Chromatin accessibility at the HIV LTR promoter sets a threshold for NF-κB mediated viral gene expression

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Supplementary Information

SI Methods

Western blotting. J-Lat 6.3 cells were treated with DOX at the indicated concentrations for 4 days. Cells were pelleted and resuspended in lysis buffer containing IGEPAL (1%; Sigma), sodium dodecyl sulfate (SDS) (0.1%), phenylmethanesulfonylfluoride (0.1 mg/mL; Sigma), aprotinin (0.03 mg/mL; Sigma), and sodium orthovanadate (1mM; Sigma) in PBS. Lysate protein concentrations were quantified by BCA Protein Assay Kit (Pierce) according to manufacturer's instructions. 10 μg of protein from each lysate were electrophoretically separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were probed with anti-NF-κB p65 (C-20) primary antibody (Santa Cruz Biotechnology, sc-372) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Pierce, 31460), developed with ECL Plus (Pierce), and analyzed on the Versadoc 4000 imager (Bio-Rad).

Imaging protocol and analysis. Clone 6.3 and B6 infected with iRelA were treated with 0, 30, and 300 ng/ml DOX for 4 days. One million cells per condition were washed twice with PBS, fixed with 4% Formaldehyde (F79-1, Fisher Scientific), and applied to the well of a glass bottom 6-well plate (P06G-1.0-20, MatTek Corp., Ashland,MA) treated with 0.01 mg/ml Poly-L-Lysine Solution (SDP8920A, Fisher Scientific) to promote cell adhesion. After 30 minutes, wells were washed twice with PBS and stored in 70% Ethanol at 4° C overnight before imaging. Wells were rehydrated twice for 15 minutes with PBS. Nuclei were stained with 0.0025 mg/ml DAPI (D1306, Invitrogen Corp. Carlsbad,CA) for 10 minutes and washed with PBS, and treated with an anti-bleach solution consisting of 10 mM Tris pH 8.0, 2xSSC, 0.4% glucose with 0.037 mg/ml glucose oxidase (G2133, Sigma-Aldrich Corp.) and 0.05 mg/ml catalase (C3515,Sigma-Aldrich Corp.) prior to applying the cover slip. Wells were imaged using an automated imaging system (ImageExpress Micro, Molecular Devices Inc.) with a 40X objective. Briefly, a 20x20 grid of independent fields was established in software per well and fields were imaged with hardware autofocus and a standard FITC, TexasRed, DAPI filter set. Exposure times were determined empirically to maximize signal to noise and prevent camera saturation. CellProfiler (Carpenter Genome Biology 2006) with a custom pipeline was used to segment cells and nuclei and to determine total and localized GFP and mCherry. The MeasureImageQuality module was used to reject significantly blurry fields using an empirically determined Focus Score of 0.004.

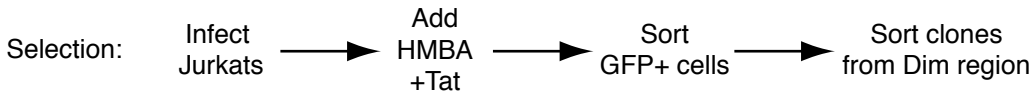
RNA Extraction and Quantification of Viral transcripts. The indicated cell lines indicated were stimulated for 24 hours at different concentrations of DOX and treated with Trizol (Invitrogen) to extract total cellular RNA. Viral and cellular mRNA were quantified using the Quantitect SYBR Green RT-PCR kit (Qiagen) and a Bio-Rad iCycler (iQ5). The following primers were used to quantify Tat transcripts: Tat-F (5'-GCATCCAGGAAGTCAGCCT-3') and Tat-R (5'-CTCCGCTTCTTCCTGCCATAG-3'). B-Actin was used as a control and quantified using the primers, β-Actin-F (5'-ACCTGACTGACTACCTCATGAAGATCCTCACCGA-3')
and B-Actin-R (5'-GGAGCTGGAAGCAGCCGTGGCCATCTCTTGCTCGAA-3'). qPCR was performed in triplicate and the error bars represent standard deviations from the mean.

Chromatin immunoprecipitation. Upstate EZ ChIP Kit Reagents (Upstate) and protocols were used with minor modifications. 10 million cells were fixed in 1% formaldehyde for 10 minutes, and the unreacted formaldehyde was quenched using 125 mM glycine for 10 minutes on ice. After extensive PBS washing, the cells were lysed with 1 mL of 1% SDS lysis buffer in the presence of a protease inhibitor cocktail. For the Ser5P-CTD of RNAPII ChIP, a phosphatase inhibitor cocktail was also added during the immunoprecipitation step. The cells were sonicated either using the Branson Sonifier 450 (Settings: 25 cycles at a power output of 2.5 and duty cycle of 25%. Each cycle consisted of 15 pulses followed by incubation on ice for at least 1 minute) or the Misonix Sonifier 3000 (Settings: 7 cycles at a power output of 4. Sonication was done for 30 sec in each cycle with 1 sec ON/OFF pulses, followed by incubation on ice for at least 1 min). DNA gel electrophoresis was used to verify that the sheared DNA fragments were within 0.1-1 kb. For the Ser5P-CTD of RNAPII ChIP, anti-mouse IgM agarose beads (Sigma) were used instead of Protein A or G beads that were used for the other ChIPs. The anti-mouse IgM agarose beads were washed extensively with RIPA buffer, then blocked with salmon sperm DNA and yeast tRNA. For the Ser5P-CTD of RNAPII ChIP, the beads were incubated with the antibody-chromatin complex for 5 hours at 4^{o}C. For all other ChIPs, the beads were incubated with the antibody-chromatin complex for 2 hours at 4^{o}C. The precipitated DNA was quantified using quantitative PCR (BioRad iCycler, iQ5) using the EpiQ Chromatin SYBR Supermix. qPCR was performed in triplicate and melt curves were run to ensure product specificity. The following antibodies were used in the immunoprecipitation step: anti-RNAPII (Millipore, Catalog # 05-623), anti-Ser5P CTD RNAPII (Covance, Catalog # MMS-134R), anti-histone H3 (Abcam, Catalog # ab1791), anti-acetyl histone H3 (Millipore, Catallog # 06-599), anti-histone H3K9me3 (Abcam, Catalog # ab8898). The following primers were used for the ChIP for AcH3, H3K9me3, and total H3 and the nuclease sensitivity assay: 5'-GGACTTTCCGCTGGGGACTTTCCAGGG-3' (forward) and 5'-GCGCGCTTCAGCAAGCCGAGTCCTGCGTCGAG-3' (reverse). Alternate primers were used for the ChIP for ChIP for RNAPII and phospho-Ser5-RNAPII: 5'-GACTTTCCGCTGGGGACTTTC-3' (forward) and 5′-GTGGGTTCCCTAGTTAGCCA-3′ (reverse).

