Chapter 10

LINE-1 Cultured Cell Retrotransposition Assay

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Abstract

The Long INterspersed Element-1 (LINE-1 or L1) retrotransposition assay has facilitated the discovery and characterization of active (i.e., retrotransposition-competent) LINE-1 sequences from mammalian genomes. In this assay, an engineered LINE-1 containing a retrotransposition reporter cassette is transiently transfected into a cultured cell line. Expression of the reporter cassette, which occurs only after a successful round of retrotransposition, allows the detection and quantification of the LINE-1 retrotransposition efficiency. This assay has yielded insight into the mechanism of LINE-1 retrotransposition. It also has provided a greater understanding of how the cell regulates LINE-1 retrotransposition and how LINE-1 retrotransposition impacts the structure of mammalian genomes. Below, we provide a brief introduction to LINE-1 biology and then detail how the LINE-1 retrotransposition assay is performed in cultured mammalian cells.

Key words LINE-1, Alu, Retrotransposition assay, *trans*-complementation assay, Mammalian cultured cells

1 Introduction

Sequences derived from Long INterspersed Element-1 (LINE-1 or L1) comprise approximately 17 % of human genomic DNA [1]. Although greater than 99.9 % of human L1s have been rendered inactive by mutational processes, a small cohort of active L1s can move to new genomic locations by a "copy-and-paste" process known as retrotransposition (reviewed in Ref. [2]). Here, we refer to these L1s as retrotransposition-competent or RC-L1s.

A full-length RC-L1 is ~6 kb in length. Human RC-L1s contain a 5′ untranslated region (UTR), two nonoverlapping open reading frames (ORFs) that are separated by a short 63 bp inter-ORF sequence, and a 3′ UTR that ends in an adenosine-rich tract [3, 4]. The human L1 5′ UTR contains an internal RNA polymerase II promoter that directs the initiation of transcription at or near the first base of the element [5–7]; it also has an ill-defined antisense

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promoter [8]. L1 ORF1 encodes an ~40 kDa RNA binding protein (ORF1p) that has nucleic acid chaperone activity [9–17]. ORF2 encodes an ~150 kDa protein (ORF2p) that has endonuclease (EN) and reverse transcriptase (RT) activities that are critical for retrotransposition [18–25]. ORF2p also contains a cysteine-rich domain (C) that is required for retrotransposition, although its function requires elucidation [24, 26]. The L1 3′ UTR contains a guanosine-rich polypurine tract, a weak RNA polymerase II polyadenylation signal, and ends in an A-rich tract [27–30]. Experiments in cultured cells have demonstrated that the L1 3′ UTR is not strictly required for retrotransposition [18, 24].

L1 elements retrotranspose by target site-primed reverse transcription (TPRT) [20, 31, 32]. As a result of TPRT, genomic L1s typically have the following structural hallmarks: (1) they are truncated at their 5′ ends; (2) they are flanked by target-site duplications (TSDs) that vary both in their length and sequence; (3) they end in an A-rich tract; and (4) they generally insert into an L1 ORF2p endonuclease consensus cleavage site (5′-TTTT/A-3′ and variations of that sequence, where "/" denotes the endonucleolytic nick) [1, 33–40].

The proteins encoded by L1s (ORF1p and/or ORF2p) occasionally act *in trans* to retrotranspose Short INterspersed Element (SINE) RNAs (e.g., the RNAs transcribed from Alu and SINE-VNTR-Alu (SVA) elements), noncoding RNAs (e.g., uracil-rich small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs)), and RNA polymerase II transcribed messenger RNAs (mRNAs) [41–51]. In aggregate, these sequences comprise at least ~11 % of human genomic DNA ([1], reviewed in Ref. [2]). Thus, LINE-1 mediated retrotransposition events account for approximately one-third, or one billion base pairs of human genomic DNA. Ongoing LINE-1 retrotransposition continues to affect both interindividual and intraindividual human genetic diversity and has caused ~100 sporadic cases of genetic disease ([52], reviewed in Refs. [2, 53]).

