Supplementary Online Material for Morris et al. "siRNA-induced transcriptional gene silencing in human cells."

Materials and Methods

Lentiviral vector and siRNAs.

FIV vector pVE-GFPwP was prepared as described (*1*). Small interfering RNAs were constructed using Ambion (Silencer™). siRNA target sites:

EF1A promoter specific EF52 5'-AAG GTG GCG CGG GGT AAA CTG-3'

GFP mRNA specific 5'-AAC GAT GCC ACC TAC GGC AAG-3'

control CCR5 specific 5'-AAT TCT TTG GCC TGA ATA ATT-3'

control HIV-1 polymerase specific 5'-AAG CCA TGC ATG GAC AAG TAG-3'.

Cell culture, lentiviral transduction and siRNA transfection.

293FT human fibroblast cells ($1x10^5$) were transduced with lentivirus in duplicate (MOI=2.5) in the presence of 8μg/ml Polybrene and transfected 24hrs later with siRNA (10nM, 3μl of Transfast™ [Promega] incubated for 15min at room temperature and added directly to previously transduced cultures). After 48hrs (72 hours post-vector transduction) the cells were collected and RNA or DNA extracted (Qiagen RNeasy and DNeasy kits). When indicated, Trichostatin A (0.05mM) (*2*) and 5-azacytidine (4μM) (*3*) were added to the lentiviral transduced and siRNA transfected cultures daily. In experiments without lentiviral transduction, 293FT cells were transfected with siRNAs (10nM) using either Transfast™ or MPG peptide at a 10:1 charge ratio

as described (*4, 5*). To generate the 293FT-pVE stable transduced cell line in Figure 3C, 293FT cells were transduced (MOI=2.5) and sorted for GFP positive cells by flow cytometry 4 days later. GFP positive cultures were grown for eight weeks prior to siRNA transfection.

RT PCR.

Cellular RNA was isolated with the Qiagen RNeasy kit and 1μg of RNA was DNase treated (~5-10 units/μg of RNA, 2hr) and used as template for the RT reaction (20 μl). RT reaction mix (*6*) contained 1 μl (2pM) of primers. The resultant cDNA (1hr/42°C and 10min/95°C, 50ng) was then used in real-time PCR (described below) or in conventional PCR reactions analyzed by gel electrophoresis. PCR reactions (*6*) for supplementary figure S2 contained 50ng of cDNA. Primer sequences:

GFP 814 5'-GGT GGT GCA GAT GAA CTT CAG GGT C-3'

FIV RRE 14 5'-TTG ATA TGG CAA TTC CTG CAT T-3'

GAPDH 59F 5'-TGG GAT TTC CAT TGA TGA CAA G-3'.

803 5'-AAG TGG GGG GAG GGG TCG GCA-3'

804 5'-GCA CTT ACC TGT GTT CTG GCG GC-3'

808 5'-GCA GTA GTC GCC GTG AAC GTT C-3'

809 5'-AAC GGG TTT GCC GCC AGA AC-3'

775 5'-GGC GCC GTC CAG GCA CCT CGA TTA GTT CT-3'

776 5'-AAC TTC AGG GTC AGC TTG CCG TAG GTG GCA TCG CC-3'

782 5'-TTC GCA ACG GGT TTG CCG CCA GAA C-3'

Real-time PCR.

Real-time PCR used 50ng of sample cDNA, or equivalent amount of non-reverse transcribed sample RNA as a control, following previously established protocols (*7*). Real-time RT-PCR results are reported as fraction of controls based on calculated copies relative to total cell number or input RNA from respective samples. Primer sequences:

GFP: 625F 5'-AGC AAA GAC CCC AAC GAG AA-3', 684R 5'-GGC GGC GGT CAC GAA-3', and 646T (Tet probe) 5'-CGC GAT CAC ATG GTC CTG CTG G-3'

FIV RRE: RRE 13 5'-AGA TAC TTC ATC ATT CCT CCT CTT TTT-3', RRE 14 (described previously), and RRE TET probe 5'-AGG AGA AAT GGT AGG CAA-3'

GAPDH: 60R 5'-TGG CAC CGT CAA GGC TGA GAA CG-3', 59F (described previously), and FAM probe 5'-CCA CCC ATG GCA AAT TCC-3'

EF1A: 1064F 5'-CTG AAC CAT CCA GGC CAA AT-3', 1128R 5'-ATG TGA GCC GTG TGG CAA T-3' and 1085 FAM 5'-AGC GCC GGC TAT GCC CCT GTA TT-3'

Vector integration assay.