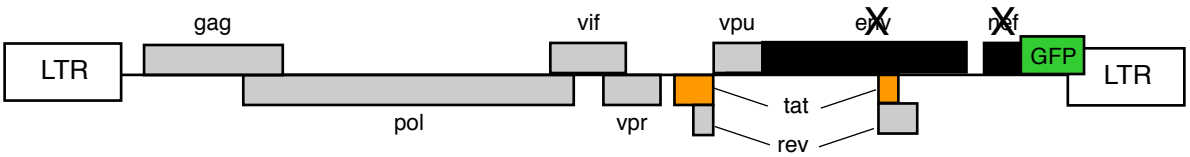
SI Figures

Fig. S1. Schematic of the HIV vector model and a brief description of the selection procedure for clones infected with a single copy of the virus for (A) LTR-GFP-IRES-Tat (LGIT) clones (21) and (B) J-Lat clones as established by Jordan et al. (12). Please refer to the published references for full details of the selection procedures.

A LGIT HIV vector and clone selection



B J-Lat HIV vector and clone selection



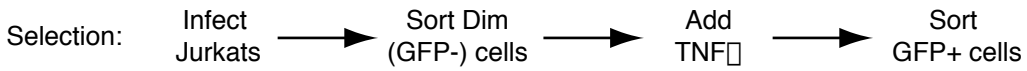


Fig S2. Clones display differential sensitivity to TNFα dose. Clones were treated with the indicated dose of TNFα for 24 hours and HIV gene expression was evaluated by flow cytometry. Data are presented as mean ± standard deviation as estimated by bootstrapping.

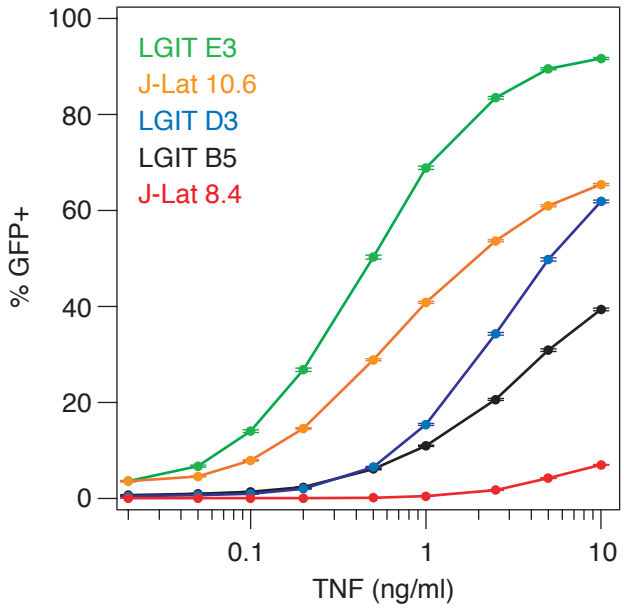

100
80
60
40
20
0
% GFP+
LGIT E3
J-Lat 10.6
LGIT D3
LGIT B5
J-Lat 8.4
0.1
1
10
TNF (ng/ml)

Fig. S3. Characterization of the iRelA vector. (A) Quantification of RelA overexpression in clone 6.3 in response to increasing DOX dosage as measured by immunoblot. Western blot was performed in triplicate and mCherry-RelA was normalized to the endogenous RelA level. Data are presented as the mean ± standard deviation. (B) For the same conditions in (A), mean mCherry fluorescence was quantified by flow cytometry and plotted against DOX dosage. Data points represent mCherry RFU mean of ~10,000 cells. (C) Histograms of mCherry fluorescence (top panels) and GFP fluorescence (middle panels) in Jurkat cells infected with WT LGIT (left) or LGIT with κB-deletions in the LTR promoter (right) and co-infected with the RelA vector in the absence (black) and presence (red) of DOX. κB-deleted LGIT vector shows negligible increase in GFP when stimulated with DOX (middle left). (Bottom panels) κB-deletion mutants also show loss of response to TNFα (green) but retain activation by TSA (blue) as compare to WT. (D) Time course of % GFP positive cells at the indicated DOX treatment concentration for LGIT B6. (E) Nuclear mCherry fluorescence as a fraction of total mCherry quantified for 650 J-Lat 6.3 cells and 1250 LGIT B6 cells from single cell microscopy data. (F) Flow cytometry histograms of mCherry fluorescence for clone B6 infected with iRelA in response to increasing DOX dosages: red (uninfected), blue (0 μg/ml), green (10 μg/ml), yellow (30 μg/ml), turquoise (100 μg/ml), and pink (300 μg/ml). (G) Density plots of GFP versus mCherry for all dosages of DOX combined for clone B6. Measurements were divided into 256 mCherry bins and % GFP positive fraction was calculated for each bin.

