

Sporadic on/off switching of HTLV-1 Tax
expression is crucial to maintain the whole
population of virus-induced leukemic cells

(HTLV-1 Tax の散発的な一過性発現はウイルス
性白血病細胞の集団の維持に必要である)

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Sporadic on/off switching of HTLV-1 Tax expression is crucial to maintain the whole population of virus-induced leukemic cells

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Abstract

Viruses causing chronic infection artfully manipulate infected cells to enable viral persistence *in vivo* under the pressure of immunity. Human T-cell leukemia virus type 1 (HTLV-1) establishes persistent infection mainly in CD4⁺ T cells *in vivo* and induces leukemia in this subset. HTLV-1-encoded Tax is a critical transactivator of viral replication and a potent oncoprotein, but its significance in pathogenesis remains obscure due to its very low level of expression *in vivo*. Here, we show that Tax is expressed in a minor fraction of leukemic cells at any given time, and importantly, its expression spontaneously switches between on and off states. Live-cell imaging revealed that the average duration of one episode of Tax expression is approximately 19 hours. Knockdown of Tax rapidly induced apoptosis in most cells, indicating that Tax is critical for maintaining the population even if its short-term expression is limited to a small sub-population. Single-cell analysis and computational simulation suggest that transient Tax expression triggers anti-apoptotic machinery and this effect continues even after Tax expression is diminished; this activation of the anti-apoptotic machinery is the critical event for maintaining the population. In addition, Tax is induced by various cytotoxic stresses and also promotes HTLV-1 replication. Thus it appears that Tax protects infected cells from apoptosis and increases the chance of viral transmission at a critical moment. Keeping the expression of Tax minimal but inducible on demand is therefore a fundamental strategy of HTLV-1 to promote persistent infection and leukemogenesis.

Significance Statement

The oncogenic retrovirus HTLV-1 encodes Tax, an activator of both viral replication and cellular oncogenic pathways. Despite the potent activities of Tax, its precise roles in pathogenesis remains unclear, since it is faintly expressed *in vivo*. This study shows that sporadic and transient Tax expression is observed in a small subpopulation of HTLV-1-induced leukemic cells. This limited Tax expression is critical for survival of the whole population through ignition of anti-apoptotic signals. Tax is induced by various stresses, suggesting that Tax efficiently protect cells from cell death and reactivates the virus from reservoirs under condition of cellular stress. It is an elaborated strategy of HTLV-1 to evade host immunity and enable persistence *in vivo*.

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Chronic viral infection is established when there is a metastable balance between host immunity and viral strategies for maintaining infected cells in infected individuals. Several human viruses cause persistent infection and are closely associated with inflammatory diseases and/or cancers (1). Human T-cell leukemia virus type 1 (HTLV-1) and human herpesviruses, such as Epstein-Barr virus (EBV/HHV-4) and Kaposi's sarcoma-associated virus (KSHV/HHV-8), are representatives of this type of viruses (2), and express a limited number/amount of viral products, enabling infected cells to survive *in vivo* by escaping from host immune surveillance. Reactivation of viral replication, such as in the lytic phase of EBV and KSHV infection, is then a critical step for facilitating viral transmission to new hosts. Human oncogenic viruses encode the regulatory factors, which are involved in viral replication and cellular oncogenesis (3); however, their expression dynamics and the roles in each phase are poorly understood.

HTLV-1 is a human retrovirus that chronically infects CD4⁺ T cells. Some infected individuals develop a malignant disease of CD4⁺CD25⁺ T cells, adult T-cell leukemia-lymphoma (ATL), and/or inflammatory diseases such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and HTLV-1 uveitis (4, 5). Unlike that of human immunodeficiency virus (HIV), the replication of HTLV-1 is at a very low level *in vivo*, and viral RNA is rarely detected in the plasma of infected individuals (6). Instead, this virus persists in the host by using two different strategies: cell-to-cell transmission of viral particles (*de novo* infection) and clonal proliferation of infected cells (mitotic expansion) (7, 8).

HTLV-1 encodes two oncogenic factors, Tax and HTLV-1 bZIP factor (HBZ), in the sense and antisense strands of provirus respectively, and these two factors counteract each other in many signaling pathways (9). Tax is not only a potent oncoprotein (10) but also an efficient transactivator of viral replication, which means that Tax is required for *de novo* infection. However, Tax expression is generally suppressed in infected cells *in vivo* (11). In contrast, HBZ is constitutively expressed in infected cells and plays important roles in viral latency (8) and proliferation of infected cells (12). These facts suggest that HTLV-1 fine-tunes the expression and function of these counteracting viral factors to establish persistent infection in infected individuals. Since Tax is highly immunogenic (13-15), HTLV-1 appears to minimize Tax expression to escape from host immunity. Therefore, the significance of Tax in leukemogenesis has been an important unsolved issue.

In this study, we show that only a small fraction (0.05-3%) of cells transiently express Tax. However, knockdown of Tax induced apoptosis in the majority of cells, suggesting that Tax expressed in a minor subset is required for survival of the whole population. At a single-cell level, Tax-expressing cells highly expressed several anti-apoptotic and NF- κ B-related genes, and Tax-negative cells were divided into two subpopulations which expressed medium or low level of the anti-apoptotic genes. Computational simulations support our hypothesis that transient Tax expression confers an anti-apoptotic property on the expressing cell, and that this effect lasts after Tax expression is diminished. This study is the first to show the dynamics of Tax expression at a single-cell level, and demonstrates novel roles of Tax in establishing persistent

infection by HTLV-1.

Results

A small fraction of MT-1 cells express Tax, and this expression is critical for the survival of the whole cell population

HTLV-1 *tax* is generally silenced or transcribed at quite low levels in ATL cells *in vivo* (16). An ATL cell line, MT-1, has an equivalent expression profile of viral genes to primary HTLV-1-infected cells (17). We carried out single-cell quantitative RT-PCR to elucidate the expression levels of *tax* and *HBZ* in individual MT-1 cells. The initial experiment showed that only one cell in 71 cells expressed a high level of *tax*, while no detectable expression was observed in remaining 70 cells (Fig. 1A). In contrast, *HBZ* was expressed in Tax-negative cells while it was not detected in the Tax-expressing cell. These results suggest that the expression of *tax* and *HBZ* is strictly and reciprocally regulated in MT-1 cells.

Our next question was, what is the role of Tax expression in a small number of MT-1 cells? To address this issue, we knocked down Tax with shRNA. Two different lentivirus vectors expressing shRNA targeting Tax (shTax1 and shTax4) were transduced into MT-1 cells, and either construct efficiently inhibited Tax expression to ~15% of the level in control cells (Fig. 1B, left). Intriguingly, progressive cell death was observed in MT-1 cells after Tax was knocked down (Fig. 1B, right), and as a mechanism, we found that Tax knockdown (KD) induced apoptosis of MT-1 cell, but not Jurkat cells (Fig. 1C and Fig. S1). To compare population dynamics between

Tax-KD cells and Tax-intact cells, a GFP competition assay was carried out (Fig. 1D, left schema). To our surprise, ~90% of shTax-transduced cells were eliminated from culture by day 21 (Fig. 1D, right), which was faster than we expected. These results indicated that Tax is indispensable for the survival of MT-1 cells even though only a small fraction of MT-1 cells express it. Since shTax-transduced cells were not rescued by the presence of non-transduced cells, it also appears that a cell's survival may depend at least partly on Tax expression within that cell, rather than depending on Tax expression by neighboring cells.

Tax is transiently expressed in MT-1 cells

We wished to further distinguish between these two possible hypotheses: one, that a small population of cells constantly expresses Tax and supports the survival of the whole population, or two, that all cells transiently express Tax by turns. To monitor Tax expression, we established a reporter subline of MT-1, MT1GFP, which expresses destabilized enhanced green fluorescent protein (d2EGFP; the half-life of the modified EGFP is 2 hours) under the control of 18 copies of the Tax-responsive element (TRE) (Fig. 1E, upper schema) (18). It was confirmed that d2EGFP expression in MT1GFP cells was closely correlated with Tax expression by intracellular staining with anti-Tax antibody, and a very small population (~0.5%) of MT1GFP cells expressed d2EGFP (Fig. 1E, bottom plot). Several stable clones of MT1GFP were established, and all clones possessed a small Tax-expressing subset. Using one of the MT1GFP clones, we evaluated the dynamics of Tax expression in individual cells by a time-lapse imaging.

The result revealed that d2EGFP expression was transient in most d2EGFP-positive cells (Fig. 1*F* and Movie S1), indicating that Tax is expressed temporarily in MT-1 cells. We could also see cells with fluctuating and continuous patterns of d2EGFP expression (see legend to Fig. 1*G* for definitions of these terms), although the percentages of cells with those patterns were lower than cells with transient expression (6% for fluctuating and 18% for continuous cells; Fig. 1*G*). Analysis of 87 cells with transient d2EGFP expression showed that the median and mean duration of Tax expression was approximately 22 and 18 hours, respectively (95% CI, 19.3, 24.7) (Fig. 1*H*).

It has been reported that two other ATL cell lines, KK-1 and SO-4, have expression levels of Tax and HBZ similar to those of fresh ATL cells (17). A small fraction of KK-1 and SO-4 cells each expressed Tax, and a subline of KK-1 reporting Tax expression, KK1GFP, was successfully established (Fig. S2*A* and *B*). Since DNA hyper-methylation in 5' long terminal repeat (LTR) is known to suppress *tax* transcription, we evaluated its methylation level and *tax* expression in fresh ATL cells and three ATL cell lines (MT-1, KK-1, and SO-4). In approximately half of ATL cases and all cell lines, DNA methylation level of 5'LTR was low and *tax* mRNA was detectable, suggesting that *tax* expression is inducible in these cells (Fig. S2*C*).

Computer simulation represents the dynamics of Tax expression

It was an incomprehensible observation that knockdown of Tax in a minor fraction (~0.5%) of MT-1 cells induced progressive apoptosis. To estimate the duration that all MT-1 cells experience Tax expression, we employed a computational simulation based

on a mathematical model. A previous study on HIV elegantly established a mathematical model of 5'LTR activation by Tat in latently infected cells (19). We adapted this model to simulate the regulation of the HTLV-1 5'LTR by Tax (See Fig. S3A for a schematic representation and Note S1 for details.) We tested several parameters used in the HIV study and adjusted them to fit our experimental data of transient Tax expression (Fig. S3B-D, Table S1, and Note S2). The simulation could then successfully reproduce the dynamics of Tax expression in MT-1 cells, and the calculated mean duration of Tax expression was 13.6 hours, consistent with experimental observations (Fig. S3C), suggesting that the parameters we used were suitable for the model for activation of the HTLV-1 LTR. Using this model, we then estimated the distribution of the interval between two successive Tax expression episodes (Fig. S3E), and interestingly, our simulation reproduced the single-cell level expression pattern of Tax (Fig. S3F). Furthermore, we calculate that it takes approximately 150 days for 90% of MT-1 cells to experience Tax expression (Fig. S3G). This result means that the loss of Tax-KD cells occurs much faster than the Tax expression (Fig. 1D). The details of our single-cell level computational simulations are described in the SI Notes.

This simulation suggests a possible mechanism that short-term expression of Tax by a cell confers some effects on not only to the cell during its period of Tax expression but also to the cell and its progeny even after Tax expression is lost.

Distinct transcriptional profiles in Tax-expressing cells

To clarify the effects of Tax on both Tax-expressing cells and non-expressing cells, we analyzed the transcriptional profiles of each population. Tax-expressing MT1GFP or KK1GFP cells (Fig. 1E or Fig. S2B, respectively) were purified from bulk cells by a cell sorter using d2EGFP as a marker for Tax, and RNAs from each fraction were subjected to RNA-Seq. Many T-cell activation-associated genes were differentially regulated in Tax-expressing cells compared to non-expressing cells (Fig. 2A and B). Upstream regulator analysis was carried out using the Ingenuity Pathway Analysis program and revealed that NF- κ B-related pathways were significantly affected by Tax in both cell lines (Fig. 2B).

To analyze the correlation between Tax expression and that of other genes at a single-cell level, we chose ~90 representative genes in addition to *HBZ*, and compared their mRNA levels in sorted Tax-positive vs. Tax-negative MT-1 cells by single-cell quantitative RT-PCR. As expected, clustering analysis could clearly separate Tax-positive cells from negative cells (Fig. 2C). Expression of NF- κ B target genes and apoptosis-related genes was positively correlated with that of *tax* in each MT-1 cell (Fig. 2D and Fig. S4). Interestingly, violin plots of several anti-apoptotic genes, such as *CFLAR*, *GADD45B*, *TRAF1*, and *TNFAIP3*, showed 2 peaks in Tax-negative cells; one with almost no expression and another with lower expression than Tax-positive cells (Fig. 2D). In addition, a 3D principal component analysis (PCA) plot generated by the expression levels of these genes revealed that there were 2 clusters in Tax-negative cells (Fig. 2E). These findings suggest that Tax influences the expression of anti-apoptotic genes in Tax-negative cells, there are 2 subpopulations -- those with medium and low

levels of anti-apoptotic factors and thus different degrees of sensitivity to apoptosis.

Tax expression is induced by cytotoxic stresses and contributes an anti-apoptotic property to cells

Since Tax expression was associated with the upregulation of anti-apoptotic genes, we hypothesized that Tax might be induced in response to cytotoxic stress. First, we evaluated the effect of the phases of cell growth on Tax expression. As shown in Fig. 3A, the percentage of d2EGFP-positive (Tax-expressing) cells increased stepwise from 1.2% at day 1 to 11.5% at day 7, if cells were cultured without passage. In contrast, the ratio of d2EGFP-expressing cells remained less than 3% when cells were properly passed, suggesting that stresses associated with the cell overgrowth triggered Tax expression. Importantly, under stressed conditions, the viability of Tax-expressing (d2EGFP+) cells was strikingly higher than that of Tax-negative (d2EGFP-) cells (Fig. 3B). These observations suggest that stress-induced Tax expression confers anti-apoptotic capacities on MT-1 cells.

Similarly, an inducer of oxidative stress, H₂O₂, induced Tax (Fig. 3C, left), and the viability of Tax-expressing cells was significantly higher than that of non-expressing cells (Fig. 3C, right). It has been reported that one of the platinum-containing anti-cancer drugs, cisplatin, induces production of reactive oxygen species (ROS) in cancer cells and contributes to cytotoxicity (20). Cisplatin induced Tax in MT1GFP cells, while an inhibitor of ROS, N-acetyl-L-cysteine (NAC), completely canceled its effect (Fig. 3D). These results suggest that Tax functions as a safeguard against

apoptosis induced by various cytotoxic stresses.

Similar mechanisms for latency of HTLV-1 and HIV

It has been known that cytotoxic stresses, such as DNA damage and oxidative stress, activate the HIV LTR (21, 22). Several HIV reactivating reagents (disulfiram, panobinostat, SAHA, and JQ-1) could also induce Tax expression in MT1GFP cells (Fig. 3E). JQ-1 is an inhibitor of bromodomain-containing 4 (BRD4) and robustly reactivates HIV transcription when it is used with phorbol 12-myristate 13-acetate (PMA) and ionomycin (23). A combination of JQ-1 and PMA/ionomycin exhibited potent activity on Tax induction in MT1GFP cells (Fig. 3F), suggesting overlapping mechanisms for latency of HTLV-1 and HIV.

It has been reported that minimum feedback circuit between HIV Tat and the HIV 5'LTR is sufficient to establish HIV latency (19). When a lentivirus vector expressing Tat through the HIV 5'LTR was transduced into Jurkat cells, gene expression from the 5'LTR was highly transactivated in most cells, while a small population of infected cells became latent (24). Since our results suggested that Tax has roles similar to Tat in latency, it was tested if the HTLV-1 LTR-Tax circuit can generate latency in T cells. We found that in Jurkat cells expressing Tax and d2EGFP through the HTLV-1 5'LTR (Jurkat/LTRd2EGFP-Tax cells), a very small population expressed d2EGFP -- just as was the case for MT1GFP cells (Fig. S5A, right). In contrast, a similar construct containing HIV Tat and HIV LTRs induced continuous expression of Tat and d2EGFP in more than 95% of cells, while a remaining subset was dormant (Fig. S5B); this

observation was compatible with the previous report (24). Treatment by JQ-1, PMA and ionomycin together could activate expression of d2EGFP in all Jurkat/LTRd2EGFP-Tax clones (Fig. S5C), and time-lapse imaging revealed transient expression of d2EGFP in some untreated Jurkat/LTRd2EGFP-Tax cells (Fig. S5D). These findings suggest that the Tax-5'LTR circuit is a basic unit for modulating Tax expression flexibly in response to various stimulations.

