycin, 20 μM Resveratrol, 0.7 μM Cytarabine, and 10 μM 17β-Estradiol. For investigation of GFP-degradation rates, LTR-d2GFP isoclone 20 was treated with 10 μg/ml Cycloheximide. 3 μM Flavopiridol was used for the investigation of transcriptional bursts using LTR-d2GFP isoclone 20 (Figure S2). All chemicals were obtained from Cayman Chemicals except for TNF (R&D Systems).

Staining of nuclei using DAPI
JLat 9.2 was treated for 14h with 10ng/ml TNF in combination with either 2.5μM SAHA or 3μM Prostratin. Cells were washed once with 200μl PBS and fixed with 3.9% Formaldehyde (Miltenyi Biotec Inc., CA, USA) for 10 min at room temperature (RT). Afterward, cells were centrifuged at 500 x g for 5 min and 4 °C and permeabilized for 30 min on ice using ice cold permeabilization buffer A (Miltenyi Biotec Inc., USA). Two additional washing steps with PBS were performed after cell permeabilization, and cell nuclei were then stained with 0.1μg/ml DAPI (Sigma-Aldrich, St. Louis, USA) for 5 min in the dark at RT. After two washes with PBS, 1.5 × 105 cells were seeded into each experimental well of a half-area glass bottom 96-well plate (Corning) and imaged by taking 36 images per well using a 40x objective.

Ratio of cytoplasm volume to nucleus volume for GFP+ cells
Equivalent whole cell and nucleus diameters were used with the following equations under the assumption of a spherical cell:

\[
V_{\text{nucleus}} = \frac{\pi}{6} (d_{\text{DAPI}})^3, \quad V_{\text{total}} = \frac{\pi}{6} (d_{\text{GFP}})^3
\]

\[
V_{\text{cytoplasm}} = V_{\text{total}} - V_{\text{nucleus}} \quad \text{and} \quad \text{Ratio}_{CN} = \frac{V_{\text{cytoplasm}}}{V_{\text{nucleus}}}
\]

Here d_{DAPI} and d_{GFP} are the equivalent diameter calculated from their respective segmented cell areas. It is worth noting that DAPI staining involves fixation and measurements have shown that changes in the total cell area of unfixed populations are correlated with their fixed counterparts (Padovan-Merhar et al., 2015).

Automated Fluorescence Imaging
Fluorescent imaging experiments using JLats and primary CD4+ T cells were performed using Cell-Tak adhesive (Corning Life Science) and half-area glass-bottom 96-well plates (Corning #4580). For imaging experiments, 1.5 × 10^5 cells of JLat 9.2, 15.4, and 6.3
were adhered to half-area glass bottom 96-well plates using Cell-Tak adhesive (BD) and imaged for at least 20h. Long duration imaging of single primary CD4+ T cells has been proven to be challenging, and devices tailored for this purpose have been designed (Razooky et al., 2012). We used a modified sample preparation with increased amounts of Cell-Tak in the glass coating layer and a decreased amount of CD4+ T cells per well (7.5 × 10³) to achieve 12-15h imaging experiments of stimulated CD4+ T cells from the two donors before the cells started to disengage from the glass-bottom well. Single-cell imaging of resting CD4+ T cells appeared more stable with imaging lasting 22h using increased amounts of Cell-Tak. All cell lines were imaged with a fully automated and environmentally controlled inverted Nikon Ti-Eclipse Microscope with Perfect Focus System (PFS), 20x or 40x objective, 100ms exposure, 16-bit ORCA-FLASH 4.0 CMOS camera (Hamamatsu) using 2x2 binning for 12-28h durations. Cell media with activator treatment was added at the start of imaging. Imaging utilized a stage top environmental control at 37 °C and 5% CO₂ (Tokai Hit). Periodic and automated image acquisition of multiple points within multiple wells at 10 min imaging intervals was executed using a high-speed linear motorized stage controlled by Nikon Elements software (Nikon Instruments Inc.). To reduce background fluorescence and increase imaging sensitivity, phenol-red-free RPMI 1640 medium (GIBCO) was substituted for the culturing RPMI 1640 media at the final step of imaging in all experiments.