The development of the cultured cell retrotransposition assay represented a seminal moment in LINE-1 biology because it allowed the experimental study of LINE-1 retrotransposition in "real time" [24]. The rationale of the assay is built upon experimental strategies to examine retrotransposon mobility in yeast and mammalian cells [54–57]. Briefly, a retrotransposition indicator cassette is introduced into the L1 3′ UTR in the opposite orientation of the L1 transcript [24]. The reporter cassette consists of a reporter gene (e.g., neomycin phosphotransferase in the *mneoI* reporter cassette) equipped with a heterologous promoter and polyadenylation signal (Fig. 1a). The reporter gene is interrupted by an intron that is in the same orientation with respect to L1 transcription. Thus, the reporter gene can only be expressed upon transcription of the L1, splicing of the intron in the reporter cassette, and the reverse transcription of the L1 RNA to introduce a

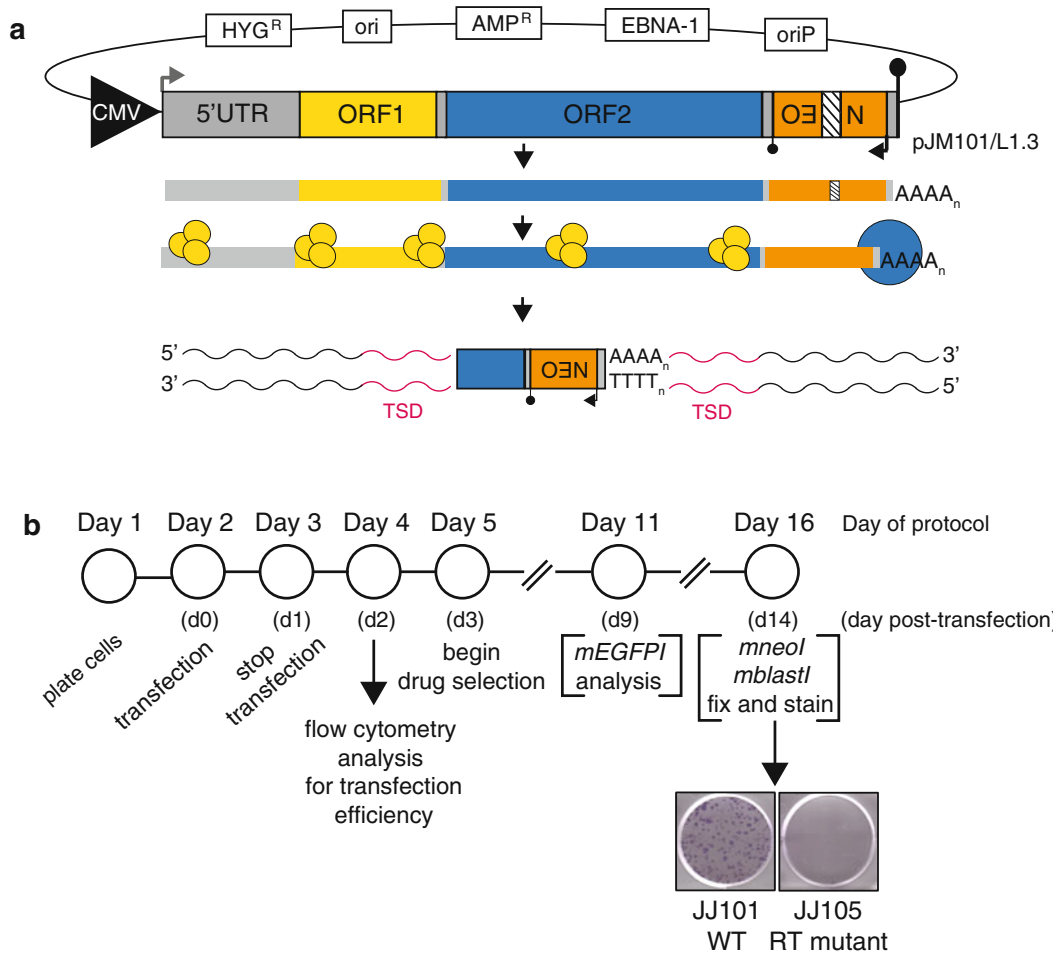

Fig. 1 LINE-1 retrotransposition assay. (**a**) A full-length retrotransposition-competent LINE-1 contains the *mneoI* reporter cassette (*orange box*) in the 3′ UTR in the opposite orientation with respect to LINE-1 (pJM101/L1.3) transcription. The reporter gene, neomycin phosphotransferase (*backwards* NEO) is interrupted by an intron (*hatched box*), which is in the same orientation with respect to LINE-1 transcription. The reporter cassette has its own promoter (*upside down black arrow*) and polyadenylation signal (*upside down small black lollipop*). The pCEP4 plasmid backbone encodes for the EBNA-1 (EBNA-1) viral protein and contains an origin of viral replication (oriP) and hygromycin B-resistance gene (HYG^R) for plasmid replication and hygromycin-selection, respectively, in mammalian cultured cells. The backbone also has a bacterial origin of replication (ori) and an ampicillin-resistance gene (AMP^R) for replication and ampicillin-selection, respectively, in *E. coli*. The LINE-1 is transcribed from the CMV promoter (*black triangle* labeled "CMV") or its promoter in the 5′ UTR (*gray arrow*) and transcription is terminated at an SV40 polyadenylation signal (*large black lollipop*). Once transcribed, the intron is spliced out and L1 ORF1p (*yellow circles*) and ORF2p (*blue circle*) are translated from the L1 mRNA (*gray*, *yellow*, *blue*, and *orange line* followed by "AAAA$_n$"). Only upon reverse transcription and integration into a genomic locus (5′ truncated *blue* and *orange box*), which is flanked by target-site duplications (TSD, *red wavy lines*), can the NEO gene be expressed to confer drug-resistance. (**b**) A timeline of the assay is depicted and described in