Tax suppresses the cell cycle transition from S to G2/M

A possible alternate explanation for the big impact of Tax KD on MT-1 cell survival was that Tax-expressing cells might proliferate faster than non-expressing cells. To test this hypothesis, the effect of Tax on the cell cycle was analyzed using a method combining DAPI staining and EdU incorporation (Fig. 4A) -- a method by which we can evaluate temporal cell cycle progression without synchronization (25). We found that the proportion of cells in S and G2/M phases two hours after the start of an EdU pulse was comparable between Tax-positive and Tax-negative cells. But at 6 hours, the proportion of G2/M cells within the Tax-positive population was only ~80% of that within the Tax-negative population (G2/M cells comprised 39.4% and 48.7% of Tax-positive and negative cells, respectively), suggesting that if anything, Tax retarded, rather than sped up, the G2/M transition of MT-1 cells (Fig. 4B and C). This result implies that Tax does not promote faster proliferation of expressing cells; rather, the anti-apoptotic property of Tax is responsible for the survival of MT-1 cells.

The carryover of anti-apoptotic factors into the Tax-negative interval is important for maintaining the MT-1 cell population

Based on the results of the Tax KD and single-cell analysis experiments, we hypothesized that transient Tax expression triggers anti-apoptotic genes in a small number of MT-1 cells, that this effect is carried over in a significant number of these cells when they enter the Tax-negative phase, and that such carryover is critical for the survival and expansion of the cell population. To check this hypothesis, we constructed a population level agent-based model (ABM) that can represent our experimental observations. More precisely, the ABM simulates the population dynamics of MT-1 cells transduced with shNC or shTax4 as an inhomogeneous (birth-death) Poisson process with a stage transition (See Fig. 5A for a schematic representation and Note S3 for details.). We carried out ABM simulations and confirmed that the time-course of experimental data for the number and fraction of shNC and shTax4 cells were well reproduced in Fig. 5B and C. Based on our ABM simulations, we reconstructed the time-course of the frequency of Tax-positive cells under normal conditions. As shown in Fig. 5D, our simulation predicted that a small number of MT-1 cells would express Tax (i.e., around 3% at the steady state). In addition, we calculated the predicted subpopulation dynamics of shNC and shTax4 cells (Fig. 5E and F, respectively). The numbers of cells that are Tax positive and anti-apoptotic gene positive (i.e., T_{on}), Tax negative and anti-apoptotic gene high (i.e., $T_{\text{off}}A_{\text{high}}$), and Tax negative and anti-apoptotic gene low (i.e., $T_{\text{off}}A_{\text{low}}$) are shown. Under normal conditions (Fig. 5E), the fraction of cells that are T_{on} remains small, but the number of cells in each

subpopulation increases and the total cell population expands, since MT-1 cells are able to express Tax and this expression protects them from apoptosis. In fact, our simulation predicts that the major subpopulation will be $T_{\text{off}}A_{\text{high}}$, which is consistent with our detection of the expression of apoptosis related genes in Fig. 2D. On the other hand, under Tax knockdown conditions (Fig. 5F), the fraction of cells that are T_{on} decreases until there are almost none left, because there is no further transition of cells from Tax negative to Tax positive. The collapse of the T_{on} subpopulation leads to a corresponding decrease in anti-apoptotic gene expression, renders more cells susceptible to apoptosis, and decreases the degree to which the total cell population expands.

To examine whether having experienced Tax expression prolongs cell survival time, we also calculated the survival probabilities of the subpopulations of shNC cells in our ABM simulations. (See Note S4 for details.) For this calculation, we defined the subpopulations of shNC cells based on whether Tax had been expressed at least once or had never been expressed (solid and dashed lines in Fig. 5G, respectively). Interestingly, in this simulation, Tax expression significantly increases the average lifespan of the cell.

Taken together, these simulations support a novel model for ATL persistence: transient expression of Tax in cells is responsible for cell population-level maintenance and expansion (Fig. 6). In a steady state (Fig. 6, left), T_{on} cells turn successively into $T_{\text{off}}A_{\text{high}}$ and then $T_{\text{off}}A_{\text{low}}$ cells. Although $T_{\text{off}}A_{\text{low}}$ cells are sensitive to apoptotic signals and gradually die in culture, Tax-negative cells proliferate more rapidly than Tax-positive cells. In response to cytotoxic stresses, Tax can be induced in some

Tax-negative cells, and thus T_{on} cells are replenished, a process which is critical for the persistence of the population. Tax knockdown (Fig. 6, right) causes a decrease in T_{on} cells, resulting in a shortage of apoptosis-resistant cells and the induction of massive apoptosis. This model enables us to explain how such a small number of Tax-expressing cells has a big impact on the dynamics of the whole population.

Discussion

Persistent viruses have evolved shrewd strategies to propagate *in vivo* while evading host immune surveillance. Here, we demonstrate that HTLV-1 utilizes a unique way to enhance survival and proliferation of infected cells: the transient expression of Tax confers an anti-apoptotic property to cells and maintains the whole population. Since it has been reported that continuous expression of Tax induces DNA damage and senescence in the expressing cells (26, 27), it is suggested that short-term expression of Tax is beneficial to cells. Interestingly, a similar phenomenon of cell survival being promoted by transient gene expression was reported in mouse embryonic stem cells (mESCs). *Zscan4*, which functions in the maintenance of telomeres, is transiently expressed in only ~5% of mESCs at any given time. Knockdown of *Zscan4* induces massive cell death before all cells experience its expression (28). Moreover, expression of *Zscan4* is linked to activation of an endogenous retrovirus, MERV1 (29). Those studies and our observations suggest an association between the transient activation of retroviral LTRs and the maintenance of cell populations.

Tax expression is essential for *de novo* infection by HTLV-1, since viral transcription

depends on Tax (30, 31). However, Tax expression strongly induces expression of viral proteins, including Tax, Env and Gag, resulting in attacks by cytotoxic T lymphocytes (CTLs). Therefore, intermittent Tax expression is a clever strategy of HTLV-1 to evade the host immune response most of the time, yet maintain the ability to cause *de novo* infection under certain conditions. A recent study has reported that *tax* is induced by hypoxia (32); it is compatible with the previous observation that high *tax* expression was detected in the bone marrow, which is physiologically hypoxic (33). As another example, HTLV-1 can be transmitted through breast-feeding -- a process in which HTLV-1 infected cells have to pass through the alimentary tract with acidic conditions and bile acids. Stress-induced Tax expression would be beneficial for *de novo* infection in these conditions. It is known that low pH and hypoxia in the physiological environment suppress adaptive immunity (34, 35), suggesting that infected cells may be able to “get away with” expressing Tax for a limited time in such immunological niches. To clarify the *in vivo* dynamics of Tax expression in immunocompetent hosts, further studies using animal models will be required.

Tax expression is suppressed by genetic/epigenetic mechanisms in 50% of ATL cases (16, 36). The other half of ATL cases have the potential to express Tax, and indeed, it is known that nearly 50% of ATL cases express viral antigens after *ex vivo* culture (37). In this study, we show that survival of MT-1 cells, which express a level of *tax* similar to that of primary ATL cells (17), depends on Tax, suggesting that clinical ATL cases are divided into at least two classes: Tax-dependent and -independent ATL. Previous finding that the arsenic/interferon treatment induced apoptosis of several Tax-expressing cell

lines including MT-1 through degradation of Tax protein implies that those established cell lines still possess the characteristics of Tax-dependent ATL (38). It is known that the activity of Tax-specific CTLs is an important factor in inhibiting the onset of ATL (15). A recent study provided more evidence that Tax plays an important role in ATL: a clinical trial of a dendritic cell vaccine targeting Tax was efficacious (39). Tax expression was faint in fresh ATL cells of the enrolled patients, but it was inducible by *ex vivo* culture. These reports support our hypothesis that transiently expressed Tax plays critical roles in the development and maintenance of ATL even if its expression is limited to a small fraction of leukemic cells. Recent comprehensive genomic studies of clinical ATL cases showed that genetic/epigenetic alterations accumulate in Tax-associated genes (40, 41), suggesting that the effects of these changes can substitute for Tax functions in leukemogenesis.

It is noteworthy that expression of *tax* was contrary to that of *HBZ* even at the single cell level ($r=-0.8$, $p<0.0001$, Fig. 2D). A similar observation in HTLV-1-infected T-cell clones was published while our study was under consideration for publication (42). That study showed the heterogeneity in the expression levels of *tax* and *HBZ* mRNA in fixed cells, whereas we here demonstrate the dynamics of Tax expression using live-cell imaging. Tax and HBZ have opposing functions in many signaling pathways (9); however, the significance of their contrary activities has not been clarified. In this study, we found that Tax induces anti-apoptotic genes, such as *CFLAR*, *TNFRSF9*, *TRAF1*, and *TNFAIP3* (43-45). In contrast, pathways associated with cell proliferation, such as CD3, CD28, and melanogenesis associated transcription factor (MITF) (46, 47), are

significantly activated in Tax-negative cells that express HBZ (Fig. 2B). This finding is compatible with the previous studies that showed that HBZ promotes T-cell growth by enhancing signals through CD3, and that knockdown of HBZ suppresses the proliferation of ATL cells including MT-1 cells (12, 48, 49). The alternation of expression of Tax and HBZ seems to execute cooperative programs for survival and proliferation rather than causing them to interfere with each other. It has been reported that hyper-activation of the NF- κ B pathway by Tax triggers senescence in HeLa cells, while HBZ could cancel this suppressive effect (27), suggesting that the collaboration of Tax and HBZ is important for the expansion of HTLV-1-infected cells. Transgenic mouse models have demonstrated that both Tax and HBZ are oncogenic (50); however, their roles in HTLV-1-infected individuals are thought to be more complicated, due to immune surveillance and the fact that human cells are more resistant to malignant transformation than rodent cells (51, 52). HBZ plays important roles in determining the immunophenotype of HTLV-1-infected cells, and HBZ itself has low immunogenicity (53, 54). It has been reported that the expression level of *HBZ* is higher in ATL cells than in non-leukemic infected cells, implying that infected clones with higher *HBZ* expression are selected during leukemogenesis (17). These findings suggest that constant expression of HBZ drives proliferation of cells with this specific immunophenotype, while transient Tax expression engages to inhibit of cell death caused by various stresses. The different functions and expression patterns of Tax vs. HBZ are thought to be important in the malignant transformation of human T cells.

Recent studies show that many pathogens can persist in their reservoirs during both

acute and chronic infections, and re-emerge in the host under stressful conditions (55). In this study, we demonstrate that the HTLV-1 5'LTR is activated by various cytotoxic stresses and several reagents that reactivate latent HIV (Fig. 3). These results suggest that HTLV-1-infected cells utilize mechanisms analogous to those of latent HIV-infected cells to act as reservoirs. One example is the antagonism of Tax and Tat to BRD4. BRD4 is a negative regulator of positive transcription elongation factor b (P-TEFb). It has been reported that both Tax and Tat compete with BRD4 for binding to P-TEFb (56, 57), and indeed, we found that a BRD4 inhibitor, JQ-1, efficiently reactivated the HTLV-1 5'LTR (Fig. 3*E* and *F*, and Fig. S5*C*). These findings suggested that BRD4 can interrupt the positive feedback loop generated by the 5'LTR and Tax/Tat, and allow the establishment of latent infection by HTLV-1/HIV. Interestingly, many other viruses, including KSHV and human papilloma virus, also use BRD4 and/or P-TEFb to regulate viral life cycles and pathogenesis (58-60). Identification of the cellular factors acted on by diverse viruses will contribute to exploring common mechanisms of viral latency and reactivation. In the case of HTLV-1, several repressors of viral replication, such as p30, Rex, and HBZ are encoded in the provirus (9). These viral factors may be able to regulate the duration and/or timing of Tax expression in infected cells more elaborately. Additional investigation will be required to understand the precise roles of each protein in HTLV-1 latency.

Since the discovery of HTLV-1, a number of studies on its pathogenesis have been conducted; however, the prevention and treatment of HTLV-1-induced diseases are still unsatisfactory (61). In this study, we demonstrated that sporadic and transient Tax

expression in a small subset of cells has significant influence on gene expression of their progeny cells, and consequently maintains the whole population of ATL cells. This is a novel mechanism of the retrovirus for persistence and latency *in vivo*, and elucidation of this mechanism can contribute to a better understanding of viral pathogenesis and the development of new strategies for treatment and prophylaxis of viral-associated diseases.

Materials and Methods

Cells

An IL-2 independent ATL cell line, MT-1 (62), two IL-2 dependent ATL cell lines, KK1 and SO4 (63), and an HTLV-1 negative T-cell line, Jurkat, were used in this study. MT-1 and Jurkat cells were maintained in RPMI supplemented with 10% (vol/vol) FBS. The MT1GFP cell line was maintained as MT-1 cells were, with the addition of 500 ug/ml G418 (Nacalai). KK1 and SO4 cells lines were maintained in RPMI supplemented with 10% FBS and IL-2 (100 U/ml) (PeproTech).

Clinical samples

Fresh ATL cells were obtained from 20 aggressive type ATL cases, and used for extraction of genomic DNA and total RNA. Use of the clinical samples in this research was approved by the ethics committee of Kyoto University (approval number: G204). Written consent was obtained from the patients. Using genomic DNAs from primary ATL cells and ATL cell lines, DNA methylation level of 5'LTR was analyzed by Combined Bisulfite Restriction Analysis (COBRA) method as previously described (36).

Expression level of *tax* in fresh ATL cells was analyzed by a conventional quantitative RT-PCR (16).

GFP Competition assay

The GFP competition assay (64) was carried out to observe the long term effect of Tax knockdown on MT-1 or Jurkat cells transduced with pLKO-GFP lentivirus expressing shNC, shTax1 or shTax4. Cells were infected with concentrated lentivirus at an MOI of 0.5 to adjust the ratio of transduced cells to around 50%. The effect of shRNAs on target cells was evaluated by measuring the percentage of GFP+ cells using a FACSverse flow cytometer (BD Biosciences).

Single cell quantitative PCR (qPCR)

The C1 Single-Cell Auto Prep Array for PreAmp (Fluidigm) was used for harvest of RNA, cDNA synthesis, and pre-amplification of cDNA (18 cycles of PCR for the target genes) from single cells according to manufacturer's instructions. After loading cells onto an integrated fluidic circuit (IFC), we checked all 96 chambers by microscope to verify capture of a single cell. Thereafter, pre-amplified cDNA was harvested and subjected to qPCR. The Biomark HD system (Fluidigm) combined with EvaGreen chemistry (Bio-Rad) was used for the qPCR assay. To increase specificity, we used nested primers (one pair for the pre-amplification step and another pair for the subsequent qPCR of 30 cycles). The sequences of the primers used in this study are indicated in Table S3. Raw data was processed by Fluidigm Real-Time PCR analysis software and the melting curve was used to determine the Pass/Failure call of qPCR. Data was analyzed with the R program using the Singular Analysis Toolset package

(Fluidigm). Any chamber which contained more than one cell was excluded from analysis; outlier cells that had low global expression were also excluded. The level of detection value was set to 24 cycles according to manufacturer's recommendation.

Time lapse imaging

For live cell imaging, 8×10^4 MT1GFP cells were seeded in a 5 mm glass bottom dish (Matsunami) pre-coated with Poly-D-lysine (Sigma), and incubated at 37°C in 5% CO₂. Images in the DIC and GFP channels were captured with an LCV110 microscope (Olympus) every 20 minutes for 96 hours. Semi-automated cell tracking was done by Fiji software with the Trackmate plugin (65). Cells, which had already expressed d2EGFP at beginning of observation, were excluded from analysis because the starting point for expression was unknown. To analyze single-cell dynamics of d2EGFP expression, normalized fluorescence intensities are plotted against time. The starting time ($t=0$) is the time at which the cell started expressing d2EGFP above background level.

RNA-seq

MT1GFP or KK1GFP cells were sorted into d2EGFP⁺ and d2EGFP⁻ populations with a FACS Aria III (BD Biosciences), and RNA was then extracted using the RNeasy mini kit (Qiagen). Single-end RNA sequencing was performed (BGI). A quality check was done with FastQC, and then Tuxedo pipeline was used for RNA quantification (66). Upstream regulator analysis was carried out by Ingenuity Pathway Analysis (Qiagen).

Cell cycle analysis

To measure cell the cycle dynamics of MT1GFP cells without cell synchronization, a

method combining Edu incorporation and DAPI staining was carried out as previously described (25) using the Click-iT Plus EdU Alexa Fluor 647 kit (Thermo Fisher). Initially 1×10^7 cells were cultured in complete RPMI medium containing 10 μ M Edu for 2 hours and washed thereafter. At that point (time=2 hours), half of the cells were fixed and stained for Edu and DAPI according to the manufacturer's protocol. The other half of the cells were re-cultured again for 4 hours without adding Edu. At the end of the assay (time=6 hours) those cells were washed, fixed and stained. Cell cycle status in the d2EGFP+ or d2EGFP- population was analyzed using a FACSVerse based on the levels of Edu and DAPI.

Statistical analysis

Statistical analysis was done using Microsoft Excel, GraphPad Prism, R or Python. Data obtained by flow cytometry was analyzed with FlowJo. Two-sided t-test was used to compare between two different groups.

Computational simulations

A simple deterministic two-state model of Tax positive feedback was developed (see the chemical reaction scheme in Fig. S3A) (19), and simulated by the Gillespie algorithm (67) to investigate the stochastic dynamics of Tax expression, especially to estimate the length of Tax expression (t_{period}) and the interval between Tax expression episodes (t_{interval}). In addition, an agent-based model (ABM) of cell population dynamics (birth-death Poisson process) with stage transitions as described in Fig. 5A was constructed based on the individual-based Gillespie algorithm to confirm the experiment-based hypothesis that the transient expression of Tax is a critical event for

the persistence of the MT-1 cell population. See SI Notes for further details.