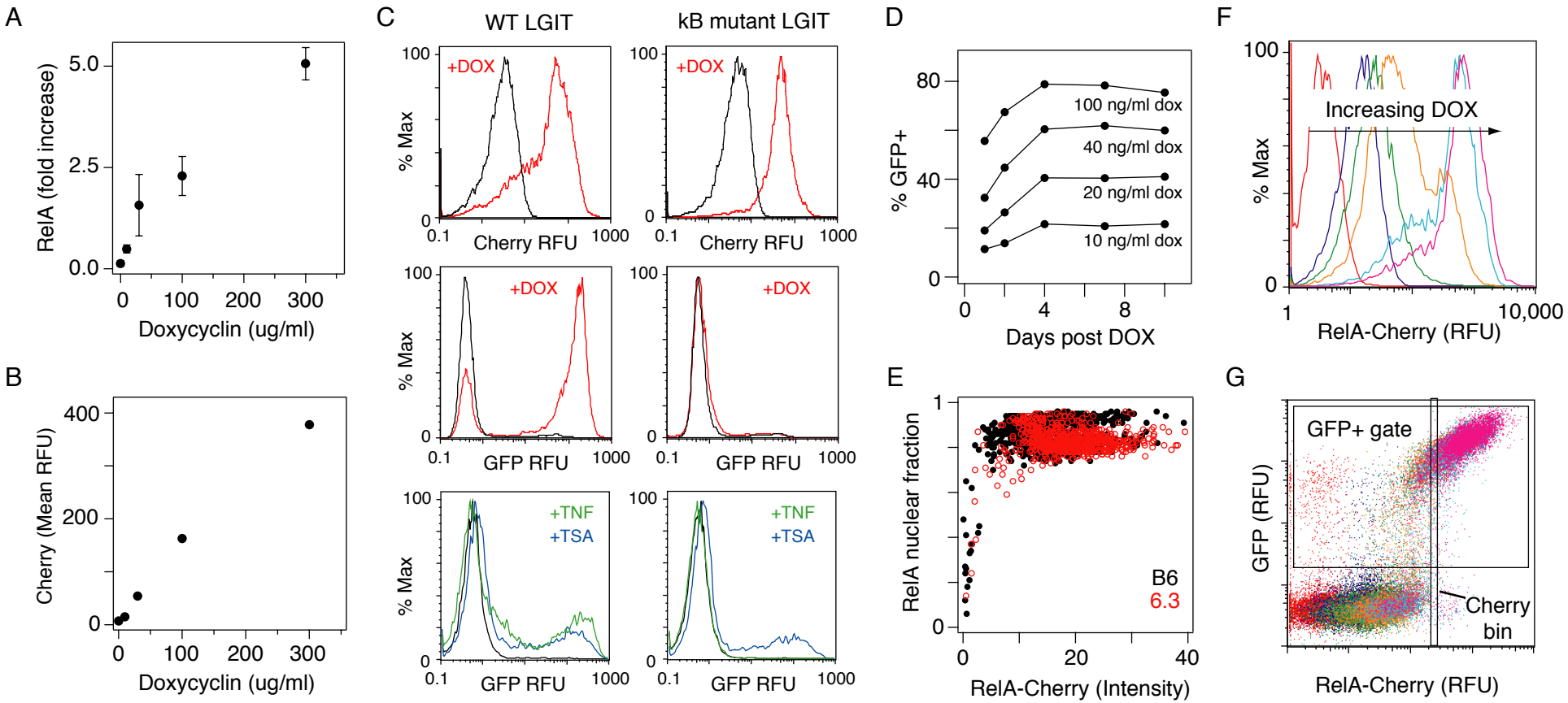

A
RelA (fold increase)
5.0
2.5
0.0
0
100
200
300
Doxycyclin (ug/ml)
B
Cherry (Mean RFU)
400
200
0
0
100
200
300
Doxycyclin (ug/ml)
C
WT LGIT
kB mutant LGIT
+DOX
% Max
100
0
0.1
1000
Cherry RFU
GFP RFU
+TNF
+TSA
D
% GFP+
80
40
0
0
4
8
Days post DOX
100 ng/ml dox
40 ng/ml dox
20 ng/ml dox
10 ng/ml dox
E
RelA nuclear fraction
1
0
0
20
40
RelA-Cherry (Intensity)
B6
6.3
F
100
0
1
10,000
% Max
Increasing DOX
RelA-Cherry (RFU)
G
GFP (RFU)
GFP+ gate
Cherry
bin
RelA-Cherry (RFU)

Fig. S4. Least-squares fits of gene activation functions for the panel of clones. Flow cytometry data obtained from stimulating iRelA clones at different DOX levels were pooled together and binned into 256 GFP and mCherry channels. Each blue circle quantifies the metrics shown on the *x*- and *y*-axis, obtained from the mean %GFP+ and mCherry expression within each channel. The black line shows the best fit obtained from least-squares fitting after log transforming the Hill equation in to a linear equation. The clone IDs and the goodness of fits for each clone are indicated. Parameter estimates from the best-fit line were used to compute the induction threshold and activation coefficient shown in Fig. 2E-F.