Subheading 3. Days of the protocol are noted above and the corresponding days post-transfection (d0–14) are noted below. The end result of the assay is depicted under d14. The pJJ101/L1.3 construct is a wild type LINE-1 with a blasticidin deaminase (*mblastI*) reporter cassette and its retrotransposition results in many drug-resistant colonies. The pJJ105/L1.3 construct contains a LINE-1 reverse transcriptase mutant, cannot complete retrotransposition, and does not result in any drug-resistant colonies

new L1 copy and the reporter cassette into the genome [24]. Once integrated into the genome, the cells harboring new retrotransposition events can be identified by either selecting or screening for reporter gene expression. The number of colonies or cells expressing the reporter genes allows the quantification of the LINE-1 retrotransposition efficiency.

Since the initial publication of the LINE-1 retrotransposition assay [24], several adaptations have made the assay more efficient [58] and applicable to study an array of biological questions. For example, the *mneoI* cassette and a derivative of the cassette, neo-*Tet*, which allows the detection of a retrotransposed RNA polymerase III transcribed RNA, have been used to study the retrotransposition of Alu and SVA SINE RNAs, U6 snRNA, and cellular mRNAs [33, 43, 45–47, 49, 51]. Modified versions of the *mneoI* cassette have been used to recover LINE-1 retrotransposition events from genomic DNA, enabling detailed analyses of how LINE-1 retrotransposition events impact the genome [33, 34, 38]. Indeed, these studies revealed that de novo retrotransposition events from engineered L1 constructs resemble endogenous L1 insertions in their structure. Moreover, they revealed that L1 is not simply an insertional mutagen, but that LINE-1 retrotransposition events also can generate intrachromosomal deletions, intrachromosomal duplications, and perhaps interchromosomal translocations [33, 34, 38].

Other variations on the retrotransposition reporter cassette include incorporation of an enhanced green fluorescent protein (EGFP) reporter gene (*mEGFPI*), which has been used to assay LINE-1 retrotransposition both in vitro and in vivo [59–66]. The subsequent development of the blasticidin S-resistance reporter cassette (*mblastI*) was used to examine DNA repair deficient Chinese hamster ovary (CHO) cell lines for retrotransposition [36, 67–69]. Blasticidin S kills cells more efficiency than G418 and may be used for cell lines that have a high tolerance for G418. More recently, luciferase-based retrotransposition indicator cassettes, which may be amenable for high-throughput screening, have been developed to assay for retrotransposition [70].