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Author contributions

M. Mahgoub, J.Y., K.S., and M. Matsuoka designed, performed, and/or analyzed all experiments. S.I., S.N., and Y.K. performed computational simulations. M. Mahgoub, J.Y., S.I., and M. Matsuoka wrote the manuscript.

Figure legends

Fig. 1. Significance and dynamics of Tax expression at a single-cell level in MT-1 cells

(A) Single-cell qPCR for *tax* and *HBZ* expression in MT-1 cells (n=71).

(B) Left: Efficiency of Tax knockdown by shRNA. Right: The percentage of cells those are dead after Tax knockdown. MT-1 cells were infected with a bicistronic lentivirus vector expressing GFP and shRNA (shTax1, shTax4, or shNC). Cells were stained with LIVE/DEAD reagent and the ratio of dead cells to shRNA-transduced (GFP+) cells was measured by flow cytometry.

(C) AnnexinV/7-AAD double staining in shRNA-transduced MT-1 cells at day 15 post-transduction.

(D) Left: Schematic depicting the concept behind the GFP competition assay. The ratio

of GFP+ cells changes over time depending on the effect of shRNA upon transduced cells. Right: Serial measurements of the percentage of GFP+ cells among the whole MT-1 cell population after shNC or shTax4 transduction. For (B) and (D), error bars show SD for three experiments.

(E) Upper: Scheme of Tax reporter cassette that expresses d2EGFP. MT1GFP is a stable subline of MT-1 transduced with this cassette. Lower: Intracellular Tax staining of MT1GFP.

(F) Live cell imaging of Tax expression in MT1GFP cells. This montage of time-lapse images shows changes in d2EGFP expression.

(G) Expression pattern of Tax in MT1GFP. Cells were categorized based on their pattern of d2EGFP expression during the observation period. Transient: cell has single spontaneous episode. Fluctuating: cell has multiple episodes. Continuous: cell continuously expressed d2EGFP until the end of observation period.

(H) Single-cell dynamics of d2EGFP expression for 87 cells with transient expression. Mean $\pm$ SD is shown.

Fig. 2. Differences in gene expression between Tax-expressing and non-expressing MT1GFP cells

(A) RNA-Seq analysis comparing Tax+ and Tax- MT1GFP and KK1GFP cells. Read coverage plots for two Tax associated genes: TNFRSF9 (upper panel) and TRAF1 (lower panel).

(B) Ingenuity Pathway Analysis for dysregulated genes. Inclusion criteria are FPKM ≥ 1 and ≥ 2 fold expression change. Upper panel: The number of dysregulated genes

common to both MT1GFP and KK1GFP. Lower panel: Top-scoring activated or inactivated upstream regulators.

(C) Heatmap of single-cell gene expression for viral and cellular genes in single MT1GFP cells. Two experiments were performed; either d2EGFP⁺ enriched or un-enriched (whole) populations were subjected to single-cell analysis. Ninety-four cells from each experiment were analyzed.

(D) Violin plots comparing expression of viral and apoptosis-related genes in the Tax⁺ vs. Tax⁻ populations. Based on the Apoptotic process gene list (GO:0006915, Gene Ontology database), 9 apoptosis-related genes, whose expression levels were significantly correlated with that of *tax* (*r* value > 0.5 by Pearson's correlation test), were identified.

(E) 3D PCA plot showing single-cell data clustering based on expression of apoptosis related genes from (D).

Fig. 3. Induction of Tax in MT1GFP cells by cytotoxic stresses and HIV-reactivating reagents

(A) Effect of cellular overgrowth on Tax expression. On day 4, cells were either passaged or allowed to overgrow. Upper panel shows cell count and bottom panel shows percentage of cells that are d2EGFP⁺ (Tax⁺).

(B) Effect of cellular overgrowth on cell viability in d2EGFP⁺ (Tax⁺) and d2EGFP⁻ (Tax⁻) cells. Viability is measured by LIVE/DEAD staining.

(C) Induction of Tax expression by H₂O₂ treatment. Left: Percentage of cells that are

d2EGFP⁺ (Tax⁺); right: viability (LIVE/DEAD staining) of d2EGFP⁺ (Tax⁺) and d2EGFP⁻ (Tax⁻) cells after 48 hours.

(D) Induction of Tax expression by cisplatin treatment is reversed by the antioxidant N-acetylcysteine (NAC).

(E and F) Effect of HIV-reactivating reagents on Tax expression in MT1GFP cells. (E)

The effect of HIV-reactivating reagents on Tax expression. MT1GFP cells were treated for 18 hours with the indicated drugs that have been previously reported to reactivate HIV expression. (F) The effect of a combination of JQ-1 and PMA/I on Tax expression.

The percentage of cells that are d2EGFP⁺ was measured by flow cytometry. Each figure (A-F) is a representative for two independent experiments. Error bars show SD for three replicates in one experiment.

Fig. 4. Cell cycle transition in d2EGFP⁺ (Tax⁺) and d2EGFP⁻ (Tax⁻) cells

(A) Scheme for analysis of cell cycle transition without cell synchronization.

(B and C) Cell cycle phase distributions for EdU⁺ cells among d2EGFP⁺ (Tax⁺) and d2EGFP⁻ (Tax⁻) cells at 2 and 6 hours. (B) Upper: gating strategy for EdU⁺ cells; lower: linear flow cytometric analysis of cell cycle with DAPI staining. Distribution of EdU⁺ cells is shown in red, and that of whole cells is blue.

(C) Bar charts showing the percentage of EdU⁺ cells in the indicated phase.

Fig. 5. Agent-based simulations of cell population dynamics under normal and Tax-knockdown conditions

(A) Scheme for modeling of cell population dynamics as a birth-death (Poisson) process with a stage transition. The ABM simulates the population dynamics of MT-1 cells transduced with shNC or shTax4. Top panel: normal conditions; bottom panel: Tax knockdown conditions.

(B and C) The time-course of the absolute number (B) and fraction (C) of shNC (control) and shTax4 (Tax knockdown) cells in a simulation resembling the experiment in Fig. 1D. In this simulated experiment, some MT-1 cells are shRNA⁺ and some are shRNA⁻. The simulated population dynamics of the shRNA⁺ cells in the mixed population are shown. The blue and green lines give the best-fit solutions for the agent-based simulations based on Tax-period sampling from experimental values (i.e., black bars in Fig. S3C) and Tax-interval sampling from simulated values (i.e., Fig. S3G). All data were fitted simultaneously.

(D) The simulated dynamics of the frequency of Tax-expressing cells in the normal MT-1 cell population. A steady state is reached in which about 3% of cells are Tax⁺.

(E and F) The simulated dynamics of subpopulations of cells transduced with shNC (E, blue lines) or shTax4 (F, green lines).

(G) The survival probabilities for subpopulations of shNC cells which have expressed Tax at least once and have never expressed Tax are described in solid and dashed lines, respectively.

Fig. 6. Proposed model of the dynamics and significance of Tax expression

Left: under normal conditions; right: under Tax-knockdown conditions.

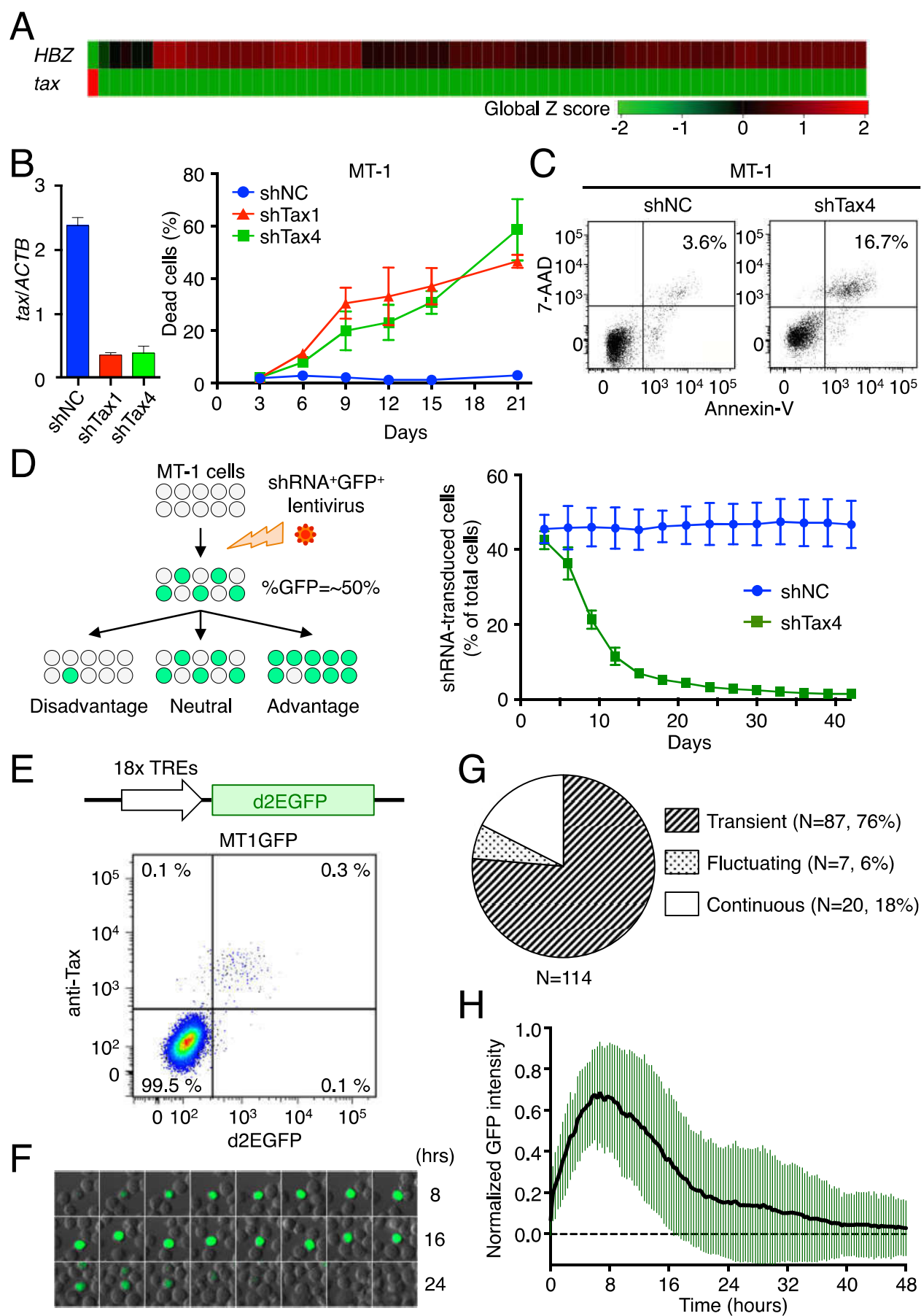


Figure 1

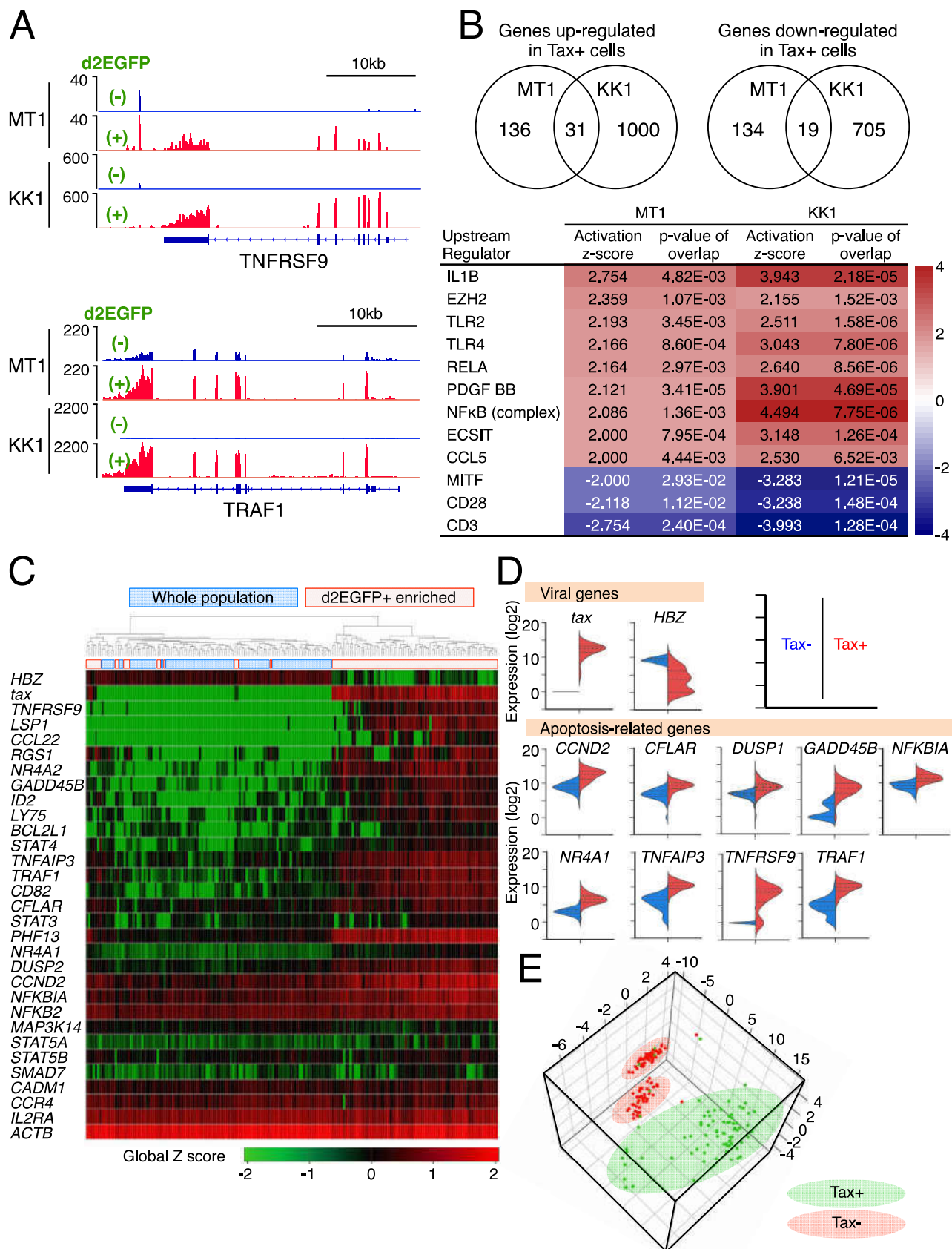


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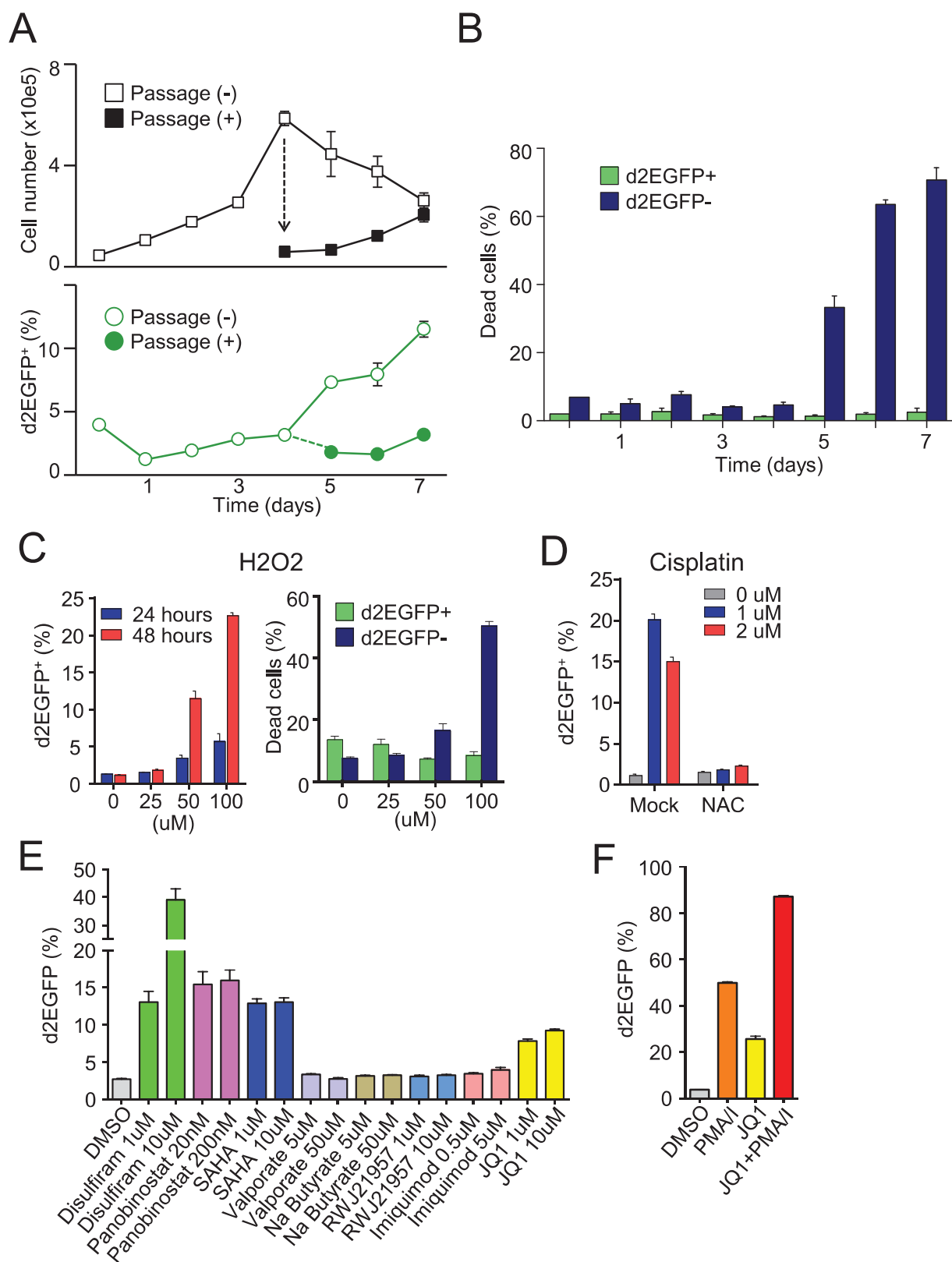