Data processing
Image processing and tracking of identified single-cells was performed using Nikon Elements software with high content imaging module (Nikon Instruments Inc.). Exported data files including tracked single-cell intensity trajectories and equivalent cell diameters calculated from the processed image binary areas were processed and quantified for reactivation event detection using custom MATLAB codes (Mathworks) (Dar et al., 2012). The total number of GFP+ cells within multiple wells at various time points of imaging were calculated using segmented binary cell areas. Quality controls were performed for cells that were both DAPI and GFP positive to ensure the overlapping area is at least 95% of the total DAPI area. Although similar, autofluorescence and detection thresholds were defined for each day of imaging.

Cell-cycle analysis of reactivated latent T cells
For cell-cycle analysis, drug treated JLat cells were harvested, counted, and washed twice in PBS. Cells were resuspended in flow cytometry staining buffer (R&D Systems, MN, USA) at a concentration of ≤ 5 × 10⁵ cells/ml and fixed with 3.9% Formaldehyde (Miltenyi Biotec Inc., CA, USA) for 10 min at RT. Cells were centrifuged at 500 x g for 5 min at 4 °C, permeabilized by adding ice cold permeabilization buffer A (Miltenyi Biotec Inc., CA, USA) and incubated on ice for 30 min. After two washing steps with PBS, cells were stained with NUCLEAR-ID® Red DNA Stain (ENZO Life Sciences Inc., NY, USA) using a 1:1,000-fold dilution. Stained cells were incubated at RT for 30 min. At least 3k reactivated stained cells were collected and analyzed with a BD LSR Fortessa flow cytometer. Cell-cycle distributions are shown as the percentage of cells containing G1, S, and G2 DNA by PE-Cy5-A staining analyzed by FCS Express cell-cycle analysis software (De Novo Software, CA, USA).

In silico quantification of infected primary CD4+ T cells
In silico gating of small and large cells was carried out for HIV infected ON-sorted primary CD4+ T cells treated with different drugs for 24 to 48h. Live cell populations containing 10k cells were defined as small and large cells by gating ~3k cells for each region of interest using low and high forward-scatter (FSC) from flow cytometry. For ON-sorted primary CD4+ T cells, mean fluorescence intensity was calculated by creating an ON-gate for the live cell population. The small and large FSC gated subpopulations were part of the ON-gate. Using an ON-gate allows the exclusion of cells with dim fluorescence intensities which could be sorted into the ON-sorted population. The mean GFP intensity versus FSC for each subpopulation was plotted.

CD69 receptor staining of T cell activation
JLat 9.2 and 15.4 were incubated 24h with 10 ng/ml TNF and washed twice with flow cytometry staining buffer (R&D Systems, MN, USA). Cells were pre-incubated with human AB serum to block nonspecific binding of the monoclonal antibody, followed by CD69 receptor staining (CD69 PE clone FN50, Thermo Fisher Scientific, MA, USA) for 30 min at 4 °C using 1x10⁶ cells/100μl. The cells were washed twice in PBS and centrifugated at 500 x g for 5 min. Samples were then analyzed using a BD LSR Fortessa flow cytometry analyzer (BD Biosciences). 10k cells were collected and defined as LIVE cells. This population was used to determine small and large cells by gating ~1k cells for each region of interest using low and high forward-scatter (FSC) from flow cytometry. The mean fluorescence intensity of GFP+ cells was calculated by creating an ON-gate for the LIVE cell population. Both activated cell populations were examined for their CD69 expression and mean FCS versus PE was plotted. Previous studies using CD25 and CD69 activation markers of CD4+ T cells have also shown a lack of correlation between cell activation and cell size (Waysbort et al., 2013).