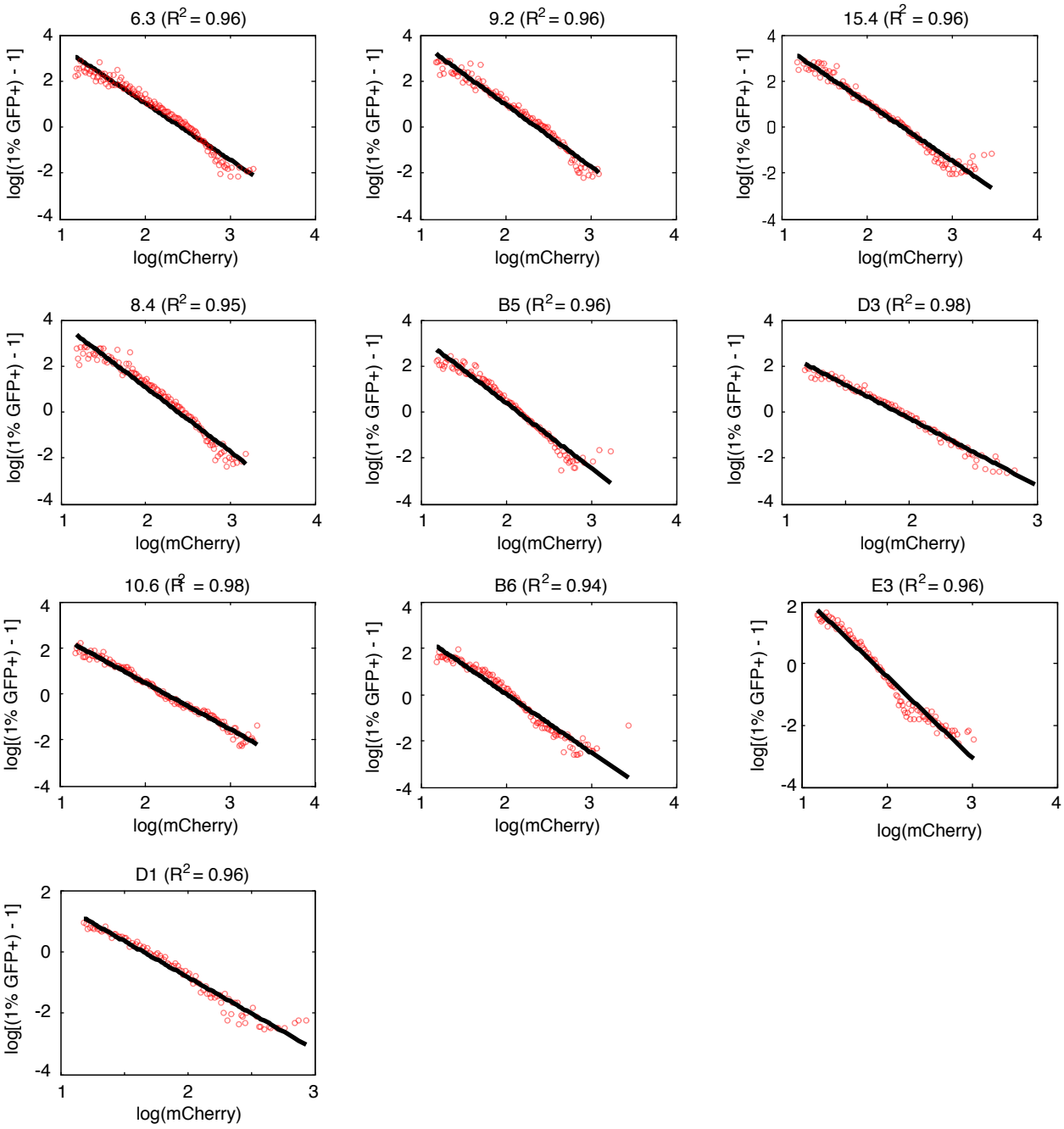

6.3 (R^2 = 0.96)
9.2 (R^2 = 0.96)
15.4 (R^2 = 0.96)
8.4 (R^2 = 0.95)
B5 (R^2 = 0.96)
D3 (R^2 = 0.98)
10.6 (R^2 = 0.98)
B6 (R^2 = 0.94)
E3 (R^2 = 0.96)
D1 (R^2 = 0.96)
log[(1% GFP+) - 1]
log(mCherry)

Fig. S5. Analysis of the variation in induction threshold and K values. (A) Values of RelA induction threshold were calculated for different fractions of GFP+ population and compared to the chosen induction fraction of 5%. GFP+ fractions: 1% (black), 3% (blue), and 8% (red). (B) Values for the fit parameter K (half-max induction). Error bars represent standard deviations and were calculated by bootstrapping. (C) Comparison of K and 5% induction threshold.

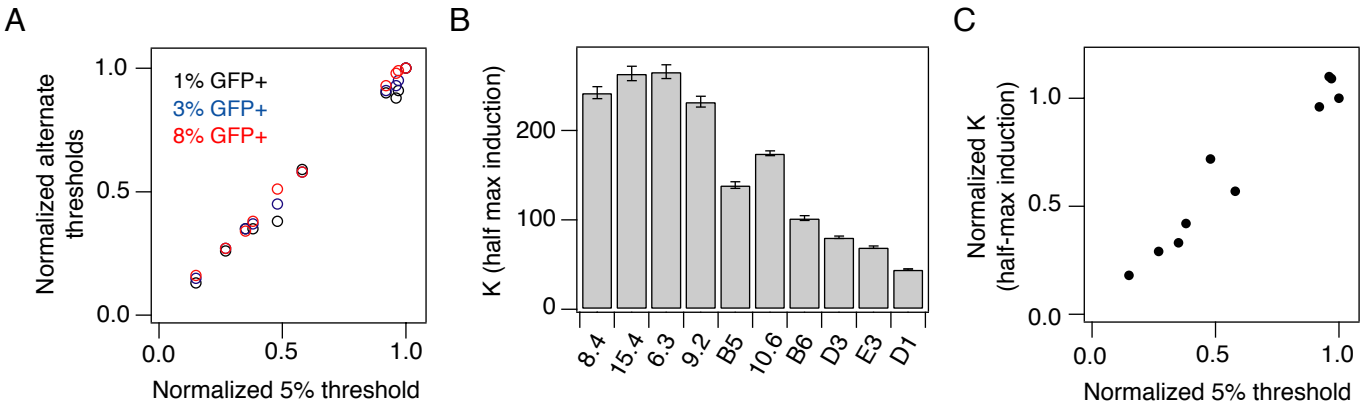

A
1.0
0.5
0.0
Normalized alternate thresholds
1% GFP+
3% GFP+
8% GFP+
0.0
0.5
1.0
Normalized 5% threshold
B
K (half max induction)
200
100
0
8.4
15.4
6.3
9.2
B5
10.6
B6
D3
E3
D1
C
Normalized K (half-max induction)
1.0
0.5
0.0
0.0
0.5
1.0
Normalized 5% threshold

Fig. S6. Tat transcript levels are undetectable until threshold is reached. RNA was isolated from clones 15.4, 8.4 and E3 in the basal state or in iRelA-infected cell lines after treatment with DOX for 24 hours at the indicated concentrations and Tat transcripts were measured by RT-PCR. β-actin transcription was used as a normalization control. Data are presented as mean ± standard deviation. N.D. indicates non detectable. Corresponding fraction of GFP+ cells for each condition is indicated above each bar. Basal Tat transcription was also measured in LGIT B5, LGIT D3, and J-Lat 10.6 and found to be N.D.