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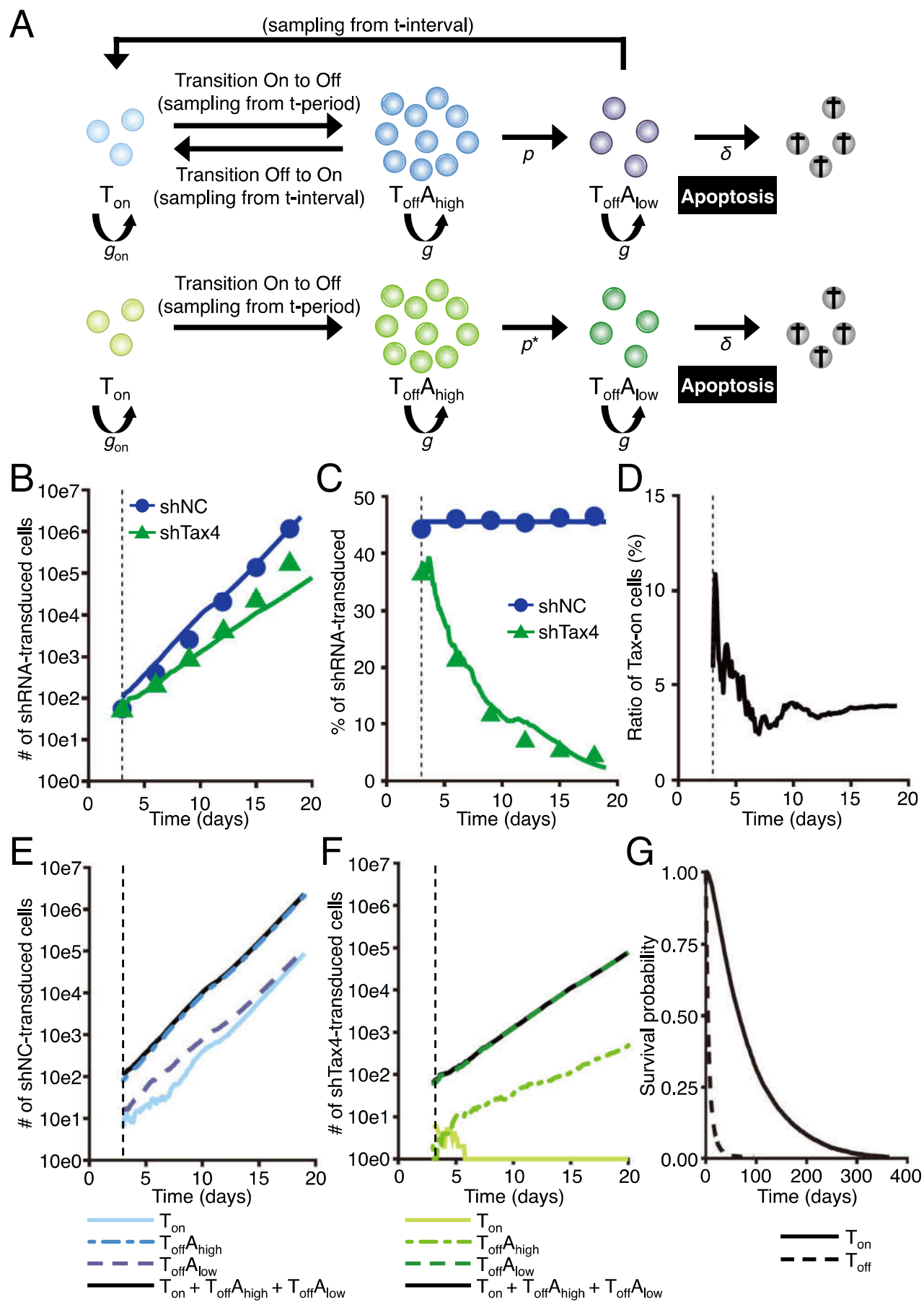


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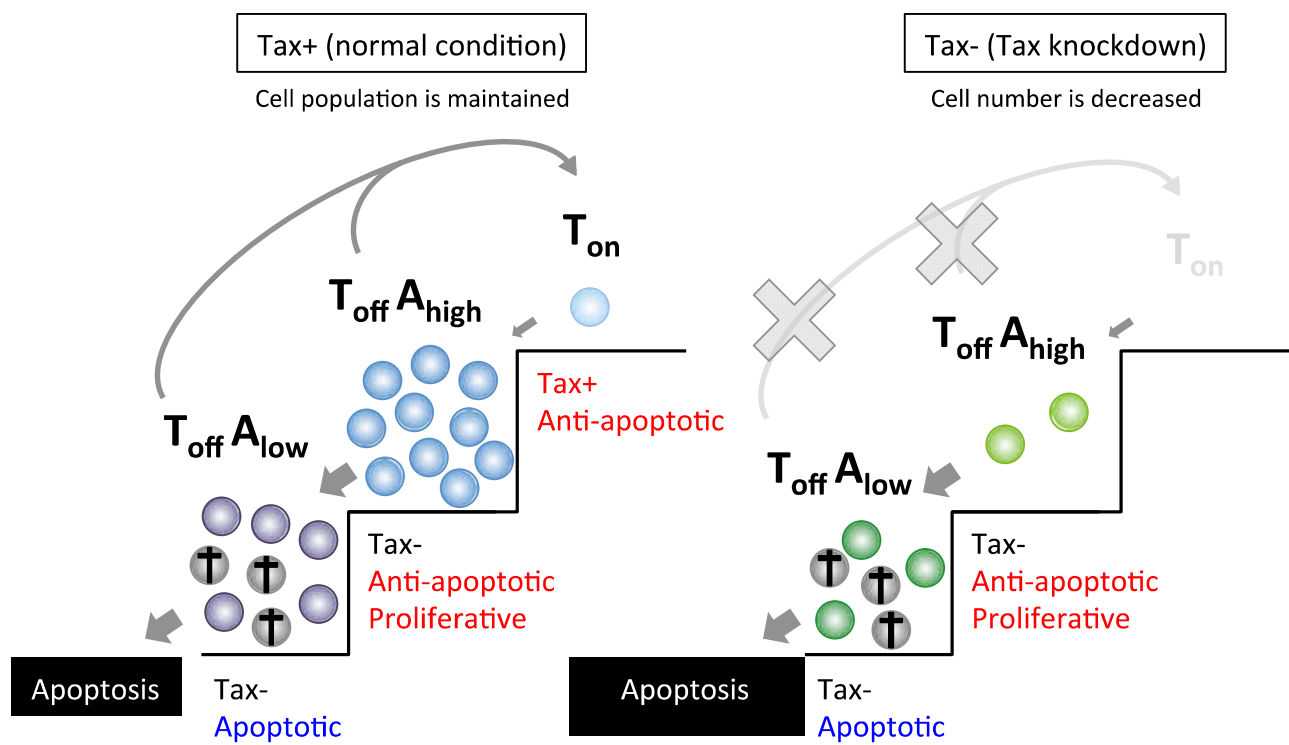


Figure 6

Supplementary Material

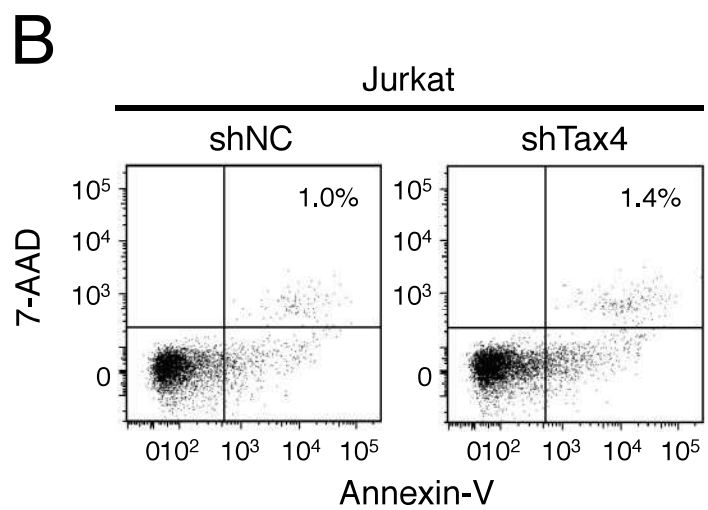
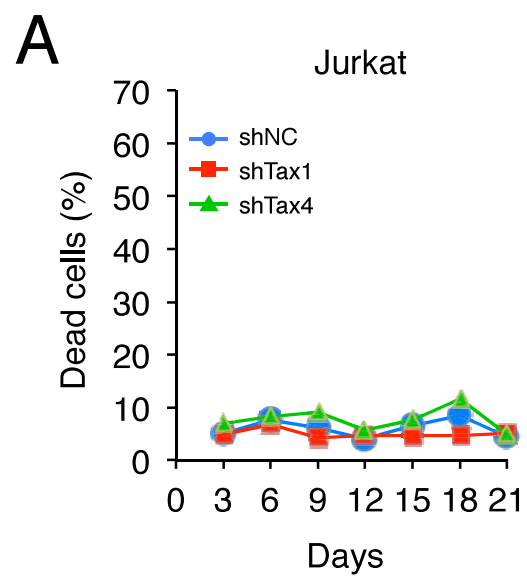


Fig. S1. Effects of shRNAs against Tax on HTLV-1-uninfected cells

(A) Tax knockdown does not cause apoptosis in uninfected cells. Jurkat cells were transduced with anti-Tax shRNA or control non-target shRNA. The percentage of dead cells in the transduced (GFP+) population was measured by flow cytometry using LIVE/DEAD reagent.

(B) AnnexinV/7-AAD double staining in shRNA-transduced Jurkat cells at day 15 post-transduction.

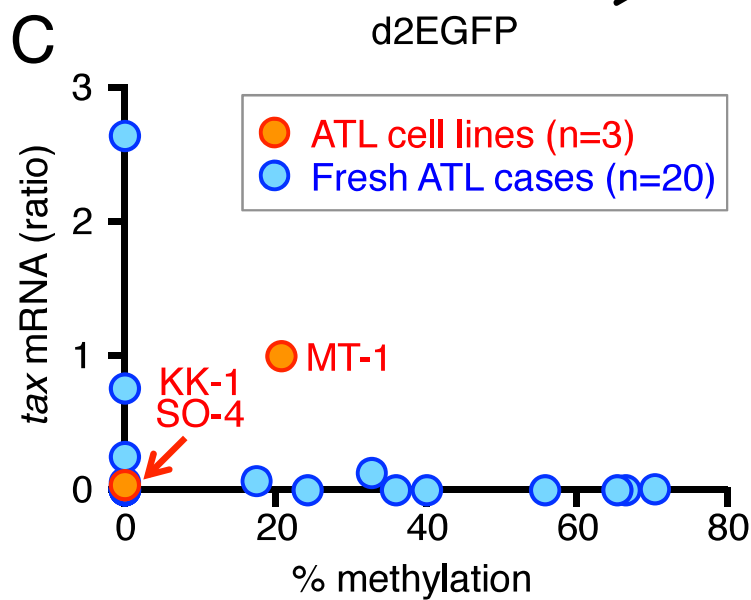
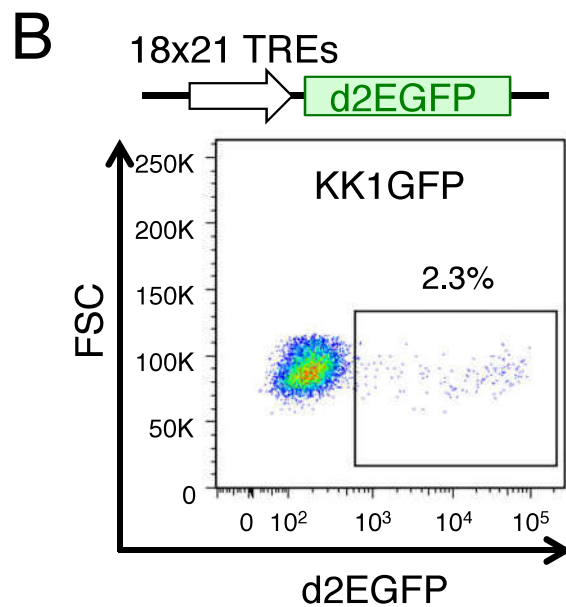
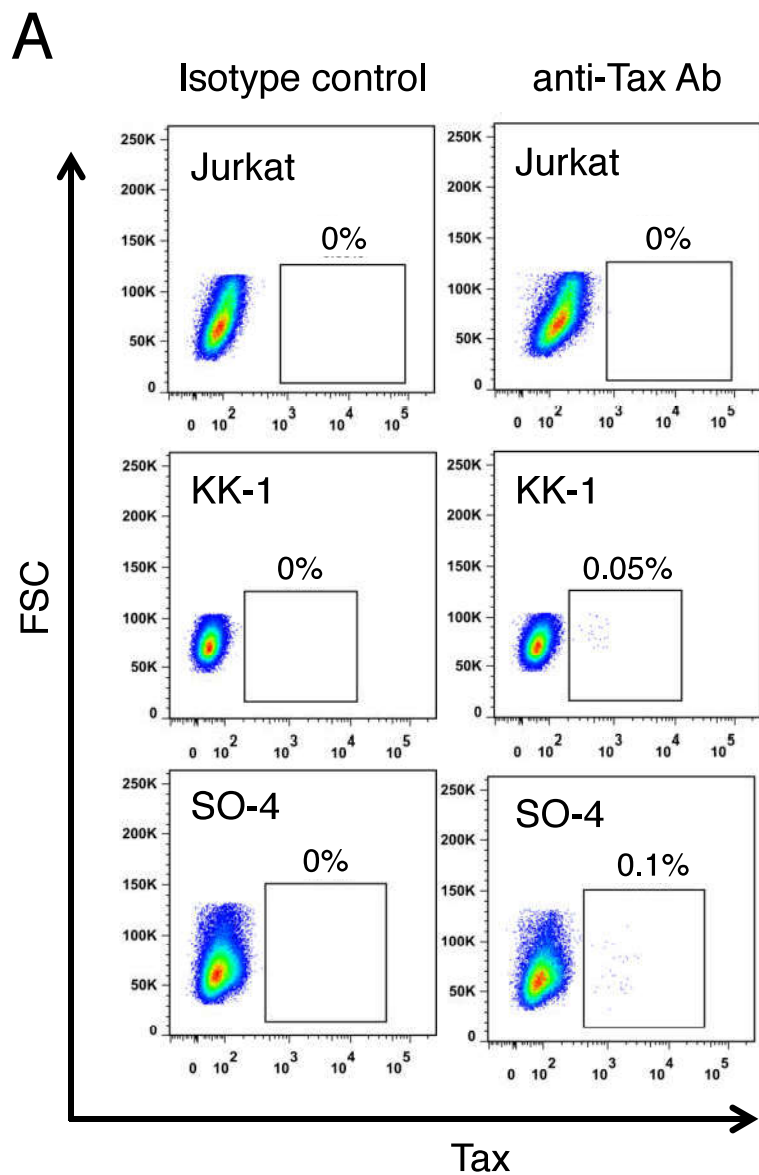


Fig. S2. Tax expression in several ATL cell lines

(A) Intracellular Tax staining in ATL cells lines KK1 and SO4, and in an HTLV-1 negative T-cell line, Jurkat.

(B) Generation of a Tax reporter subline of KK1 (KK1GFP). Upper panel: Scheme of the reporter cassette expressing d2EGFP under the control of tandem repeats of TRE. Lower: Expression of d2EGFP in a representative subclone of KK1GFP (#11).

(C) Correlation between levels of *tax* mRNA and DNA methylation of 5'LTR in fresh ATL cells and ATL cell lines. Fresh ATL cells from aggressive type ATL (n=20) and three ATL cell lines (MT-1, KK-1, and SO-4) were subjected to the analysis.