In sum, the LINE-1 cultured cell retrotransposition assay has been used successfully in multiple human and other mammalian cell lines (summarized in Ref. [71]). The following protocol has been adapted from previously published assays [24, 36, 43, 58, 66] and is optimized for HeLa cells. Importantly, the necessary experimental controls required to correctly interpret retrotransposition assay results (e.g., transfection efficiency, cell viability, and off-target effects) are highlighted in Subheading 4 of this protocol.

2 Materials

2.1 Cell Culture Media and Transfection Reagents

1. Two clonal HeLa cell lines support retrotransposition. HeLa-JVM cells are used to assay LINE-1 retrotransposition [24]. HeLa-HA are used to assay either LINE-1 or Alu retrotransposition [72].
2. Cultured cell growth medium for HeLa cells.
   (a) To assay LINE-1 retrotransposition (*in cis*), HeLa-JVM cells are grown in DMEM (4.5 g/L d-glucose) containing 10 % FBS, and 1× Pen–Strep, glutamine (100 U/mL penicillin, 100 μg/mL streptomycin, and 292 μg/mL glutamine). This is called HeLa-JVM DMEM growth medium in the protocol below.
   (b) To assay Alu retrotransposition (*in trans*), HeLa-HA cells are grown in MEM (with Earle's salts) containing 10 % FBS, 1× MEM nonessential amino acids, and 1× Pen–Strep, glutamine (100 U/mL penicillin, 100 μg/mL streptomycin, and 292 μg/mL glutamine). This is called HeLa-HA MEM growth medium in the protocol below.
3. 1× Phosphate-buffer saline (PBS), pH 7.4, sterilized.
4. 0.05 % Trypsin–EDTA.
5. A cell counter (e.g., Countess® Automated Cell Counter, Life Technologies) or a hemocytometer.
6. Tissue culture dishes or flasks (6-well plates, T-75 flasks, or 10 cm dishes).
7. FuGENE® 6 transfection reagent (Promega).
8. Opti-MEM® I (Life Technologies).
9. Geneticin (G418, 50 mg/mL stock), blasticidin S-HCl (10 mg/mL stock), or puromycin (10 mg/mL stock).
10. A flow cytometer (e.g., the Accuri C6 Flow Cytometer).

2.2 Plasmids

1. To assay LINE-1 retrotransposition, *in cis* (Fig. 1):
   (a) The pCEP4 mammalian expression episomal plasmid (Life Technologies), which generally is the backbone of the LINE-1 expression plasmids.
   (b) A LINE-1 expression plasmid that is tagged with a retrotransposition reporter cassette (e.g., pJM101/L1.3 (*mneoI*), pJJ101/L1.3 (*mblastI*), or pLRE3-mEGFPI (*mEGFPI*) [67, 73–76]).
   (c) A reporter plasmid (e.g., hrGFP (Agilent)) to monitor transfection efficiency.
   (d) A plasmid expressing a cDNA of interest. This plasmid should not contain the same selectable marker as the L1 reporter cassette.

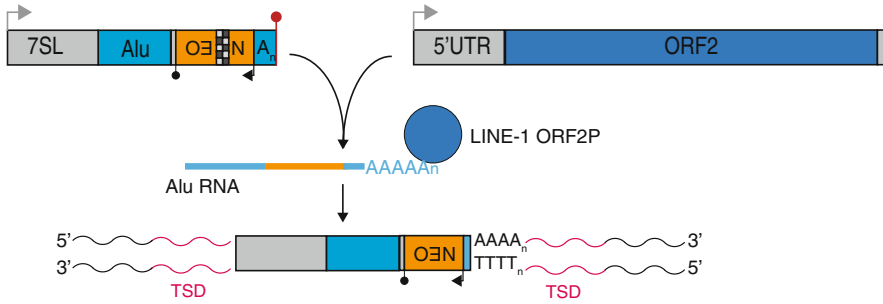

Fig. 2 Alu retrotransposition (*trans*-complementation) assay. Retrotransposition of Alu requires the LINE-1 ORF2 protein [43]. For the Alu *trans*-complementation assay, the reporter plasmid contains the Alu sequence (*light blue box*) and is tagged with a modified NEO retrotransposition indicator cassette (neo*Tet*) interrupted by a self-splicing group I intron (*checkered box*) [81]. The neo*Tet* cassette is followed by a variably sized poly A tract (A_n) and the RNA pol III terminator sequence (*red lollipop*). The Alu plasmid must be co-transfected with a plasmid (pCEP5′UTR-ORF2pΔneo) expressing LINE-1 ORF2p (*blue circle*) to detect retrotransposition in HeLa cells. The resulting G418-resistant colonies generally contain de novo full-length Alu retrotransposition events flanked by target-site duplications (TSD, *red wavy lines*)