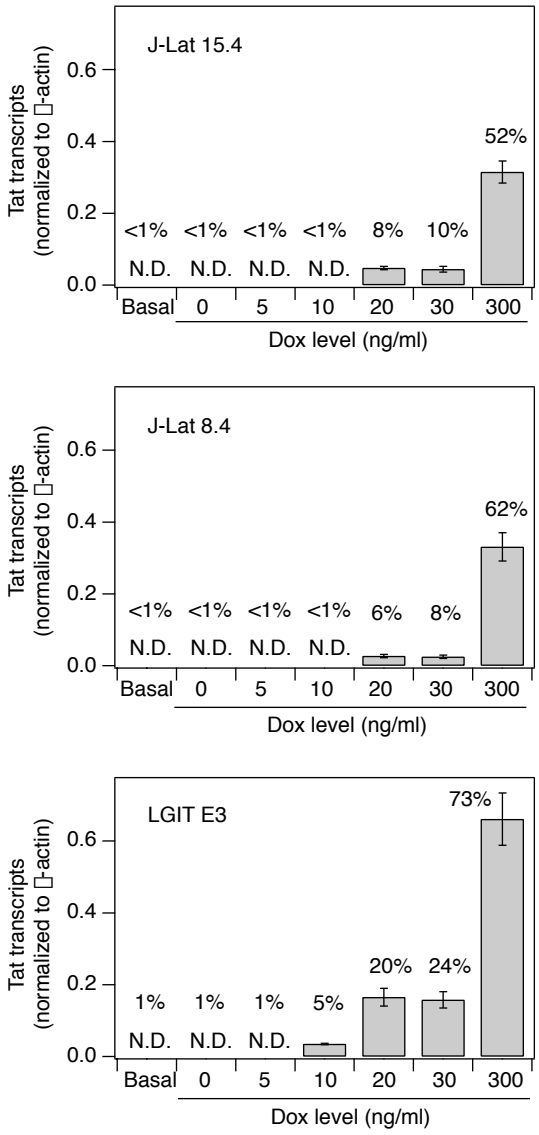

J-Lat 15.4
Tat transcripts (normalized to β-actin)
0.0
0.2
0.4
0.6
<1% <1% <1% <1% 8% 10%
52%
N.D. N.D. N.D. N.D.
Basal 0 5 10 20 30 300
Dox level (ng/ml)
J-Lat 8.4
Tat transcripts (normalized to β-actin)
0.0
0.2
0.4
0.6
<1% <1% <1% <1% 6% 8%
62%
N.D. N.D. N.D. N.D.
Basal 0 5 10 20 30 300
Dox level (ng/ml)
LGIT E3
Tat transcripts (normalized to β-actin)
0.0
0.2
0.4
0.6
1% 1% 1% 5% 20% 24%
73%
N.D. N.D. N.D.
Basal 0 5 10 20 30 300
Dox level (ng/ml)

Fig. S7. Total H3, H3K9me3 and acetylated H3 bound to the HIV promoter in unstimulated clones was measured by chromatin immunoprecipitation and correlated to the activation coefficient that describes the gene activation function. Quantitative PCR was performed in triplicate and normalized to an input control. Data are presented as mean ± standard deviation.

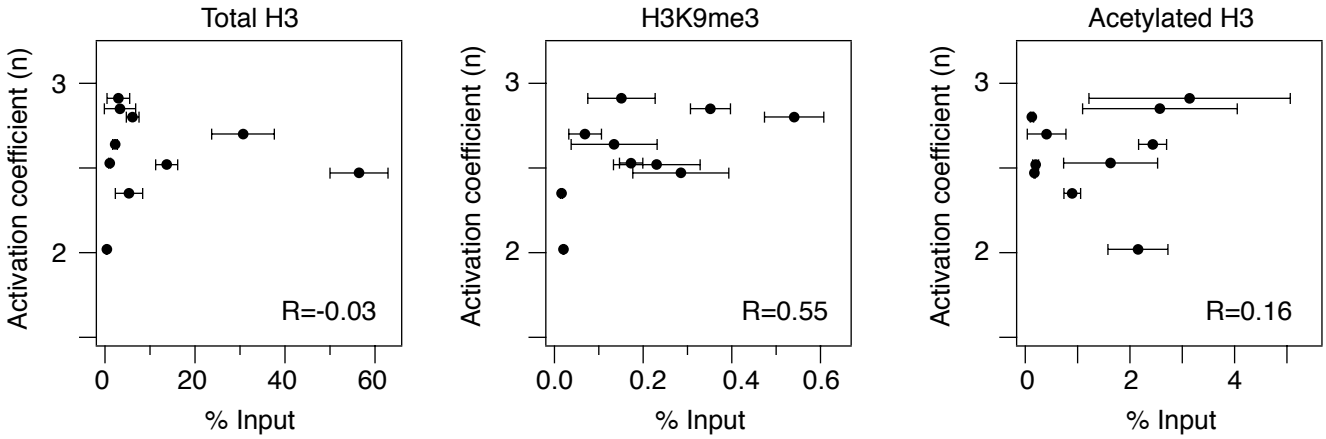

Total H3
Activation coefficient (n)
3
2
R=-0.03
0
20
40
60
% Input
H3K9me3
Activation coefficient (n)
3
2
R=0.55
0.0
0.2
0.4
0.6
% Input
Acetylated H3
Activation coefficient (n)
3
2
R=0.16
0
2
4
% Input

Fig. S8. Total RNAPII and RNAPII phosphorylated at Ser5 (RNAPII-pSer5) are present at low levels at the HIV LTR promoter of inactive clones. Chromatin immunoprecipitation (ChIP) for (A) total RNAPII and (C) RNAPII-pSer5 bound to the HIV promoter in unstimulated clones. ChIP for (B) RNAPII and (D) RNAPII-phospho-Ser5 was compared between clone E3 and a polyclonal population of Jurkat cells singly-infected with an LGIT vector and sorted for GFP-expressing cells. Quantitative PCR was performed in triplicate and normalized to a GAPDH control. Data are presented as the mean ± standard deviation.