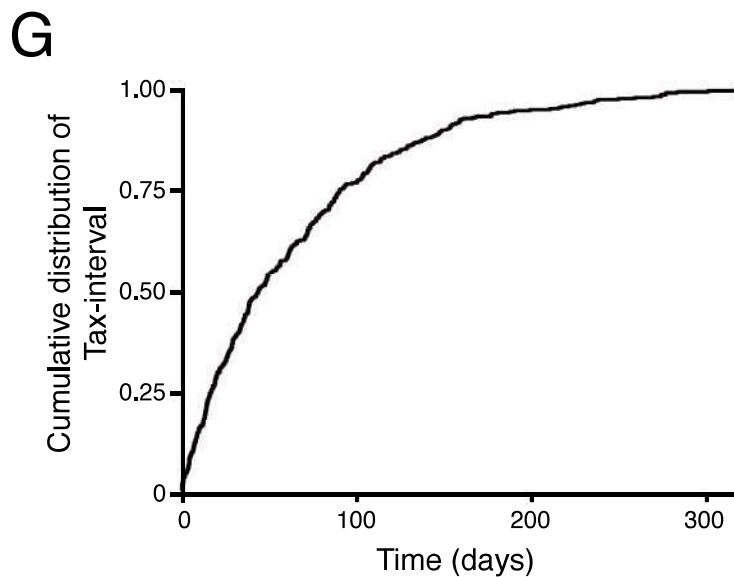
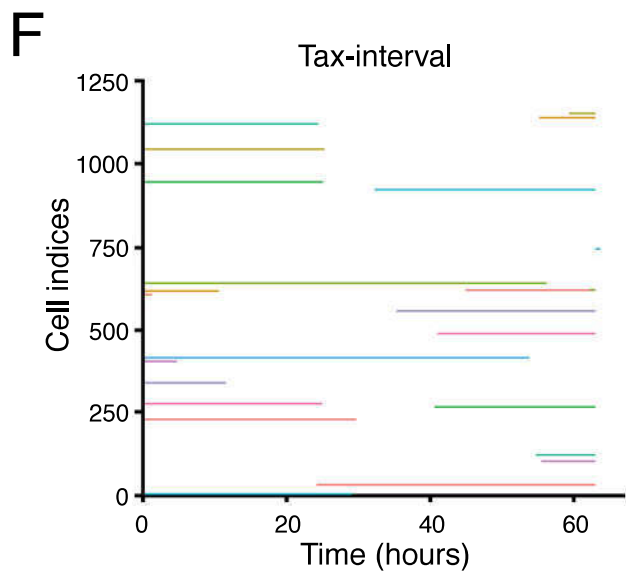
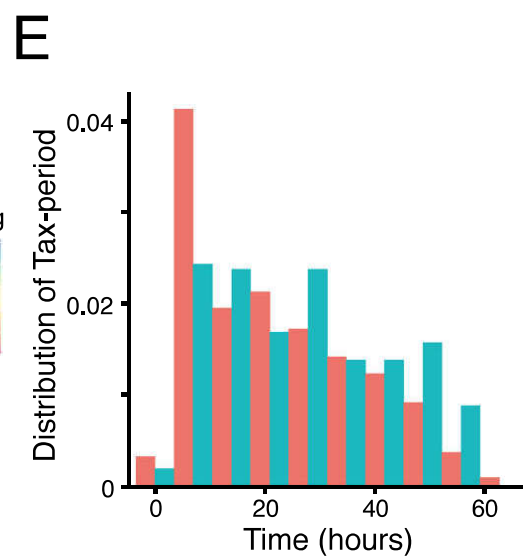
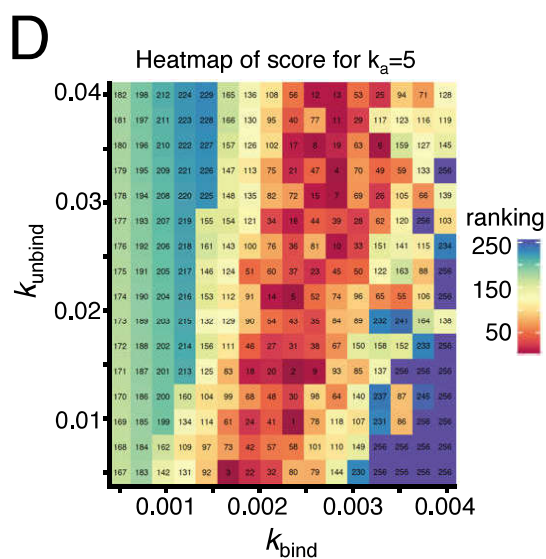
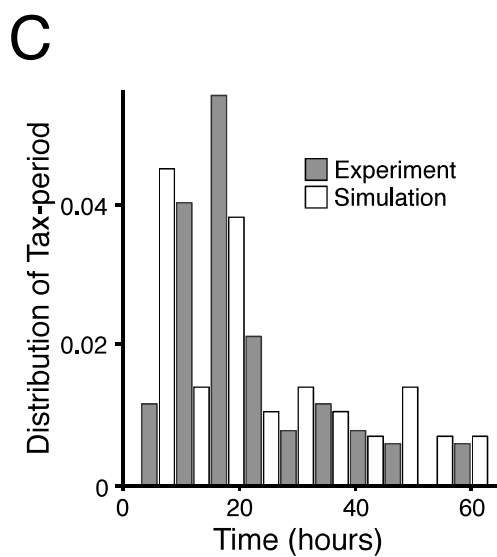
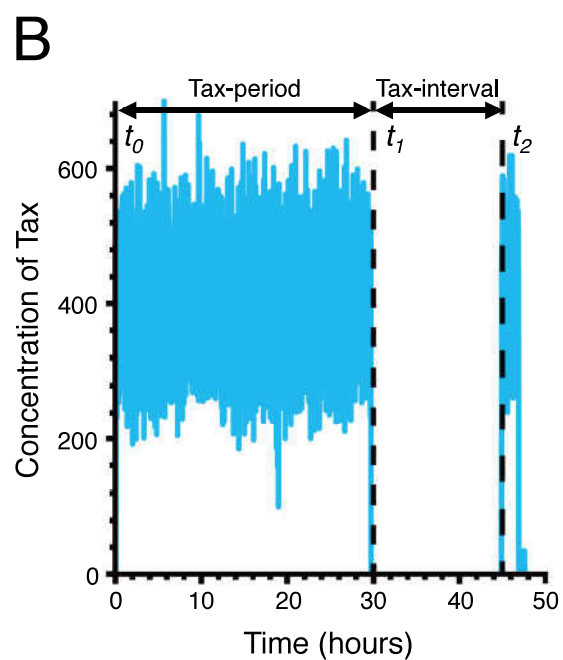
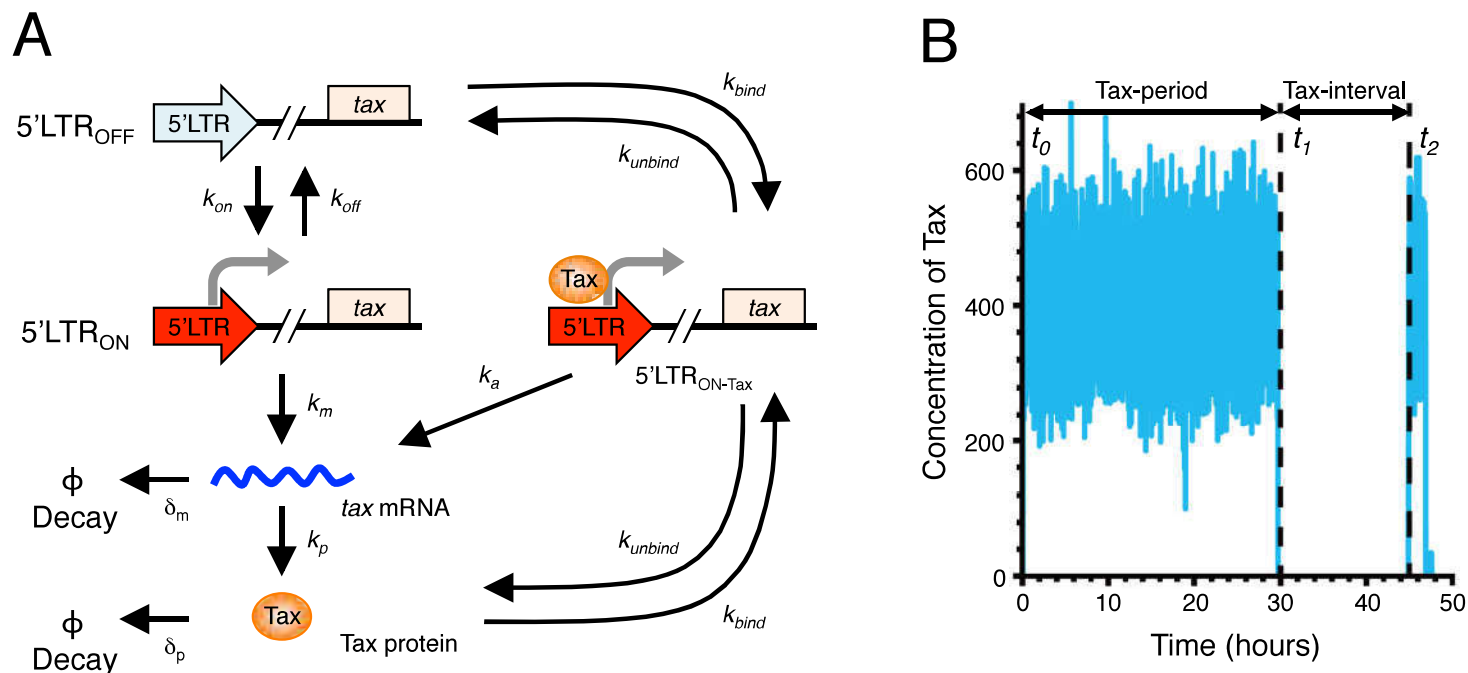


Fig. S3. Computational simulation of intracellular Tax expression dynamics

(A) Scheme for the mathematical modeling of the Tax expression process.

(B) A sample path of the stochastic simulation of the Tax expression process. The *Tax-period* is defined as the time period of sustained Tax expression between the start point (the first time to reach the burst threshold of 200 copies from no expression) and the end point (the time to return to no expression); i.e., $t_{\text{period}} = t_1 - t_0$. The *Tax-interval* is defined as the time from the end of one Tax-period to the beginning of the next; i.e. the time during which the Tax concentration never reaches the burst threshold (i.e., $t_{\text{interval}} = t_2 - t_1$).

(C) The experimental and simulation-predicted distributions of Tax-period durations are shown.

(D) The ranking of feasible parameter sets determined by computing the closeness of simulated and experimentally determined Tax-periods. For each choice of the two parameters k_{bind} and k_{unbind} , a distribution of Tax-periods was generated by a single long-run stochastic simulation (up to 1.0×10^8 hours). The closeness between simulated and experimental Tax-periods was then numerically computed by the discretized Kullback-Leibler divergence (R package entropy). The ranking is determined by the closeness: the box with the minimum KL divergence value is ranked 1. If no tax-periods are found, the maximum KL divergence value is assigned (boxes with Ranking 256).

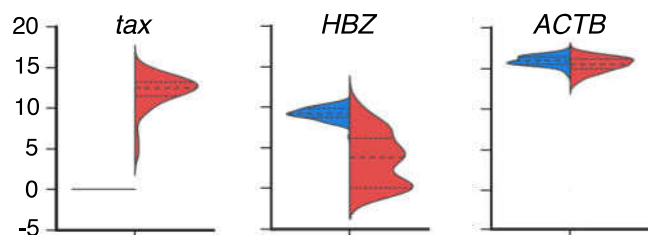
(E) Comparison of histograms for the distribution of Tax-periods between the collection of short-run (up to 65 hours) and long-run (65000 hours) stochastic simulations. A total

of 362 Tax-periods from 10,000 short-run simulations are used (red bars), while 407 Tax-periods were obtained from a single long-run simulation (blue bars).

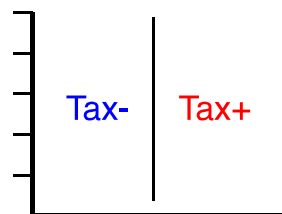
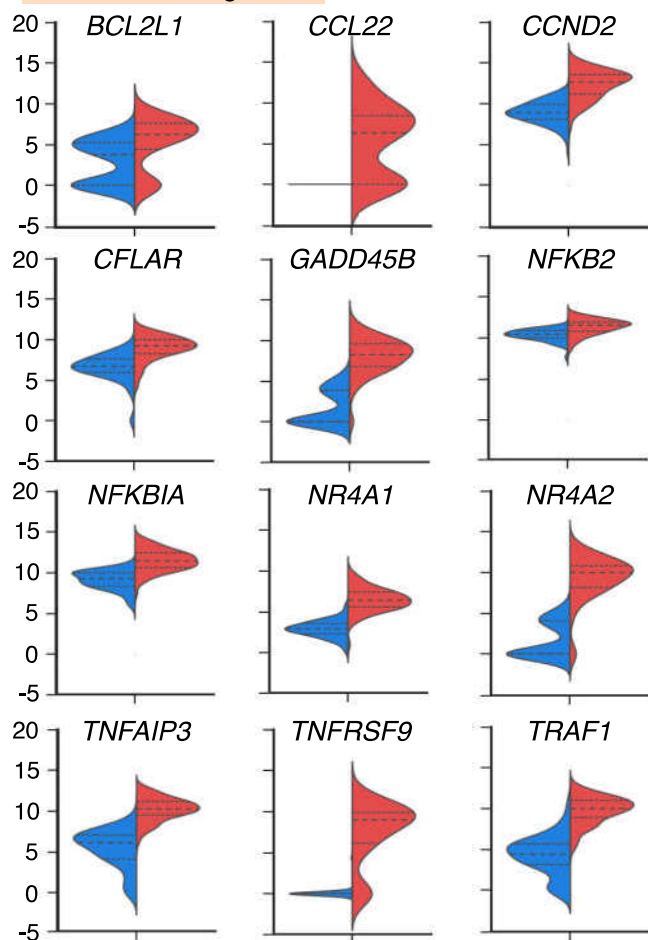
(F) Plots of Tax-periods generated from short-run stochastic simulations. Twenty four samples that exhibit a Tax-interval are mapped on the time axis.

(G) The simulation-predicted cumulative distribution of Tax-intervals is shown. 50% of Tax-intervals are longer than about 40 days.

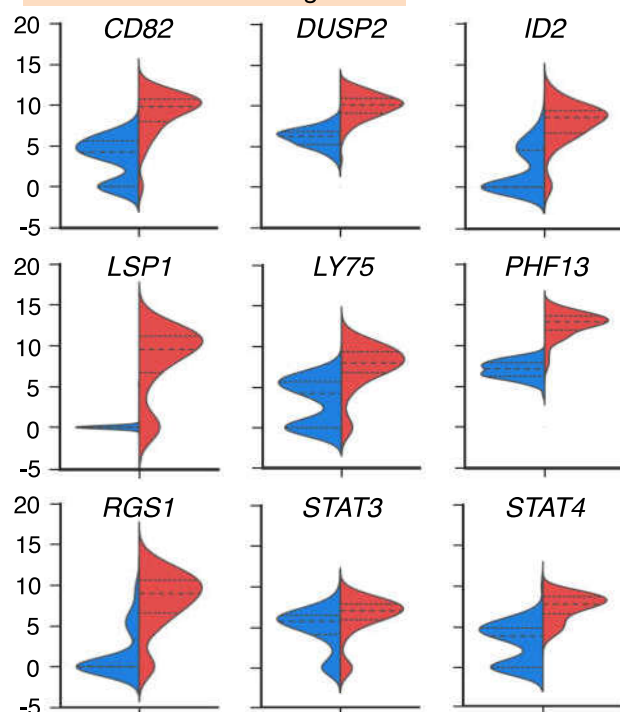
Viral genes and house keeping gene



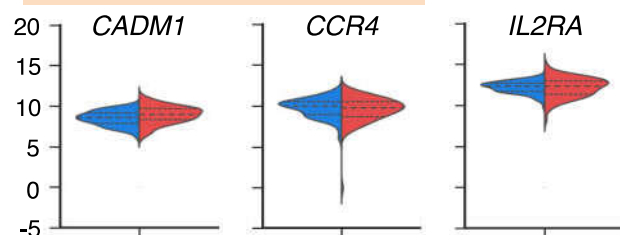
NF-κB related genes



Other cancer-related genes



HTLV-1-infected cell markers



Expression (log2)

Fig. S4. Violin plots of genes expressed differentially in Tax+ and Tax- MT1GFP cells

Results of single cell qPCR for the selected genes are shown as violin plots. Inner lines represent the population's distribution percentiles: upper: 75th; middle: 50th; lower: 25th.