QUANTIFICATION AND STATISTICAL ANALYSIS
Mean and standard deviation were used for all cells collected in a cell size bin in Figure 2. Bar plots of subsequent figures contain mean calculation of measurements with standard error based on the replicates noted in the figure caption.
For Figure 1C, a two-sample Student’s t test with unequal variance between the simulated cell diameter results (red bar graph) and the measured MOXI measurement (orange curve) was carried out. The p value of the test is $2.16 \times 10^{-124}$, which shows that the difference in mean diameters between the whole population and the simulated population is highly significant.

For Figure 2E, an ANOVA test was performed after taking natural log on both x- and y axis values on our fitted model. The resulting statistical tests showed that the slope (corresponds to the power in the original non-log formula) has a p value of $7.85 \times 10^{-10}$ using MATLAB built-in function anova() on our model.

**Calculation of p values**

A two-sample t test was used to check for significance of cell-cycle-dependent differences in synergistic reactivation with TNF treatment. The first result for the significance of cell-cycle phases G1, G1/S (n = 11, SD = 2.97) and S (n = 2, SD = 0.25) shows a p value of 0.003. The second result of p = 0.03 for the same hypothesis is based on the same samples for G1, G1/S and G2, G2/M (n = 5, SD = 2.77). Differences were considered statistically significant if the p value was less than 0.05. Significance was indicated as * for p < 0.05 and ** for p < 0.01.
Supplemental Information

Cell Size-Based Decision-Making
of a Viral Gene Circuit

Kathrin Bohn-Wippert, Erin N. Tevonian, Yiyang Lu, Meng-Yao Huang, Melina R. Megaridis, and Roy D. Dar
Figure S1. Burst size increases with cell reaction volume. Related to Figure 1 and 2. (upper) Cell-free gene expression in chambers and vesicles, mRNA count of GAPDH and EEF2 in human fibroblasts, and expression burst size increase with reaction volume and cell size (Caveney et al, 2016; Padovan-Merhar et al, 2015). (lower) Increase of total fluorescence and burst size with cell diameter in Jurkats harboring the LTR and UbC housekeeping promoter. (lower left) Single-cell microscopy by cell size of UbC-d2GFP and LTR-d2GFP polyclonal populations (Dar et al, 2012) demonstrates a comparable increase in total cell fluorescence for both housekeeping and LTR promoters. (lower right) Noise processing of size sorted cell-clusters shows an increase in burst size for both the LTR and UbC polyclonal populations. LTR displays larger burst sizes across all cell sizes in comparison to the UbC population, consistent with noise shifts in previous studies (Dar et al, 2012). Increase size-dependent burst size across polyclonal populations for two very different promoters suggests the burst increase is global for most promoters and integration sites. Error bars represent the standard deviation for each individual size cluster.
Figure S2. GFP-degradation rate does not change with cell size, and transcriptional bursts dominate in small, medium and large cells. Related to Figure 2 and 3. (A) Representative FSC gating of flow cytometry measurements using FSC versus SSC to quantify large, medium and small cells. 10k cells of the LTR-d2GFP isoclone 20 were collected and 3k FSC cells of the 10k cells were gated for each of the large, medium and small regions. (B) Mean GFP fluorescence versus duration of Cycloheximide treatment to arrest translation (time in hours) for small, medium and large cells. Decay of mean d2GFP after translational block is exponentially fit and GFP half-life is calculated for each size subpopulation. (C) d2GFP half-life of about 2.5 hours for small, medium and large cells. These results suggest d2GFP decay is not dependent on cell size. Similarly measurements have shown that mRNA half-life is also constant with cell size (Padovan-Merhar et al, 2015). (D) Similar to the gating in (A), 50k live cells were collected in the LIVE gate and GFP expression was measured. Gates for small, medium and large cells for the LIVE cell population were defined with 3k cells in each gate and the noise before and after transcriptional block was quantified. Transcriptional block was performed with 3µM Flavopiridol for 10h using a Jurkat T lymphocyte LTR-d2GFP population (isoclone 20). The noise ratio with and without transcriptional block has been previously reported to distinguish between the dominance of promoter fluctuations (ratio = 1) versus mRNA birth-death processes (ratio = ~3 at 10h arrest)(Singh et al, 2012). The results show that noise is primarily due to promoter fluctuations and transcriptional bursting from the viral promoter in all cell sizes. The results strengthen that total burst measured (BxB) is dominated by B across all Jurkat cell sizes, changes are more sensitive to B, and consistent with the observation that whole cell volume is dominated by nucleus volume in Figure 3. All measurements were performed in duplicate with mean and standard error plotted.
Figure S3. Cell growth during imaging is minimal and average Jurkat cell diameter measured by microscopy and an automated cell counter are consistent. Related to Figures 2, 3 and 4.