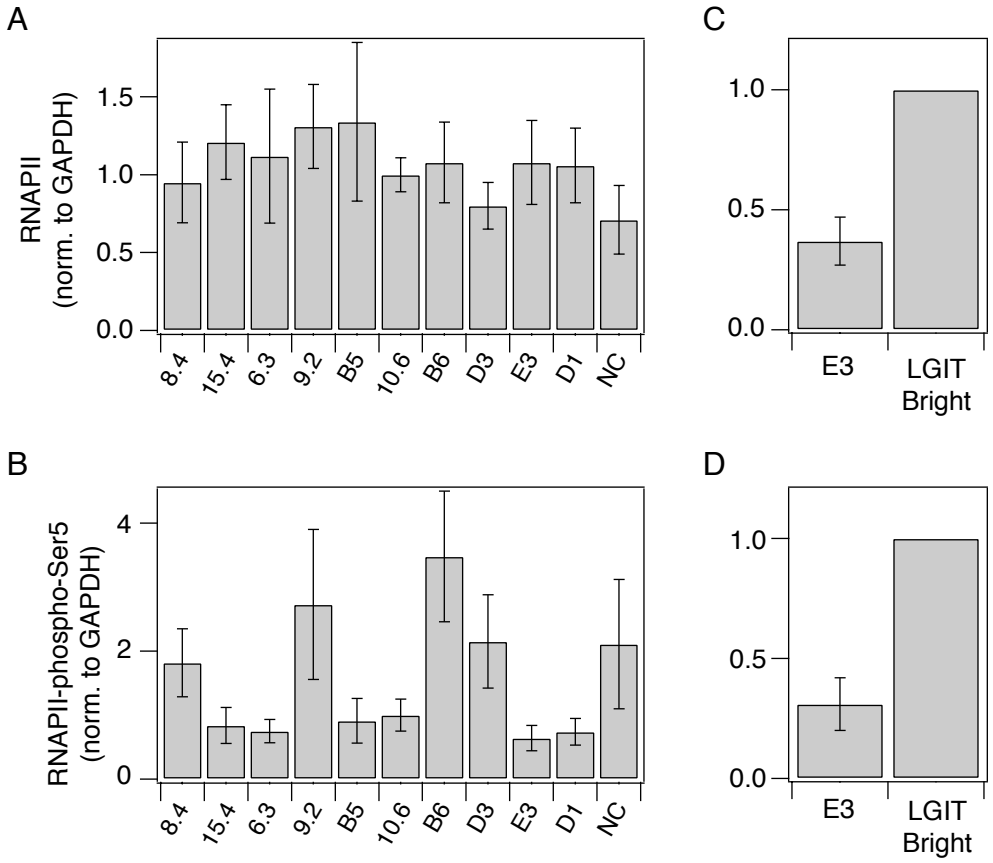

A
RNAPII
(norm. to GAPDH)
1.5
1.0
0.5
0.0
8.4
15.4
6.3
9.2
B5
10.6
B6
D3
E3
D1
NC
C
1.0
0.5
0.0
E3
LGIT
Bright
B
RNAPII-phospho-Ser5
(norm. to GAPDH)
4
2
0
8.4
15.4
6.3
9.2
B5
10.6
B6
D3
E3
D1
NC
D
1.0
0.5
0.0
E3
LGIT
Bright

Fig. S9. (A) Heterochromatin fraction for clone 15.4 was quantified with a DNAse I sensitivity assay following stimulation with TSA (40 or 400 nM) for 4 hours or with 5-aza-dC (5 μM) for 48 hours. Quantitative PCR was performed in triplicate and normalized to the HBB reference gene. Relative heterochromatin fraction was calculated by normalizing clone 15.4+drugs to the unstimulated 15.4 control. (B) Threshold and (C) activation coefficient extracted from fitting 15.4+iRelA gene activation functions in the presence of TSA and 5-aza-dC for the conditions in (A). Data are presented as the mean ± standard deviation. Standard deviation error bars for the threshold and activation coefficients were calculated by bootstrapping. Changes are labeled as significant (*) if $p < 0.05$.

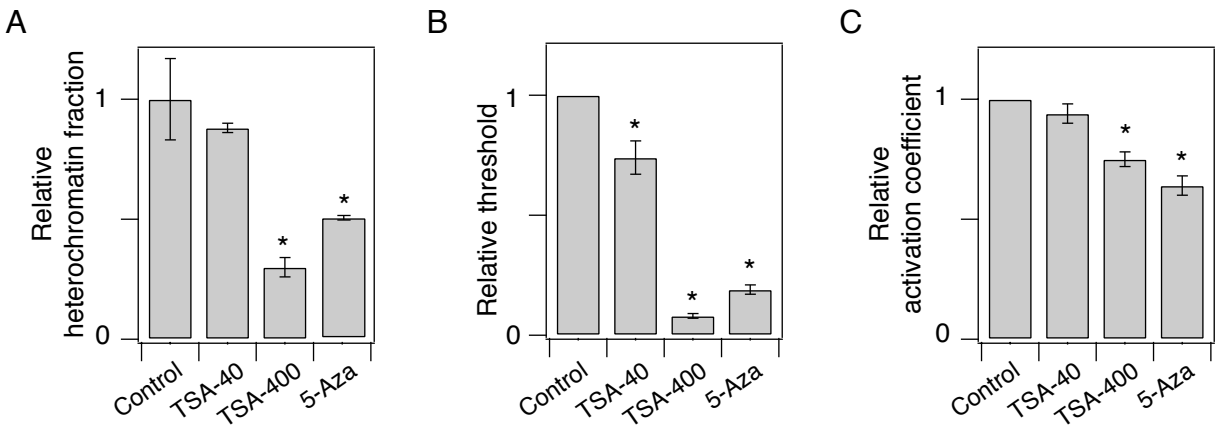

A
Relative heterochromatin fraction
1
0
*
*
Control
TSA-40
TSA-400
5-Aza
B
Relative threshold
1
0
*
*
*
Control
TSA-40
TSA-400
5-Aza
C
Relative activation coefficient
1
0
*
*
Control
TSA-40
TSA-400
5-Aza