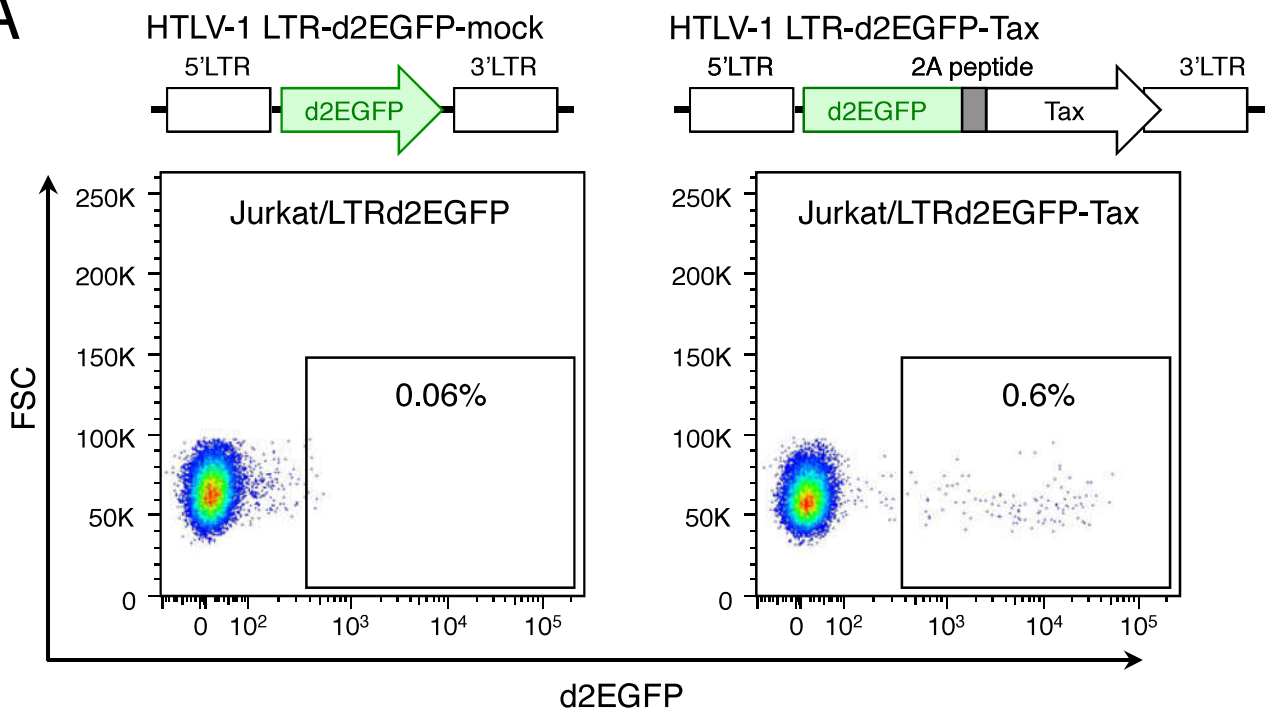
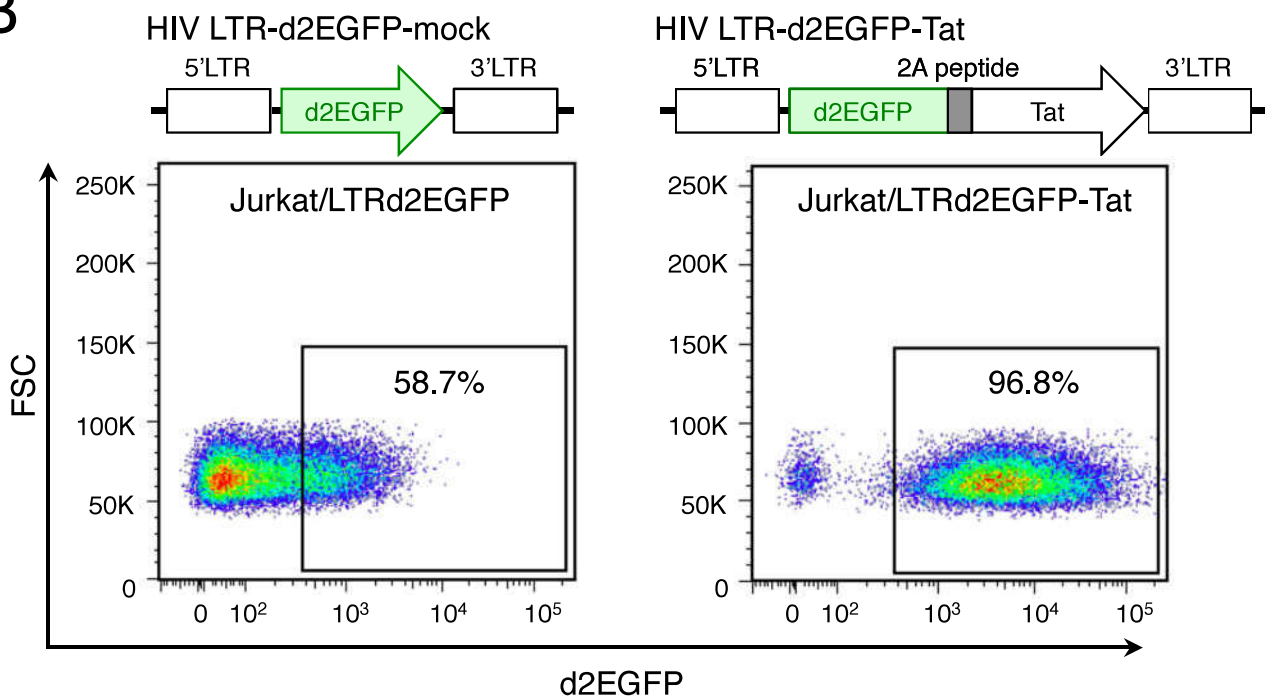
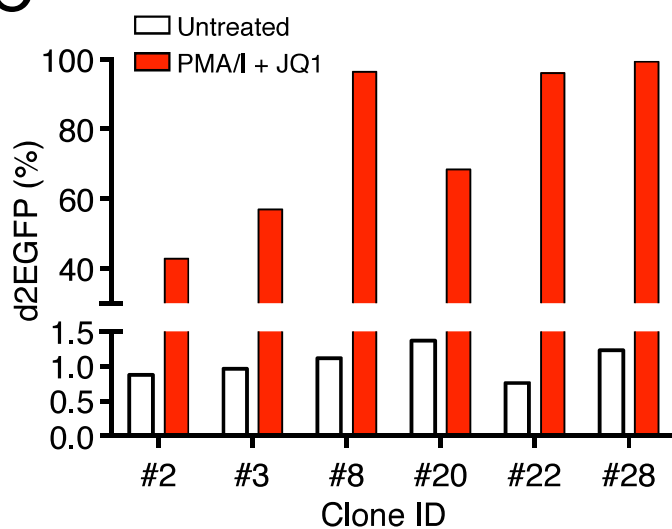
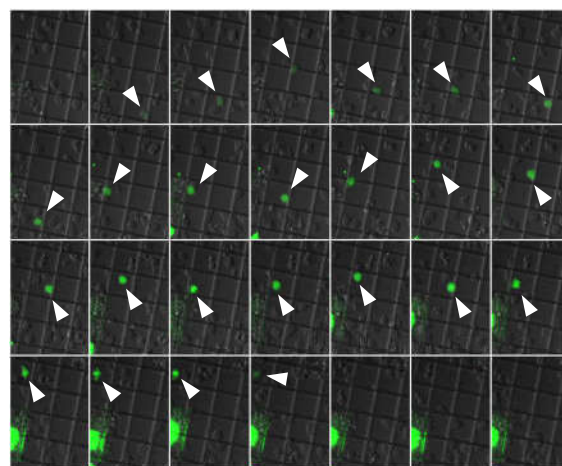
A**B****C****D**

Fig. S5. Latent phenotype established by HTLV-1-LTR-Tax minimal circuit

(A and B) d2EGFP expression under the control of the LTR promoter from HTLV-1 or HIV-1 in presence or absence of transactivator (Tax or Tat respectively) in stably transfected Jurkat cells. (A) d2EGFP expression from the HTLV-1 LTR: basal transcription (left) and transcription in the presence of Tax positive feedback (right). (B) d2EGFP expression from the HIV-1 LTR: basal transcription (left) and transcription in the presence of Tat positive feedback (right).

(C) Reactivation of LTR-Tax latency. Several Jurkat subclones stably transfected with HTLV-1-LTR-d2EGFP-2A-Tax (Jurkat/LTRd2EGFP-Tax cells) were established. All subclones had a similar latent phenotype, and could be drastically reactivated by PMA/Ionomycin + JQ1 treatment.

(D) Transient expression of Tax and d2EGFP in Jurkat/LTRd2EGFP-Tax cells. White arrowheads indicate cells transiently expressing Tax.

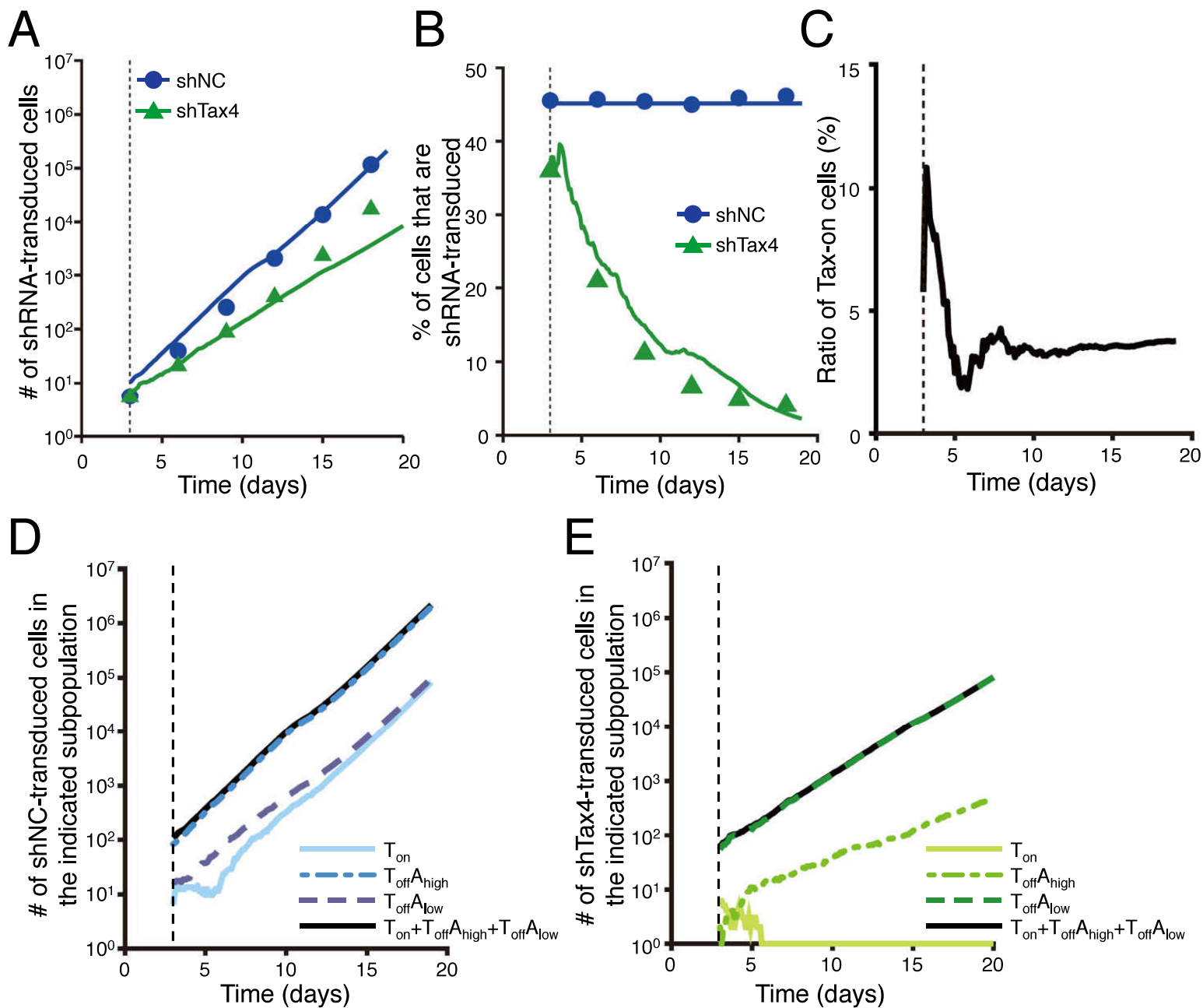


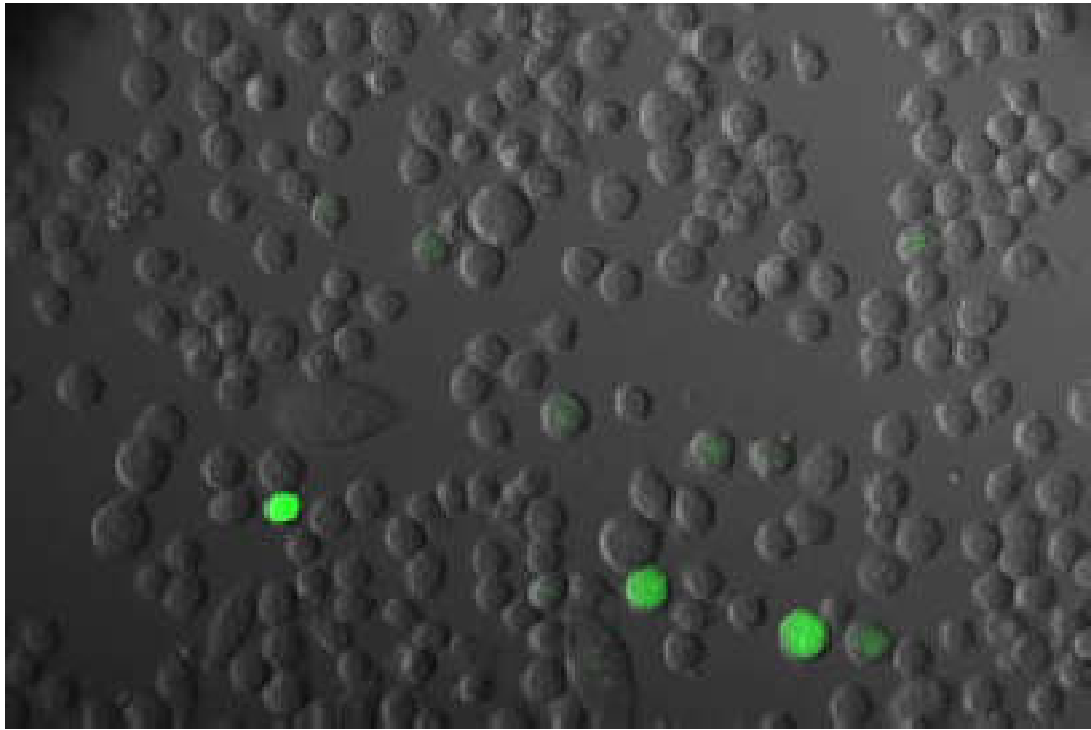
Fig. S6. An agent-based simulation using Tax-period sampling from previously simulated values

The simulation in this figure resembles the simulation in Fig. 5, except that Tax-period sampling is done from simulated values (i.e., the gray bars in Fig. S3C) and Tax-interval sampling from simulated values (i.e., Fig. S3G).

(*A* and *B*) Time-course of the number and fraction of shNC and shTax4 cells.

(*C*) The simulated dynamics of the frequency of Tax-positive cells in the normal MT-1 cell population.

(*D* and *E*) The simulated dynamics of subpopulations of cells transduced with shNC (*D*, blue lines) or shTax4 (*E*, green lines).



Movie S1. Time-lapse imaging of MT1GFP cells

Tax is transiently expressed in a small subpopulation of MT1GFP.

Table S1. Chemical reaction scheme and parameter values for stochastic simulation of the intracellular Tax expression model.

Reactions	Description	Rate and Value [*]
$5'LTR_{OFF} \leftrightarrow 5'LTR_{ON}$	Promoter toggling from active to inactive state	${}^{\dagger}k_{ON} = 3.0 \times 10^{-6}$, ${}^{\dagger}k_{OFF} = 1.0 \times 10^{-2}$
$5'LTR_{ON} \rightarrow 5'LTR_{ON} + mRNA$	Transcription of mRNA encoding Tax	${}^{\ddagger}k_m = 0.1$
$mRNA \rightarrow mRNA + Tax$	Translation	${}^{\ddagger}k_p = 10$
$5'LTR_{OFF} + Tax \leftrightarrow 5'LTR_{ON-Tax}$	Tax binding/unbinding to 5'LTR	${}^{\ddagger}k_{bind} = 2.5 \times 10^{-3}$, ${}^{\ddagger}k_{unbind} = 1.8 \times 10^{-2}$
$5'LTR_{ON-Tax} \rightarrow 5'LTR_{ON-Tax} + mRNA$	Transactivated rate of transcription	${}^{\ddagger}k_a = 5$
$mRNA \rightarrow \varnothing$	mRNA decay	${}^{\ddagger}\delta_m = 1$
$Tax \rightarrow \varnothing$	Tax decay	${}^{\ddagger}\delta_p = 0.125$

^{*} Time in simulation is arbitrary units.

[†] These parameter values which are specific to HTLV-1 are estimated from experimental data by fitting.