(A) d(equivalent diameter)/dt measured for 3037 single cells from a Ld2G isoclone population (iso 20 from Figure 2) after applying a 2h moving average over 14h of imaging. (B) Growth ratio for each single-cell is defined by the ratio of mean diameter during hours 12 - 13 divided by mean diameter during hours 1 – 2. The distribution is centered with a mean close to 1. (C) Resulting cumulative distribution function shows that less than 1 in 10 cells changes its diameter by over ~35% over the course of the 14h quantified. The other ~90% of cells remain roughly the same size. (D) Comparison of average cell diameter of Jurkat cell lines LTR-d2GFP (Ld2G) isoclone 20, polyclonal Ld2G, and polyclonal LTR-d2GFP-IRES-Tat (Ld2GIT)
using fluorescence microscopy and automated MOXI cell analyzer (ORFLO Technologies). Comparison of cell size measurements allow calibration and estimation of error in measuring cell sizes. 10µm polystyrene calibration beads served as a control. The results reveal similar diameter values using both devices. The cell size distribution of the population and average cell diameter of 12µm for JLat isoclines (Figures 3C and 3D) was determined using the cell analyzer. Size distribution shifts observed for detected reactivation events in Figures 3 and 4 are much larger than the overlapping standard deviation error bars seen here (i.e. diameter measurement error cannot explain the shifts observed throughout the study). All measurements were performed in duplicate with mean and standard error plotted.
Figure S4. Drug screens provide evidence of drug induced modulation of noise and cell size. Related to Figure 2. (A) A 40 drug moving average applied to 345 FDA-approved drugs common to two independent screens for average cell size for treated Jurkat cells (Miettinen & Bjorklund, 2015) and a noise drug screen for enhanced coefficient of variation relative to untreated controls ($\Delta CV^2$ or $\Delta$Noise) of the LTR in Jurkats (Dar et al, 2014). A positive correlation of noise enhancement with cell size for diverse drug treatments ($p < 0.001$). Here the average cell size (%) axis is defined as the amount of change in average size for a drug-treated Jurkat cell population in relation to its untreated control (no size change would receive a value of 100%). A 40 drug moving average was performed on the 345 drugs found in common between the two drug screens shown above. (B) (lower left) The 40 drug window was determined by testing a moving average with window sizes from 1-100 data points in order of ascending average cell size. The sum of absolute differences was calculated for each window size used to smooth the data, and plotted versus its window size. The sum of absolute difference calculated is between the smoothed data and the original data. Although the trend does not completely plateau at larger window sizes, a pivot to a shallower slope is observed around a window size of 40 data points and was chosen as the filter size to use. (lower right) Alternatively, a moving median filter of 50 drugs produces a comparable trend with increasing noise enhancement at lower cell sizes to a constant. The decrease at larger cell sizes is due to edge effects.
Figure S5. Reactivation events of JLats are detected at a higher rate with higher intensity in larger cell sizes at later time points of TNF-treatment. Related to Figure 3. (A) Detected reactivation events for 3 JLats as a function of TNF treatment duration in hours (colorbars) reveals higher reactivation intensity in larger cells with increasing TNF treatment duration. Highest reactivation intensity occurs at the latest treatment durations. (B) Distribution for the number of reactivation events detected over time for JLats 9.2 (red), 15.4 (blue) and 6.3 (green) treated with TNF at t=0 and imaged for 20-26h. Cells display an increasing reactivation rate that reaches a constant rate at later time points. (C) Among the large
GFP+ reactivated cells, larger cells reactivate at a higher frequency at later time points and are brighter than smaller reactivated cells. Mean intensity of cells over 20h of TNF-treatment shows that cells with a size range between 16-19 µm reactivate at later stages of drug treatment with increased fluorescence intensity compared to smaller cells with a diameter between 12-14 µm that only slightly increase intensity (still with sizes above the population average).
Figure S6. Detection of small LTR-d2GFP and JLAT GFP+ cells with microscopy and reactivation of JLat 15.4 exclusively in larger cells. Related to Figure 3. (A) Control LTR-d2GFP population used in Figure 2 is imaged and distribution of the average equivalent cell diameter calculated from time-dependent single cell area in pixels is plotted. Compared to both reactivated JLat and CD4+ JLat-d2GFP cells, the control shows that a lower diameter range is captured with the imaging system. (B) As an additional control, JLat 15.4 cells were pre-treated with TNF 22h prior to preparing the imaging sample and adhering cells to the glass bottom well. After plating, cells were imaged for 26h. Cell doubling during pre-treatment causes the large cell distribution of detected reactivated cells in panel A to shift back to a smaller size regime with a mean diameter of 11.9 µm (red dashed line). This control shows that small JLat GFP+ cells are capable of being captured and that the detection events defined by appearance of GFP+ for a 4h window of the JLat population are first time reactivation events and not a byproduct of cell doubling or sample preparation biasing larger cell sizes. In addition the mean of 11.9 µm matches the mean diameter of the total JLat population distribution (GFP+ and GFP- cells) in panel (A), measured with automated cell analyzer. GFP+ cells as small as 3-5 µm in size are detected. Finally a shoulder is observed at 14 µm for additional reactivation events occurring after TNF is washed and replaced at the start of the 26h of imaging. (C) 24h imaging experiment of JLat 15.4 shows cell-size dependent reactivation. Using a 4h detection window cell size distributions were measured for reactivated GFP+ cells treated with TSA+TNF, SAHA+TNF, Pro+TNF, and TSA for at least 20h. Cell diameters shift to a larger cell size regime. The size distribution of a latent JLat 15.4 cell population is represented in orange (solid line) and the average cell size of this population is represented using a vertical orange dashed line. Only 124 cells were collected for TSA treatment due to low reactivation levels resulting in a low and non-symmetric distribution.
Figure S7. T-cell activation and cell cycle are independent of cell size. Related to Figure 3 and 5. (A) (upper row) Gating strategy for calculating expression of CD69+ cells in JLat 9.2 and 15.4. (middle and lower row) JLat 9.2 and 15.4 are treated with TNF for 24h and then stained for CD69 receptors using human anti-CD69 antibody conjugated to PE. LIVE cells are defined by creating a LIVE gate, and gates for low and high PE expression, using forward scatter vs. PE of LIVE cells are determined. Cell size range is presented by a FSC histogram of PE+ expressing cells for both untreated and TNF treatment for all cells, High PE+, and Low PE+ populations (right side and legend). Cell sizes of LIVE cells range from 4-20 µm, and no significant shift between the high and low activation is observed. (B) CD69 receptors are expressed in TNF treated and untreated cells. CD69+ expression increases with TNF treatment in the already active JLat cell lines. (C) Cell cycle stain of DNA content for both untreated JLat 9.2 and 12h treatment with TNF. (D) FSC-A versus Cy5-A of all live cells shows that G1 FSC-A covers the whole size range for all DNA content that overlaps the G2 FSC-range for both untreated and TNF treated cells. Consistent with previous reports this result shows that cell cycle is independent of cell size (Padovan-Merhar et al, 2015).
Figure S8. Reactivation of primary CD4+ T-cells occurs in cells larger than 10 μm without intensity increasing over 12 hours of reactivation, but show higher mean fluorescent intensity post-48h treatment. Related to Figure 3 and 4. (A) Reactivation of an OFF sort of primary CD4+ T-cells infected with a JLatd2GFP vector. Detected reactivation events using a 4h window are larger than the average size of the primary cell population (dashed curve). The distribution of diameters for CD4+ T-cells are also included on a secondary y-axis (orange). Intensity is constant with PMA+Ionomycin treatment duration (colorbar). (B) Mean population intensity trend for time-lapse microscopy of the clonal LTR-d2GFP population from Figure 2 after TNF addition. The trend accounts for 2100 cells and plateaus in the 10-14h range. This suggests that a delayed reactivation from latency by NF-kB activation using TNF and PMA+ Ionomycin in Jurkat and primary cells can account for the difference in the intensity versus treatment duration results (Figure S5 and Figures 3 and 4). (C) Representative FSC gating of flow
cytometry measurements using FSC vs. SSC of the activated JLatd2GFP CD4+ T-cell population to quantify larger and smaller cells. The highest and lowest 3k FSC cells of all 10k cells collected were selected for the large and small gates respectively. (D) Mean intensity versus size by FSC post-48h treatment reveals enhanced fluorescent intensity for larger cells. Diverse transcriptional activators were used and measured in duplicate. All measurements used the same gating strategy and represent infected primary cells that are already GFP+ at the time of both treatment and flow cytometry. All drug treatments were performed using two donors and the mean and standard error were plotted for each treatment.
Figure S9. Flow cytometry gating strategy for quantifying cell cycle distribution. Related to Figure 5 and 6.