The semi-quantitative FIV vector integration assay is based on previously published protocols (*8*), and consists of a nested PCR using an Alu repeat specific primer 637 5'-TCC CAG CTA CTC GGG AG-3' and an FIV LTR specific primer 670 5'-GAG ACT CCT CGA AGT TTC ACA-3'. A second, nested PCR was then performed using FIV primers 669 5'-GGG ACT GTT TAC GAA CAA ATG-3' and 670 (above) in multiple parallel dilutions (10 μl first dilution, 10-

fold). The number of copies per cell was calculated from endpoint dilutions of the second PCR, based on a standard curve established with pVE-GFPwP plasmid. Controls for amplification of non-integrated viral DNA forms consisted of equivalent amounts of input genomic DNA (not amplified in the first PCR step).

Nuclear run on assay.

A total of $2.0x10^6$ 293FT cells were plated, lentivirally transduced and siRNA transfected as described. 48 hours after siRNA transfection, cultures were collected in 1xPBS and lysed in 4ml of NP-40 lysis buffer (10nM Tris.CL, pH 7.4, 10mM NaCl, 3mM $MgCL_2$, 0.5% NP-40) on ice. Nuclei were extracted by centrifugation, washed in NP-40 Lysis buffer and resuspended in 200μl of glycerol storage buffer and frozen in liquid N_2. The nuclear run-on assay was performed on the N_2 frozen nuclear pellets following pre-established protocols (*9*) with slight modifications; namely detection of bound probe with the Ambion BrightStar™ BioDetect™ nonisotopic detection kit instead of radioactive label. Primer sequences for probe amplification:

GFP: #775 and 776

GAPDH: 83 5'-TGG TAT CGT GGA AGG ACT CAT GAC-3' and 84 5'-GCT GAA CGG GAA GCT CAC TGG CAT-3'.

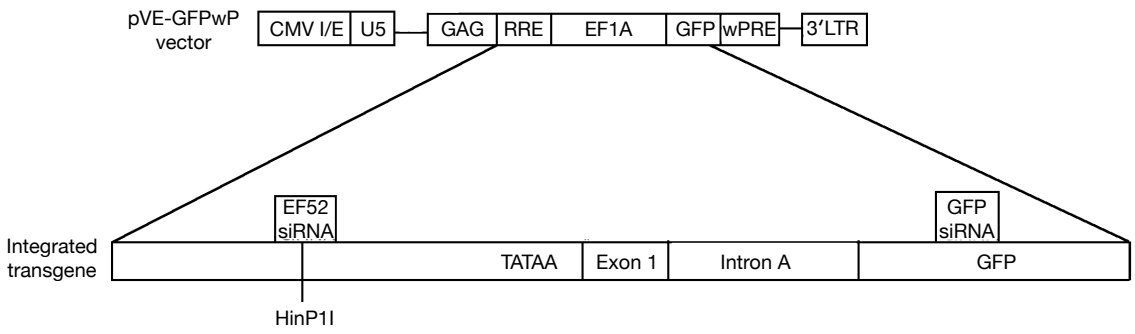
Promoter methylation analysis.

Genomic DNAs (1-2μg) were digested for 2 hours with HinP1I (New England Biolabs) in a 20μL reaction, 2μls of which was used as template for promoter specific PCR with primers 803 and 804 (see Figure S2A). The control was 0.1μg of pVE-GFPwP plasmid methylated with Sss-I methylase (New England Biolabs). PCR amplification indicates that the HinP1I site within the

EF52 targeted sequence is methylated and protected from digestion.