[‡] These parameter values are fixed as estimated in (Razooky et al., 2015).

Table S2. Parameter values for agent-based simulation of cell population dynamics under normal and Tax knockdown conditions.

Parameters	Description	Unit	Value
g_{on}	Growth rate of Tax-expressing cells	day ⁻¹	[†] 0.54
g	Growth rate of Tax-negative cells with or without anti-apoptotic gene expression	day ⁻¹	[†] 0.68
p	Decay rate of anti-apoptotic gene expression for $T_{\text{off}}A_{\text{high}}$ for shNC cells	day ⁻¹	[†] 0.02
p^*	Decay rate of anti-apoptotic gene expression for $T_{\text{off}}A_{\text{high}}$ for shTax4 cells	day ⁻¹	[†] 0.40
δ	Apoptosis rate for $T_{\text{off}}A_{\text{low}}$ cells	day ⁻¹	[†] 0.25

[†] These parameter values are estimated from experimental data by fitting.

Table S3. Primers used in the single-cell quantitative PCR.

Pre-amplification primers			qPCR primers	
Gene	Forward	Reverse	Forward	Reverse
<i>ACTB</i>	CCTTCAGCAGATGTGGATCAG	TCATCTTTCTTCGCGCAAGTTAG	AAGCAGGAGTATGACGAGTCC	CAAGAAAGGGTGTAAACGCAACT
<i>ATF3</i>	GCTGTCTTCCTGTGTACCTCTA	AAATCCTGGAGTTAGGCAAAAG	CAGCAGACAGAAACCATCAAG	CTGGTCCAAGACCCCACTCTG
<i>BCL2</i>	GGATTGTGGCCTTCTTTGAGTT	CCAGCCTCCGTTATCCTGG	TGGGTCATGTGTGTGGAGAG	GGTGCCGGTTCAGGTACTCA
<i>BCL2A1</i>	CGTCCAGAGTGTACAAAATGT	CCGTCCTTCAAACTCCTTTTCCA	GAAGTGGAAGAAATCTGAAGTCATGC	TCACATTGGTTGAATAGTGTTCGGC
<i>BCL2L1</i>	CCTAAGCGGATTTGAATCTCTT	TATAATAGGGATGGGCTCAACCAG	CTCTCCCTTCAGAAATCTTATCTTGG	ATTGTCAAAAACACCTGCTCAC
<i>BCL3</i>	CAGGATTGCACAGAGCACAT	GGAAATGGAAGATGTGGGTGG	CTCTTCTGAGCACAGATGTTCC	CATTCCGGACAGGGACTGG
<i>BIRC5</i>	GACCACCGCATCTCTACATTC	GCTCCTTGAAGCAGAGAAGAACAA	AAGAACTGGCCCTTCTTTGGAG	AAGTCTGGCTCGTTCCTCAGTG
<i>CADM1</i>	GAAGTACAGTATAAGCCTCAAGTGC	TTCATCATCGACTCTCACCCAA	CAGATGACTTATCCTCTACAAGGCT	GTTACCATCACAGGCTGGGG
<i>CASP4</i>	TGGGCTCTATCTTCATCACACA	TCATGGACAGTCGTTCTATGGT	GAAATATTCTTGGTGTGCTGCCAC	GCATTTGAGCTTTTGCCCTT
<i>CASP8</i>	CTTTTGACCACGACCTTTGAAGA	CATGGGAGAGGATACAGCAGAT	TCATTTTGGAGATCAAGCCCCAC	GTTACTGTGTGGTCCATGAGTTGG
<i>CCL20</i>	GTGCTTATCTAATTTGTGCTCACT	TAGCTAAACACAGAAACCTACAGC	CTAATTTGTGCCCTCACTGGACT	TGTGATGCTTTAAACAAAAGCAAACT
<i>CCL22</i>	CTTCTACTGGACCTCAGACTCC	GGTCATCAGAGTAGGCTCTTCA	CGTGGTGTGTCTAACCTTCAG	TTGGCTCAGCTTATTGAGAAATCA
<i>CCL3</i>	CTGCAACCAAGTTCTCTGCATC	TTAGGAAGATGACACCGGGC	CTTGCTGCTGACACGCCG	GCTGCTGCTCTCAAAAGTAGTCA
<i>CCND2</i>	GGAAGTTGAAGTGGAAACCTGG	CAAACTTAAAGTCGGTGGCACA	TGACTTCATTGAGCACATCTTTGC	AGAGCAATGAAGGTCTGACAT
<i>CCR10</i>	CTAGGGCTGCGAATCTAGAGG	GGGTTGCATCTCATTTCCATGT	GGTCGTGGGAAAGGGGAGTA	TTTAAATGTGGCAAGGCACAGA
<i>CCR4</i>	AAAGCAAGCTGCTTCTGGTTG	TGCAAGGCTTGGGGATACTTT	GCCCAGACCTGCCTTTGAG	TTTGCTGTATATGCTTTTCATCGAGGG
<i>CCR6</i>	GATACAGTCAACAGCCTGACC	CAACCTGAGCTGCTTTATCCAA	AAATGGAGGTGGAAGACAAGC	AATTGTCACCAACACCTGTTCT
<i>CCR9</i>	GGAAGTTCGACCTTCATGTG	TTTCTCCCTCCAAGTATGTCT	GGTCAACAGCATGTACAAGATCAAC	GGCAATGGCAATGTACCTGT
<i>CD48</i>	ATGGAAGATCAAGCTGCAAGTG	TTTTTCTCCCATACCAGGTGTA	GTACCCAAGCCTGTCAATCAAAA	TTTACAGACTCGCCAGGTATCA
<i>CD55</i>	CCCTAATCCGGGAGAAATACGA	GGACAGAGCTGCCTGAAATAG	AATGGTCAGATTGATGACACGG	AATAATTTGTACCCCTGTGTACATGAGAA
<i>CD69</i>	GAGCTCCAGCAAGACTTTTAC	GGACTGGTGGCATCATTTTCTT	GACTTGACCTGAGATTAACTAGGGA	TCCACTCTCCGGATGCAAG
<i>CD82</i>	GATGGGCTACGCCTGTATCAA	GGAGGTTTGCAGACAGAGAT	GTCACCAATACTTTTCTCTTCTCTTC	GAAACTGCTCTTGTGCGCCA

<i>CD99</i>	TTAATCTTGGATGTGCTTTGC	AATAATGGCAGCGCTTCACAT	GCTGGCGGATGATGTTTACTA	GGCCTTGGAGAAATGGGG
<i>CDC25B</i>	CTGGACAAGAGAGTCATCCTCA	TAGCCGCTTTTCAGGATATACA	TGTGAATTCATCTGTAGCGTG	GTAGTAGAGGCTGGGGTAGTC
<i>CDKN1A</i>	ACAGATGACAGATTCTACC	CACAAAACCTGAGACTAAGCA	CGGCTGATCTTCCAAAGAG	GAAGATGAGAGCGGGCCTTT
<i>CFLAR</i>	CCCTAGGAATCTGCCGTGATAA	TACAGGCAAAATTGGCCAAGAA	ATTGGCAATCAGACAGAGCTTC	TGGGATATACCATGCATCTAGAA
<i>COL1A1</i>	AGACATGTCAGCTTTGTGGAC	GTACCTGAGGCGCTTCTGTA	GGCTCCTGCTCCTCTTAGC	AGGTGATTTGGTGGGATGCTT
<i>CREB3</i>	GGTAGTACTACAGATGAGGA	GGCTCTCTTGAGAGATCTTTTA	AGAGTCTATTGGAGAAGAGGG	CGCACAGCTTTCAGAAATTTGTT
<i>CXCL10</i>	TGTCCACGTGTTAGATCATTG	GATTTTGCTCCCTCTGGTTTT	GGTGAGAAGAGATGCTCTGAATCC	GAGATCTTTTAGACCTTCCCTTCT
<i>CXCL11</i>	TGGGGTAAAAGCAGTGAAGTG	ATAAGCCTTGCTTGCTTCGATT	AGCCTCCATAAATGTACCCAAGT	GGATTTAGGCATCGTTGTCTTT
<i>CXCL8</i>	AAACTTCAGACACAGCAGC	CAGAGCTCAGAAAAATCAGGAAG	ACAAGCTTCTAGGACACAGACC	GCCAGCTTGGAAAGTCATGTTTA
<i>DUSP1</i>	GAAATCCTGCCCTTCTGTACC	AGGGATGCTCTTGTACTGTTAG	CAGTGGGTATCACGCTTCC	CCCTCAAAATGGTTGGGACAAT
<i>DUSP2</i>	GATCTTGCCCTACCTGTTCT	TCCACAGGGATACTCTTGTAGC	CTGCAGTCACTCGTCAGACC	GAAAAGGCCCTCAAAAGTGGTTG
<i>EGR1</i>	TAGGTGAGATGGAGGTTCTCAG	AGAACTTGACATGGCTGTTTC	CAAGTCTCCTCTCTACTGGA	GCAGCTGAAGTCAAAAGGGAATA
<i>FZD6</i>	CTTGGGTCCTGATCATTTCTCG	TTTTGCTTCCAAACCCAGAAAGAC	CCCATGTCCTTATCAGGCAAAA	TGCCAACAAATTAATGTCTATCAGST
<i>GADD45B</i>	GAGTCGCCCAAGTTGATGAATG	ACGATGTTGATGCTGTTGTCCAC	CTGCCTCTTTGGCCATTGACGA	AGCAGAAGGACTGGATGACGG
<i>HIF1A</i>	TACAGGATGCTTGCCAAAAGAG	GGAAAAATCAAGTCGTGCTGA	CTGGGTTGAAACTCAAGCAACT	ACCACTCACACACGTAATTCACA
<i>HIGD1A</i>	ACAGGTGTTTCCCTTCCTTCAT	AGTATTTCCCTGCTCTTTCAGT	GGAGATCAGGGATCAAAACTCA	CAACAATTTGCTGCAAAACCCCG
<i>ICAM1</i>	ATGTGCAAGAGATAGCCAAACC	GGTAAGGTTCTTGCCCACTG	GCTATTCAACTGCCCTGATGG	GGAGGGTGCCAGTTCCA
<i>ID2</i>	TCAAATCACACAAAGCACTGT	CCATTCAACTTGCTCCTCTTGT	GTGGCTGAATAAGCGGTGTTT	AAGTTCAGCACTTAAAGATTCCGTG
<i>IFI12</i>	TCAGCATTTATTGGTGGCAGAA	ATGGCATTTTACTTGCCGTAGG	GGAGATTTCTGAAGAGTGCAGC	CTGCTCTCCAAGGAATTCATTGT
<i>IFI13</i>	GGAGTATTTTCCCTCTCAGCAT	AGGCTTCTGATGCTCTGTTTTTC	TGAGCTTGAGGATGGGTAGTGAG	TCTGTCTCAGTTCAGTTGCTCT
<i>IKZF1</i>	ATATTGGAGACCGGAGGGTAAC	TTACAGAAAGGGATATGGGGCAA	GTGGCCCTGTTGATTACAGCTA	TGAGATCCAGGCTTTCACAT
<i>IL10R4</i>	CTCCTAACCTCTGGAGAAGTGG	AGAAATGATGACGTTGGTCCAC	GAGTTCTGTGTCCAGGTGAAAC	GTGAAATACTGCCCTGGTGAGG
<i>IL13</i>	TCATTGCTCTCACITGCGCTTG	GTTGATGCTCCATACCATGCTG	CCTGTGCCCTCCCTCTACAGC	CATTGCAGAGCGGAGCCCTTC
<i>IL15</i>	GAAGTGCTTTCTCTTGAGCTTACA	TCCTCACATCTTTTGCATCCAG	ATTTCACTTTGAGTCCGGGAGTG	GAAGACAAAACGTGTTGTTTGTCTAGG
<i>IL23A</i>	TGATGTTCCCATATCCAGTGT	AGAAAGGTCCTCTGTGAAAATA	CCCAAGGACTCAGGACAAC	TCCGATCCTAGCAGCTTCTCAT
<i>IL2RA</i>	GGNAGACAAGTGGACCCAG	TGAAAATCTGTTGTTGACGAGG	CAGCTCATATGCACAGGTGAAA	CAGGAAGCTCTACTCTCAGGAC

<i>IMMT</i>	GCTCGGATGACTTTAAACGAG	TCAATACACGATGTCATGAG	AGCTGCACAGTATTACTCCAGAA	CATCAGTAGAGAGCTTGTGAGC
<i>INSI1</i>	ACTGCATTAAACGTGTGGTGT	ACAAGGCTCAGATTGGTTTTCA	GTGAGCACAATGATTTCATTAAATGGC	TGCACTTGAAGTTTTAAGACCCA
<i>JUNB</i>	GCACTAAATGGACAGCCCTT	CTTTGAGACTCCGGTAGGGG	TACCAGGAGACTCATACACAG	GCTCGGTTTACGAGCTTTGTAG
<i>LSP1</i>	TATTGTGGCTGGAGACATGAGC	TCCACAAGCACCTTCTCATACT	AAGAAAAGCCTCTCGGAGCAGAA	CGGTGGCCACAACCTTATACCT
<i>LY75</i>	AAGAGTTGTCTGCAAAAGTCC	GAACTGATGAGAAAACCCGCC	GGCCCTGATTACACAGCAATAG	GAAGAGGAACCAAAATCAGTCCG
<i>MAP3K14</i>	GGAGCTGGAATAGAAATTATTCCTC	GAGTTTTCAGAGGCCCTTGTATG	CCTGCCCGACCAATTTTCTCT	GGTTCTTCTACTGTCATCCGA
<i>MT1X</i>	CCACGCTTTTTCATCTGTCCC	GTGCATTTGCACCTCTTTGCATT	CTGCGTGTTTTCCCTCTTGATCG	CAGGCACAGGAGCCAAACAG
<i>MT24</i>	AATGCACCTCCTGCAAGAAAAG	TGGAAGTCGCGTTCTTTTACATC	TGGGTGTGCCAAGTGTG	GGGTGTCCAGCATCAG
<i>MYD88</i>	CAATTCTGGAGATGCCAACTT	GTCTCAGCTGGTCCCTGTTCTAT	CAGAGCTGGACATCACATTTTC	GGCTGATAATCCAGCAAGTGG
<i>NFKB1</i>	CCGTGTAACCAAGCCCTAAA	GAACCAAGAAAGGAAGCCAAAGT	ATTCCACTGCGTTGTCCAC	AAATCGAGAATGATTCAGGCGG
<i>NFKB2</i>	TGTTGCATATGCCTGACTTTGA	CTAGATGCAAGGCTGTTCTGTC	CTGTATCCAGTACACCTGGCGG	CTCCACTTCAGCCCCACTGTC
<i>NFKB1A</i>	AATGCTCAGGAGCCCTGTAA	AGGTGAGTGGTAGGAGAAAT	CCTTCACCTCGCAGTGGAC	CCCTGGTAGGTAACCTCTGTGA
<i>NR411</i>	ATAGCGCCGTGCTGTAAATAAG	GAAGGAGGTTAGAAAGAGAGAG	CAGAAAGAAAAGAGCTTGAGGTG	CAGCAGGAGGCTGGAAGG
<i>NR42</i>	GAATGAATGAAGAGAGACGCGG	TTACAGGCGTTTTTCGAGGAAT	CTAAGGAGGAGATTTGACAGG	AGGTGGACAGTGTGTAAPTCA
<i>PHF13</i>	ATGATGAGGACATCATGGTGA	ATTTCCGATTTTTCGCACAGG	CTCAGATGACGATTCTCTGGAC	TGGCACTCAATTACACTCGATCA
<i>PKMYT1</i>	CCTCACCATCCTGGAAGTGG	CTCCAGCATCATGACAAGGAC	CATGCAACATGGAGCTGCC	GAAGACAGACCGGCAGTGAA
<i>PP1R21</i>	TGCGTTAAGAGCCAGGAATCTA	ACACAGAAGTGTAGTTTGTCCG	TGACAGCTGTGTTTGAAAGC	AAGAGTCCATCTGGCTGTGA
<i>PRK4CB</i>	CTGGAGATACAGCAACTTTGA	TCTCTCAACAGAGTGCAAAACAC	TGACTATGAAGAAGAGATATCCGTG	GTCACTTGTTCCTCTTTAAAATTACCC
<i>PYC4RD</i>	CCTAAGGGAGTCCAGTCCTA	CAAGCTGGCTTTTGTATATTGTG	CTGGTGGAGACCTGGAG	TCAGGATGATTTGGTGGGATTG
<i>RELA</i>	GCTTGTAGGAAGGACTGCC	TTGTTGTTGGTCTGGATGCG	GGATGGCTTCTATGAGGCTGAG	CCGCTTCTTACACACTGGATT
<i>RGS1</i>	TGCAATGGTCTCAATCTCTGGA	GCTTTACAGGGCAAAAGATCAGA	TGCCAACCAAACTGGTCAAAAT	TTATAGTCTTCACAAGCCAGCC
<i>SELPLG</i>	CTCTAACATCTCCCATGGCTCT	AATGTCCACTTCTCTTTTGGTG	CTTCTCTGGTCACTGGAGTC	CATGGCAACCAAAAGACAATGG
<i>SMAD7</i>	GCTTTCAGATTCCCAACTTCTTCT	CCATTCCTCTGAGGTAGATCAT	GGATCGGTCAACCTGGTGC	AGAAATATCCAGAGAGGCTCC
<i>SMIM4</i>	TCCAGTCTTATAGTTCGGTCATA	ATCGTTCTCTCCAGGACAAAA	GGTCGAGCATGTTTCAACCAG	AGGAACCGGTAGATGCCAAATC
<i>STAT1</i>	GACCGCACCTTCAGTCTTTTC	GCAATTTCCACCACAGTCTCAAC	TCATTACAGAGCTCGTTTGTGG	AGTGAAGTGGACCCCTGTCT
<i>STAT3</i>	CACACTAAAGTCAGTTTCTGG	CATCACTTTTGTGTTTGTGCC	TTCCCTGAGTTGAATTATCAGTTT	AATGTTAAATTTCCGGGATCCTCT