Flow cytometry gating strategy used for the determination of cell cycle of GFP+ reactivated cells. At least 3k reactivated (GFP+) and PE-Cy5 stained cells were collected for each measurement from drug treated JLat 9.2 and 15.4 cells. **(upper)** Untreated PE-Cy5+ stained cells were used to define an ON cell population gate (in red) using forward scatter versus FITC-A. **(lower)** After collecting GFP+ and PE+ cells, a gate for singlets in the PE-Cy5-W versus PE-Cy5-A scatter plot (green gate) was created using the treated and PE-stained sample. This gate excludes doublets of PE-stained cells. Finally, cell cycle distributions for GFP+/PE+ cell populations for each treatment are plotted on a linear scale (lower right).
Figure S10. Cell cycle arresting treatments show minimal JLat toxicity and no reactivation. Related to Figure 6. (A) Cell death staining post-24h was performed on activated and cell cycle arrested JLat 9.2 (left) and 15.4 (right) to confirm minimal cell death with treatments in Figure 6. Propodium iodide (PI) staining, for drug concentrations listed in the Methods shows high cell viability after drug treatment by subtracting percentage PI+ stained cells from the total percentage of LIVE cells. Compared to their untreated counterparts, percentage of LIVE cells for the treated sample is above 60%, except for 17ß-Estradiol of 15.4, indicating that a majority of the treated cells in the live gate are viable. (B) Compared to TNF, JLats 9.2 (left) and 15.4 (right) treated with cell cycle arresters alone show no reactivation.
Table S1. Simulation parameters used in Figures 1 and 5. Most parameters are adapted from Razooky et al., Cell (2015)(Razooky et al, 2015) which implemented a similar model. For this study of a latent LTR, a 15-fold increase in basal k_{off} was used to represent the stable latent state of HIV. For Figure 1, 1/3 of the change in total burst size is reflected in k_{m1} and k_{m2}, while the other 2/3 is reflected in k_{off}^2. For simulations in Figure 5, the k_{on} is reduced by half in G2 phase under the assumption that two DNA copies share the same total transcription factors and resources for activation compared to the original copy in G1.