Fig. S10. (A) Combined flow cytometry data for clone 8.4 expressing iRelA in response to a range of DOX concentrations and simultaneous stimulation with 40 nM (light blue), 400 nM TSA (dark blue) for 24 hours, with 5 μM 5-aza-dC (red) for 48 hours or without stimulation for 24 hours (black) and 48 hours (light gray). (B) Heterochromatin fraction was quantified with a DNAse I sensitivity assay following stimulation with TSA (40 or 400 nM) for 4 hours or with 5-aza-dC (5 μM) for 48 hours. Quantitative PCR was performed in triplicate and normalized to the HBB reference gene. Relative heterochromatin fraction was calculated as described in Fig. S9. (C) Threshold and (D) activation coefficient extracted from fitting 8.4+iRelA gene activation functions in the presence of TSA and 5-aza-dC for the conditions in (A). (E-H) Same conditions as described in (A-D) but repeated in clone E3. Note that 400 nM TSA induced a significant activation of clone E3 at basal RelA levels and so it was not possible to accurately fit a gene activation function. Data are presented as the mean ± standard deviation. Error bars for threshold and activation coefficient are calculated by bootstrapping. Changes are labeled as significant (*) if $p < 0.05$.

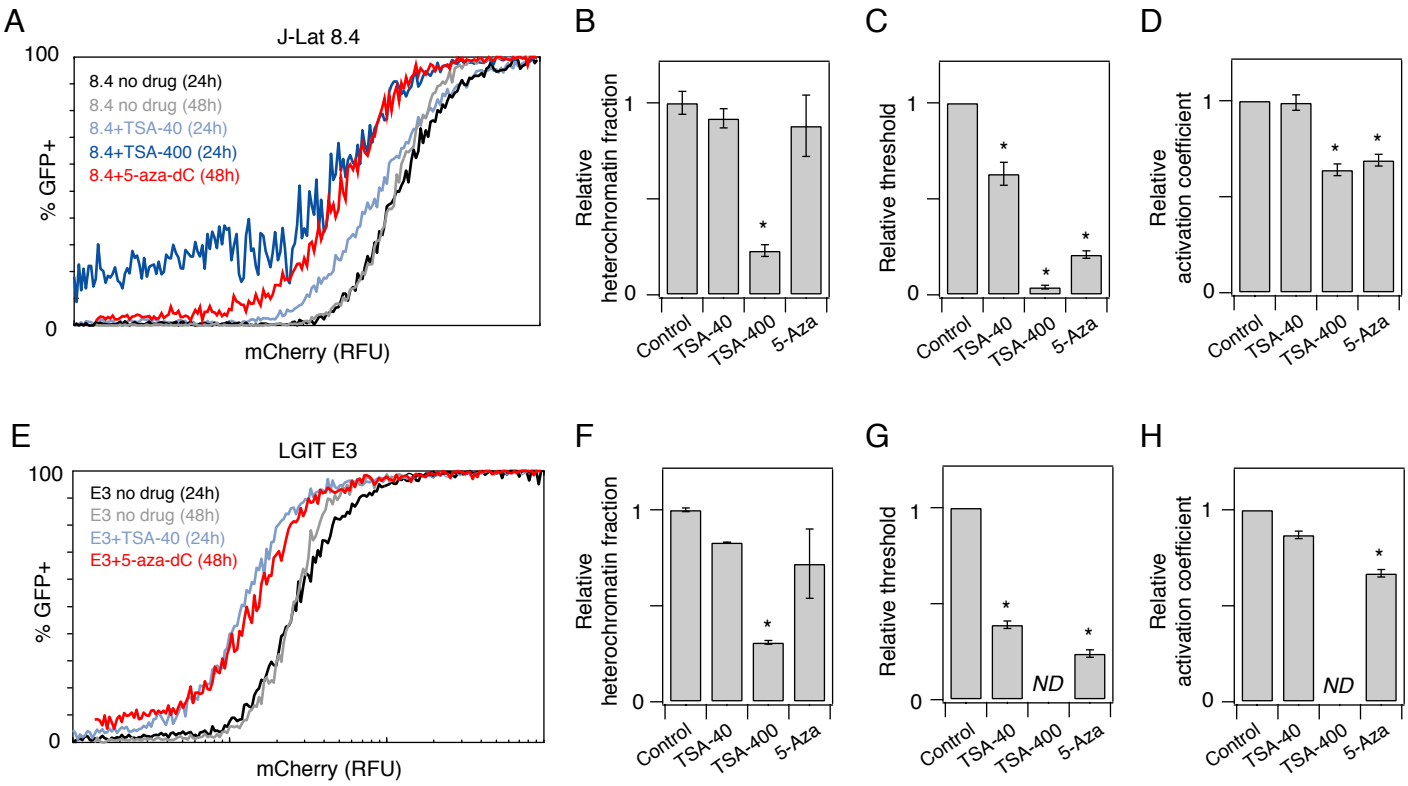

A
J-Lat 8.4
100
0
% GFP+
8.4 no drug (24h)
8.4 no drug (48h)
8.4+TSA-40 (24h)
8.4+TSA-400 (24h)
8.4+5-aza-dC (48h)
mCherry (RFU)
B
Relative heterochromatin fraction
1
0
Control
TSA-40
TSA-400
5-Aza
C
Relative threshold
D
Relative activation coefficient
E
LGIT E3
E3 no drug (24h)
E3 no drug (48h)
E3+TSA-40 (24h)
E3+5-aza-dC (48h)
F
G
ND
H

Fig. S11. Threshold function predicts drug synergy for clone 8.4. (A-B) Predicted (bars) and observed (dots) GFP+ cells following stimulation with TSA+TNFα or 5-aza-dC+TNFα for a (A) gene activation function or (B) Bliss independence model of drug activation. Experiments were performed in biological triplicate. Data are presented as the mean ± standard deviation. Methods for prediction and error analysis are described in Materials and Methods.