<i>STAT4</i>	CTAATGCTTGGGCATCCATCAT	TCTGAGTTAAGACACGACCAA	CAACGATTCCCAGAACTGGTT	ATCAAAACTGCCAGCTCATCAC
<i>STAT5A</i>	CACAGAAACCCGTGACCATGTACT	AGAGGTGAAAAGACCCGGCAG	GGATGGAGAATTCCGACCTGGAT	CGGGAGTCAAGACTGTCCATT
<i>STAT5B</i>	CTACGTGTTTCTCTGATCGGC	AGATGCGTTCAAAACTCAGG	TACTCCAAATACTACACACCACTTC	GACCATTGCTTGATCTGTGG
<i>THEMIS2</i>	AGAAGGTGGTCTGTGAGAACC	TGCTTGGAGCTCTGATTGT	CGAAGACCAGCCAGACCAT	CTCCTCCAGGGTTTCATAGCTC
<i>TNFAIP3</i>	GACACAGACTTGGTACTGAGGA	TCCAGTTCGGAGTATCATAGCA	TTCAGCACGCTCAAGGAAAC	AAGCCCGTTTCAACAAATTCC
<i>TNFRSF1B</i>	CATGCCGGCTCAGAGAACTACTA	AGTTCCAGAGCTGGGTGTATG	TGCAGAAATGTCGCGCG	CTGTCTCAGAGGAGTCACAC
<i>TNFRSF9</i>	CAGGATCGAGCATGTGTGAA	GACTTTCATCCAAAGAACAGTTTG	CAGGATTGTAAACAAGGTCAAGAAC	GACAGATGCCACAGTTTCTGAT
<i>TNFRSF10</i>	GGCATTCAATTCCTGAGCAACTT	TGGACCAATTTGTTTGTCTGTTCT	CACTTGAGGAATGGTGAAGTGG	TTTCTCTCTGAAATCGAAAGTATGT
<i>TNFRSF4</i>	GTCTGTCAACTCCTTGATGGTG	AGGACACAGAAATTCACCAGGAT	GCCTCTCTGACTTACAAAGACAA	AAGAAACAGTTCTCCGCCATTCTC
<i>TNIP1</i>	CTGGCCAAAGTCCAAGATTGAAA	GCTGGATCTCCTTTTTCCTGGTA	TGGAGGAGACCGACAAGGAG	CTCACGCTGTGCGGTGAGT
<i>TP53</i>	GCTCAGATAGCGATGGTCTGG	GTACAGTCAGAGCCAAACCTCAG	CTCCTCAGCATCTTATCCGAGT	ATAGGCACCACCACACTATG
<i>TRAF1</i>	CACTTTCCTGTGGAAGATCACC	TGCCATCTCCATTCAGGTACAG	AATGTCACCAGGCGGTGC	CATACTTGGCAGTGTAGAAGGC
<i>UBE2C</i>	ATAAGCTCTCGCTAGAGTTCCC	ATCATACAGGGCAGACCACTTT	CAGTGGCTACCCTTACAATGC	ATGTCCAGGCATATGTTACCCCT
<i>WIP1</i>	ATGGATTCACAATTCCCTCTCGG	AGGTAGTTGGTAGCAGCCATAA	CCGAGACGGTACACATCTTCAA	ACATCTTCCCATGTAGCCACT
<i>tax</i>	GATCCCAAGAAAAGACCA	CTCCAAACACGTAGACTGGGTA	GATCCCAAGAAAAGACCA	CTCCAAACACGTAGACTGGGTA
<i>hbz</i>	TAAACTTACCCTAGACGGCGGAC	ACCAATTCCTCCACCAGCAG	CAGTTCAGGAGGACCACACAG	ACAGGCAAGCATCGAAACAG
<i>Spike1</i>	GTATTTGATTACCGGCAACGGA	ACCGTCGTCAATTGAAGATATGC	GTGCATTTCTCTGGAAGGTGAAC	CTCGGGTATCCCATTTGCTGAT
<i>Spike4</i>	ATCGGTAGATAACATTCAGGCG	GTGGTGAATTCGATCCTACCG	AAGAGCTGTACACCGGTAGTGG	CCACATCTTTGGCAGCGAAT
<i>Spike7</i>	GAGAAAGGTAATGACCTGGTGC	TTTGGGTATTGAAGATCAGGGG	TCCCATATGCTGGAAGTCTGC	GATCGTCTTTACCACCAATACC

SI Methods

Plasmids

The *PiggyBac* Transposon system (System Biosciences) was used in this study. A Tax-responsive element-driven reporter plasmid, PB-18X21-RFP, and a transposase-expression vector, PB-Tase, were kindly provided by Dr. Chou-Zen Giam (1). To obtain d2EGFP under the control of the TRE's, we generated PB-18X21-d2EGFP from PB-18x21-RFP and pd2EGFP (Clontech) by replacing the coding sequence of RFP with the fragment containing d2EGFP and the neomycin-resistance gene. The 2A peptide-based bicistronic construct expressing Tax (or Tat) and d2EGFP through the HTLV-1 5'LTR (or HIV 5'LTR) replaced the CMV promoter of PB-CMV-MCS-EF1-Puro (System Biosciences) to produce PB-HTLV-1 LTR-d2EGFP-Tax and PB-HIV LTR-d2EGFP-Tat, respectively. For knockdown experiments, lentivirus vectors expressing shRNAs were used. For shRNAs, a fragment targeting a Tax coding sequence (shTax1: gccttcctcaccaatgttc, or shTax4: ggcagatgacaatgaccatga) or a non-target shRNA (shNC: caacaagatgaagagcaccaa) was inserted into pLKO.1-EGFP (Sigma-Aldrich).

Transfection and lentivirus transduction

We stably transfected MT-1 cells with the reporter transposon PB-18X21-d2EGFP using the Neon Transfection System (Thermo Fisher Scientific), thus generating MT1GFP cells. 5×10^5 MT-1 cells were electroporated with 1 μ g of PB-18X21-d2EGFP and 0.3 μ g of PB-Tase helper plasmid. 48 hours later, G418 (1000 μ g/ml) was added for selection. Stable clones were isolated by limiting dilution and expanded in 96-well plates. For

lentivirus production, $3-5 \times 10^6$ of HEK293T cells were seeded in a 10 cm dish, and 24 hours later they were transfected with 15 μ g of HIV Gag/Pol-expression plasmid (pCMV Δ 8/9), 7.5 μ g of Env-expression plasmid (pVSV-G) and 15 μ g of shRNA expressing plasmid (pLKO-GFP-shRNA). Polyethylenimine (PEI MAX 40000 from Polysciences) was used for packaging of the lentivirus vectors. 48 hours after transfection, the supernatant was collected and concentrated using ultracentrifugation. 3×10^5 MT-1 or Jurkat cells were seeded in 12-well plates and infected with the concentrated virus.

Flow cytometry

To measure GFP expression, cells were washed once with FACS buffer (PBS+ 2%FBS) and analyzed with a FACSVerse directly or after staining with LIVE/DEAD Near-IR (Molecular Probes) for viability. For apoptosis, cells were stained with Alexa Fluor 647 Annexin V and 7-AAD (Biolegend). For intracellular staining of Tax, cells were fixed and permeabilized with the Foxp3 Intracellular Staining Buffer Set (eBioscience), and then stained with anti-Tax mouse monoclonal antibody (clone MI73) and anti-Mouse IgG Alexa Fluor 647 (Life Technologies). Cells were also stained with DAPI to exclude sub G1 dead cells.

References

1. Zahoor MA, Philip S, Zhi H, & Giam CZ (2014) NF-kappaB inhibition facilitates the establishment of cell lines that chronically produce human T-lymphotropic virus type 1 viral particles. *J Virol* 88:3496-3504.

Supplementary Information

Note S1. Stochastic simulation of intracellular Tax expression model

A previous study on HIV elegantly established a mathematical model of 5' long terminal repeat (LTR) activation by Tat in latently infected cells¹. We adapted this model to simulate the regulation of the HTLV-1 5'LTR by Tax (See Fig. S3A for a schematic representation). We tested several parameters used in the HIV study and adjusted them to fit our experimental data of transient Tax expression (Fig. S3B-D, Table S1, and Note S2). A simple deterministic two-state model of Tax positive feedback was developed (see the chemical reaction scheme in Fig. S3A)¹. A stochastic simulation model corresponding to the deterministic two-state model was constructed and implemented by the Gillespie algorithm² to investigate the stochastic dynamics of Tax expression, especially for the length of Tax expression (t_{period}) and the interval between Tax expression periods (t_{interval});

$$\begin{aligned}\frac{d}{dt}[5'LTR_{OFF}] &= -k_{ON}[5'LTR_{OFF}] + k_{OFF}[5'LTR_{ON}] - k_{\text{bind}}[5'LTR_{ON}][Tax] \\ &\quad + k_{\text{unbind}}[5'LTR_{ON-Tax}], \\ \frac{d}{dt}[5'LTR_{ON}] &= k_{ON}[5'LTR_{OFF}] - k_{OFF}[5'LTR_{ON}], \\ \frac{d}{dt}[mRNA] &= k_m[5'LTR_{ON}] + k_a[5'LTR_{ON-Tax}] - \delta_m[mRNA], \\ \frac{d}{dt}[Tax] &= k_p[mRNA] - k_{\text{bind}}[5'LTR_{OFF}][Tax] + k_{\text{unbind}}[5'LTR_{ON-Tax}] - \delta_p[Tax], \\ \frac{d}{dt}[5'LTR_{ON-Tax}] &= k_{\text{bind}}[5'LTR_{OFF}][Tax] - k_{\text{unbind}}[5'LTR_{ON-Tax}].\end{aligned}$$

The description of the chemical reaction scheme and parameter values used in our simulations is summarized in Table S1. The Gillespie direct method was coded in C++ (the source code is freely available upon request) to achieve fast and efficient computations. Each stochastic simulation was performed until a computational time of 100000. An arbitrarily chosen time-scale factor 0.00065 was multiplied with the computational time to imitate the

experimental time period for the live cell imaging (65 hours as a maximum period). Initial conditions for all chemical reaction agents were set to 0, except for $5'LTR_{ON}$ (the second variable in the deterministic model), which was set to 1. A sample path of Tax protein population (the fourth variable in the deterministic model) is presented in Fig. S3B.

Note S2. Fitting mathematical model to data

Simulated Tax-periods generated from the stochastic simulation model were fitted to the distribution of experimentally observed Tax-periods (black bars in Fig. S3C). Using the “entropy” package of statistical software R³, we calculated the discretized Kullback-Leibler divergence for simulated and experimental data of Tax-periods with equal-sized 10 bins (7 hours each) to evaluate the closeness of fit between the simulation and experimental data. In fitting, all the parameters except for k_{bind} and k_{unbind} were kept fixed to the reference value (Table S1). A brute-force computation of closeness was performed on a uniform 16×16 grid of $(k_{\text{bind}}, k_{\text{unbind}})$, where the ranges of k_{bind} and k_{unbind} are set to [0.0005,0.04] and [0.005,0.04], respectively. The ranking of closeness (ranking 1-256) is presented in Fig. S3D.

Note S3. Agent-based simulation of cell population dynamics

The agent-based model (ABM) simulating the population dynamics of MT-1 cells transfected with shRNA as a Poisson (birth-death) process with a stage transition as described in Fig. S5A was constructed based on the individual-based Gillespie algorithm: an extension of the conventional Gillespie algorithm² which allows one to include non-exponentially distributed waiting-times of event occurrences as a non-homogeneous Poisson process⁴. Possible events include cell division, death, and stage transitions among three cell types. The ABM we constructed describes cell division and death events as a Poisson process with the growth rate parameter $g = 0.68$ for both Tax-negative cells expressing high levels of anti-apoptotic genes ($T_{\text{off}}A_{\text{high}}$ cells) and Tax-negative anti-apoptotic gene expression low ($T_{\text{off}}A_{\text{low}}$) cells, and the growth rate parameter $g_{\text{on}} = 0.54$ for Tax-expressing (T_{on}) cells. The lower value for the g_{on} parameter reflects our experimental observation that the transition to G2/M occurs only 80% as quickly in T_{on} cells (see Fig. 4). We used the death rate parameter $\delta = 0.25$ for $T_{\text{off}}A_{\text{low}}$ cells. The stage transition of Tax negative cells from anti-apoptotic gene expression high to low is also described by a Poisson process with rate parameters $p = 0.02$ and $p^* = 0.40$ for shNC and shTax4 cells, respectively. Note that the parameters governing the rates of other stage transitions (i.e. the transition from T_{on} to T_{off} and vice versa) need to be determined based on sampling from experimental (or previously simulated) Tax-period/Tax-interval data, which would be appropriately fitted with non-exponential distributions. In fact, our ABM allows us to include sampling of arbitrary waiting-times (Tax-periods and intervals) from experimental (or previously simulated) data. We summarize the parameter values in Table S2.

To simulate and reproduce the dynamics of shRNA-transduced cells growing in a mixture with untransduced cells, we need also to consider the growth rate of the untransduced cells. Untransduced MT-1 cells would show the same population dynamics as shNC-transduced

cells in our ABM. Numerical simulation results from our ABM exhibit transient behaviors until day 3 due to demographic stochasticity when the number of cells is small (i.e., around 10 cells). However, following this transient phase, a steady state phase is reached in which our numerical results are quite stable. Hence we started our ABM after day 3 to avoid possible artifacts due to demographic stochasticity in a transient phase. We fixed the initial number of MT-1 cells with and without shRNA at day 3 at 104 and 63, respectively. (The corresponding expected numbers of cells at day 0 are 12 and 11, respectively.) The initial conditions of ABM simulation were set as follows. For both shNC and shTax4 cells, less than 10% of the total cell population are T_{on} . However, we assumed that large and small fractions of the total cell population consist of $T_{off}A_{high}$ for shNC and shTax4 cells (78% and 2%), respectively. This is because we needed to consider the effect of Tax knockdown on the expression of anti-apoptotic genes. We also confirmed that the choice of these values is not qualitatively essential in a steady state phase (results not shown). ABM simulations for mixtures of MT-1 cells with and without shRNA were performed until day 21. Simulation results with Tax-period sampling from experimental and simulated values for shNC and shTax4 cells are shown in Fig. 5 and Fig. S6, respectively.

Since the total number of MT-1 cells without shRNA reaches 10^4 around day 10, the propensities of cell division, death and transition rarely change. In other words, the propensities required to determine the relative occurrence of an event to be selected in the Gillespie algorithm remain almost constant for a period of computational time. This allows us to skip updating the propensities, which efficiently reduces computational time. Hence we adopted an approximation algorithm to update propensities only once every 10000 events if the total cell number exceeds 10^4 (after day 10), and confirmed that this approximation does not affect population dynamics (results not shown). The ABM is coded and implemented by statistical software R. (The source code is available upon request.)

Note S4. Survival probability for subpopulations

We performed a tracking simulation for the shNC population to investigate the difference in survival time among subpopulations. Note that in this simulation, subpopulations are defined as Tax-experienced (cells in which Tax has already been expressed at least once) or Tax-naïve (cells in which Tax has never been expressed). Let a denote the age of a daughter cell, which corresponds to the time since a mother cell divided. Since we were calculating survival probabilities, we only needed to track the survival of one cohort: a subgroup of cells with the same age a . In the tracking simulation, we recorded the times of cell death and the statuses of all cells, and produced probability density distributions $f_i(a)$ for the survival time of the Tax experienced ($i = e$) and naïve ($i = n$) subpopulations. Survival probabilities are then derived as $F_i(a) = 1 - \int_0^a f_i(s)ds^5$. In this tracking simulation, we used the same parameter values that we employed in the ABM simulations (Table S2 and Note S3), except for the growth rate ($g = 0$ for the tracking simulation because tracking requires only one cohort) and the initial number of a target cohort which is set to 10000. Survival probability curves of the two subpopulations are shown in Fig. 5G: about 90% of Tax non-experienced cells die within two weeks, but Tax-experienced cells survive much longer, suggesting that Tax expression can make a significant contribution to cell survival.

Supplementary References

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- 5 Aalen, O., Borgan, O. & Gjessing, H. *Survival and event history analysis: a process point of view*. (Springer Science & Business Media, 2008).