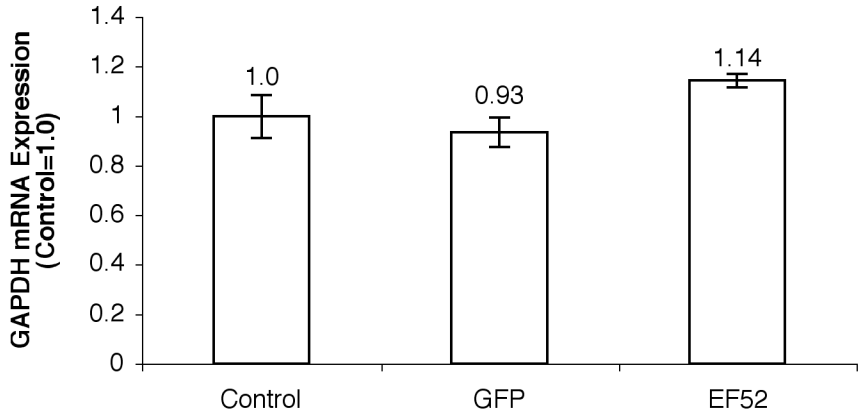
Supplementary Figure S1.

A. Lentiviral FIV vector pVE-GFPwP integrates an EF1A promoter-GFP transgene into human cells. siRNA target sites in the promoter (EF52) and coding region (GFP) of the reporter gene are shown.



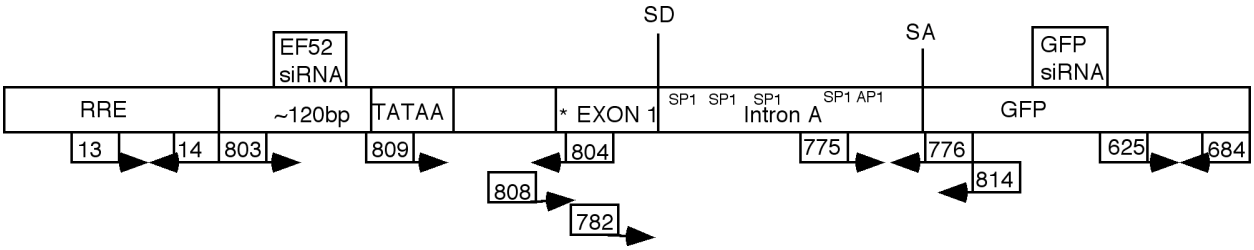
Supplementary Figure S1 continued.

B. siRNA mediated transcriptional inhibition of the EF1A promoter is specific. GAPDH expression is not altered in lentiviral transduced 293FT cells transfected with control (CCR5), GFP or EF52 siRNAs. GAPDH expression was measured by real-time RT-PCR. Standard deviations from three independent experiments are shown.



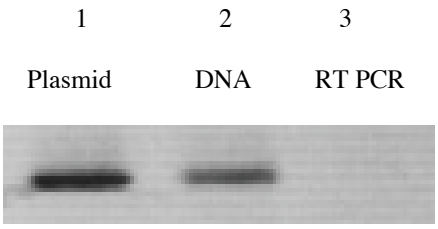
Supplementary Figure S2.

A. RT-PCR primers used to analyze transcriptional initiation from the EF1A promoter-GFP transgene. Primers are indicated below the map of the integrated transgene. The EF1A promoter begins at primer 803, and the start of exon 1 marks the site of transcriptional initiation (*12, 13*). Intron A is spliced out of the mature GFP transcript.

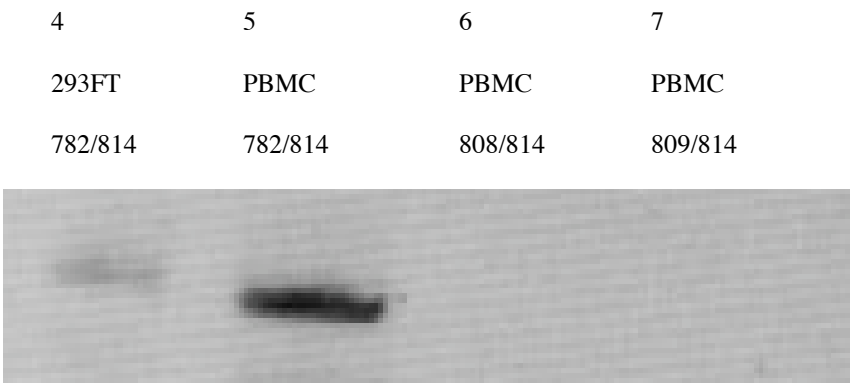


Supplementary Figure S2 continued.