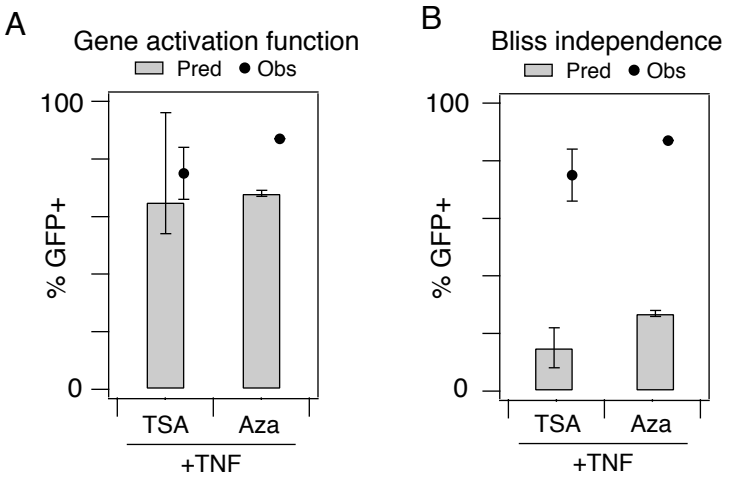
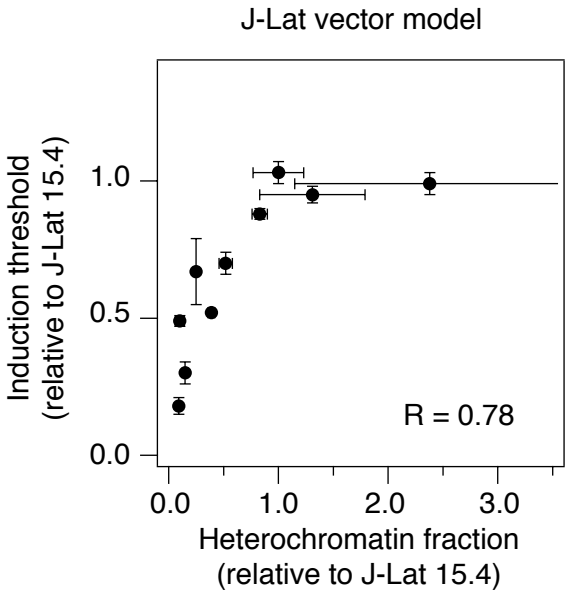
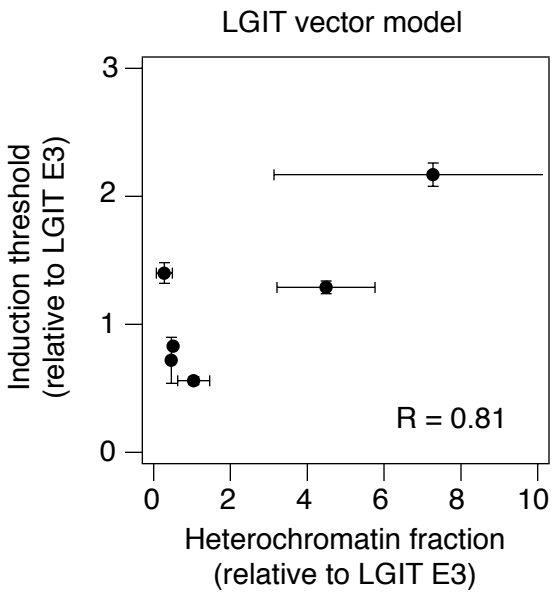

A
Gene activation function
Pred
Obs
100
0
% GFP+
TSA
Aza
+TNF
B
Bliss independence
Pred
Obs
100
0
% GFP+
TSA
Aza
+TNF

Fig. S12. Correlation between heterochromatin fraction and RelA induction threshold is independent of HIV vector type. Data relating the induction threshold and measurements of heterochromatin fraction at basal level and in the presence of small molecule inhibitors were combined from Fig. 2F, 3A, 5A-B, and Fig. S10. Data for LGIT and J-Lat clones were considered separately and normalized to a single clone (15.4 for J-Lat and E3 for LGIT) in order to combine experiments. The correlation between heterochromatin fraction and threshold is significant for both vectors (J-Lat: Pearson R = 0.78; $p = 0.01$; LGIT: Pearson R = 0.81; $p = 0.05$).


J-Lat vector model
Induction threshold
(relative to J-Lat 15.4)
1.0
0.5
0.0
0.0
1.0
2.0
3.0
Heterochromatin fraction
(relative to J-Lat 15.4)
R = 0.78


LGIT vector model
Induction threshold
(relative to LGIT E3)
3
2
1
0
0
2
4
6
8
10
Heterochromatin fraction
(relative to LGIT E3)
R = 0.81

Supplementary Tables

Table S1: Genomic locations of the integrated provirus for the clones used in this study

					Repeats		Proximity to CpG Island		
Clone ID	**Chromosome**	**Location**	**Origin**	**Gene**	**Integrated in**	**Within 200 bp**	**Left**	**Right**	**Expression[1]**
LGIT B5	12	51,557,735	–	TFCP2	SINE/Alu	LINE/L1	80,183	8,945	Below
LGIT B6	3	185,636,060	–	TRA2B	None	LTR /ERV1	91,914	18,997	Above
LGIT D1[2]	--	--	--	--	--	--	--	--	--
LGIT D3	17	5,254,712	+	RABEP1	None	SINE/Alu	68,839	67,795	Median
LGIT E3	8	61,524,041	+	RAB2A	None	LINE/L2	94,041	39,979	Median
J-Lat 6.3	19	46,884,266	+	PPP5C	LINE/L1	LINE/L1	33,588	31,536	Median
J-Lat 8.4	1	78,412,065	+	FUBP1	None	AT-rich	57,392	32,239	Above
J-Lat 9.2	19	46,884,266	+	PPP5C	LINE/L1	LINE/L1	33,588	31,536	Median
J-Lat 10.6	9	139,362,925	+	SEC16A	None	None	1,782	4,261	Median
J-Lat 15.4	19	34,932,169	–	UBA2	SINE/Alu	LINE/L1	12,199	39,881	Median

1. Refers to the expression level of the gene in the Jurkat cell line relative to the level of gene expression for all other cell lines in the NCI-60 cancer cell panel.
2. Integration position for clone D1 was not successfully characterized.