| Reactions                        | Description                                      | Rate                                      |
|----------------------------------|--------------------------------------------------|-------------------------------------------|
| LTR_{off} \leftrightarrow LTR_{on} | Promoter switching between on and off states    | \(k_{on} = 0.0001; \) basal \(k_{off} = 0.15\) (for Fig. 5C-D G1 phase, \(k_{on} = 0.0002\); G2 phase \(k_{on} = 0.00005\)) |
| LTR_{on} \rightarrow mRNA + LTR_{on} | Transcription of Tat mRNA                        | Basal \(k_{m1} = 0.01\)                 |
| mRNA \rightarrow mRNA + Tat     | Translation of Tat                               | \(k_p = 10\)                             |
| Tat + LTR_{on} \leftrightarrow Tat·LTR_{on} | Tat binding and unbinding to LTR                  | \(k_{bind} = 0.01; k_{unbind} = 0.01\)   |
| Tat·LTR_{on} \rightarrow mRNA + Tat·LTR_{on} | Enhanced transcription due to Tat positive feedback | Basal \(k_{m2} = 5\)                     |
| mRNA \rightarrow *              | mRNA decay                                       | \(\gamma_m = 1\)                         |
| Tat \rightarrow *              | Tat decay                                        | \(\gamma_p = 0.125\)                     |
Table S2: Summary of 18 noise enhancers used to investigate the relationship between cell cycle arrest and reactivation synergy in Figure 6H (Related to Figure 6).
The following table includes the reactivation levels of JLat 8.6 after 24h drug treatments for 18 different noise enhancers as reported in Dar et al., *Science* (2014)(Dar et al, 2014). For each noise enhancer the curated literature-based cell cycle arrest phase is listed. References supporting the cell cycle phase arrest by noise enhancer are also included.