B. Transcription from the transgenic EF1A promoter-GFP reporter gene initiates at a position similar to that of the endogenous EF1A gene (*12, 13*). RT-PCR was performed on RNA isolated from lentiviral transduced cells transfected with control (CCR5) siRNA. Primers 803 and 804 were used to amplify plasmid pVE-GFPwP (lane 1), genomic DNA (lane 2) and cDNA generated by reverse transcription (lane 3).

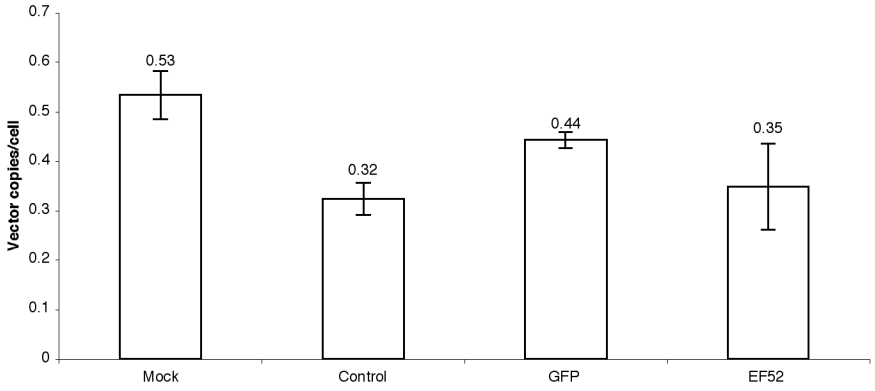


RT PCR of RNA from lentiviral transduced 293FT cells or lentiviral transduced peripheral blood monocytes (PBMC) is shown. Refer to S2A for positions of RT-PCR primers.



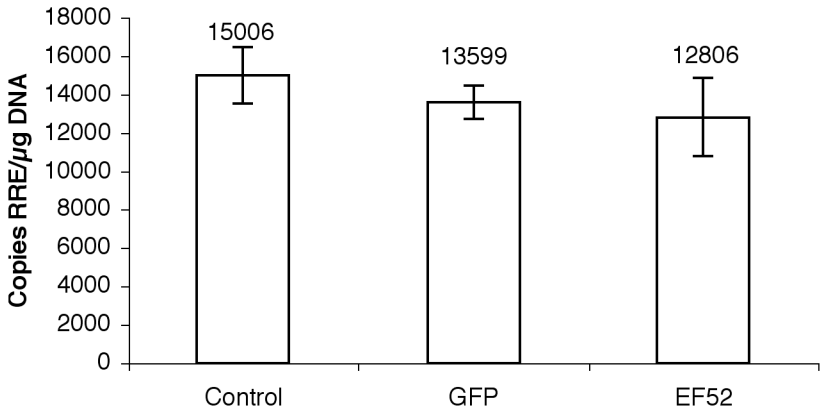
Supplementary Figure S3.

A. Treatment with siRNA does not alter vector integration frequency. An FIV integration assay (*8*) was performed on DNA from lentiviral transduced, siRNA transfected 293FT cells treated by mock transfection, or transfection with control (CCR5), GFP, or EF52 siRNA. The assay involves an Alu repeat-based PCR amplification to detect integrated transgenes, followed by a nested lentivirus-specific PCR amplification. Negative controls (10μl of equally diluted, unamplifed genomic DNA) produced no bands in the second PCR amplification (not shown).



Supplementary Figure S3 continued.

B. siRNA transfection does not alter total copy number of the lentiviral vector in transduced cells. Levels of the viral RRE sequence were quantified by real-time PCR in genomic DNA from lentiviral transduced, siRNA transfected 293FT cells. The control siRNA was human CCR5 specific.



Supplementary Table S1.

Transcription from the upstream CMV promoter is undetectable in lentiviral transduced cells. 293FT cells were transduced with pVE-GFPWP lentivirus, then transfected with siRNAs. Real-time PCR was used to measure transcription of RRE and GFP sequences 48 hours after siRNA transfection. RRE expression is only detected when the upstream CMV promoter is active; the CMV promoter is not present in the integrated transgene (see Figure 1A for diagram of the lentiviral vector).

siRNA	RRE transcription	GFP transcription
Control (CCR5)	<0.1	21.7 ±2.2
GFP	<0.1	4.9 ±0.2
EF 52	<0.1	<0.1

Supplementary references

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