| Drug (Dar et al, 2014) | Phase of cell cycle arrest | % Reactivation after 24h +TNF (Dar et al, 2014) | References |
|------------------------|----------------------------|-----------------------------------------------|------------|
| TNF                    | Control                    | 10.137                                        |            |
| Atorvastatin calcium   | G0/G1                      | 16.5                                          | (Chen et al, 2017) |
| Paraglyine hydrochloride | G1                      | 14.33                                         | (Lee et al, 2013) |
| Adapalene              | G1                         | 17.03                                         | (Shi et al, 2015) |
| Artemisinin            | G1                         | 20.1                                          | (Willoughby et al, 2009) |
| Oxytetracycline        | G1                         | 21.02                                         | (Shao & Feng, 2013) |
| Dutasteride            | G1/S                       | 13.98                                         | (Schmidt et al, 2009) |
| Dasatinib              | G/S                        | 14.63                                         | (Johnson et al, 2005) |
| Indomethacin           | G1/S                       | 16.33                                         | (Ehsan et al, 1999) |
| Mycophenolic acid      | G1/S                       | 16.63                                         | (Chaigne-Delalande et al, 2008) |
| Ethinyl estradiol      | G1/S                       | 18.87                                         | (Koroxenidou et al, 2005) |
| Pemetrexed             | G1/S                       | 23.27                                         | (Ramirez et al, 2007) |
| Clobetasol propionate  | S                          | 13.77                                         | (Guichard et al, 2015) |
| Carboplatin            | S                          | 14.13                                         | (Cruet-Hennequart et al, 2009) |
| Bleomycin              | G2                         | 10.05                                         | (Cloos et al, 2002) |
| Mebendazole            | G2/M                       | 11.6                                          | (Mukhopadhyay et al, 2002) |
| Mercaptopurine         | G2/M                       | 13.57                                         | (Kanemitsu et al, 2009) |
| Bezafibrate            | G2/M                       | 15.89                                         | (Scatena et al, 1999) |
| Docetaxel              | G2/M                       | 16.6                                          | (Nehme et al, 2001) |