2. To assay Alu retrotransposition, *in trans* (Fig. 2):
   (a) A "reporter" retrotransposition plasmid that contains an Alu element and a modified *mneoI* cassette (e.g., pAlu-neo*Tet*) that allows the detection of a retrotransposed RNA polymerase III transcribed RNA [43].
   (b) A "driver" LINE-1 expression plasmid (i.e., either a full-length LINE-1 or a plasmid that expresses LINE-1 ORF2p (e.g., pJM101/L1.3Δneo or pCEP5′UTR-ORF2pΔneo [51, 77])) that lacks the retrotransposition reporter cassette.
   (c) A reporter plasmid (e.g., hrGFP) to monitor transfection efficiency.
3. To test if cellular proteins affect LINE-1 retrotransposition (Fig. 3; [78–80]):
   (a) A LINE-1 expression plasmid that is tagged with a retrotransposition reporter cassette (e.g., *mneoI*, *mblastI*, or *mEGFPI*).
   (b) A non-LINE-1 control plasmid (e.g., pU6iNEO [79]) that expresses an intact (i.e., intronless) version of the same selectable or screenable marker present in the LINE-1 retrotransposition reporter cassette.
   (c) An expression vector containing the cDNA that is being tested for its effect on retrotransposition (e.g., pK_A3A that expresses APOBEC3A [78, 79]).
   (d) A negative control cDNA expression vector that expresses a protein that does not significantly affect retrotransposition (e.g., pK_β-arrestin [78, 79]).

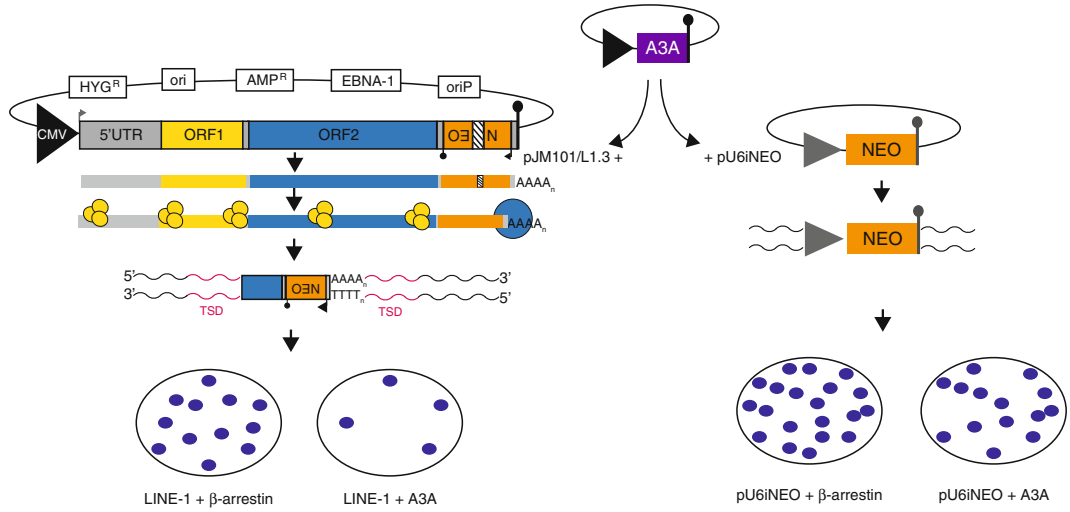

Fig. 3 Co-transfection of LINE-1 with cDNAs. The LINE-1 retrotransposition assay is carried out as described in Fig. 1 except that a plasmid expressing pK_A3A (*purple box*) is co-transfected with a LINE-1 expression construct (pJM101/L1.3, *see* Fig. 1a for details). In parallel, pK_A3A is co-transfected with a reporter control, pU6iNEO, which expresses the neomycin phosphotransferase gene without the requirement for retrotransposition (i.e., the neomycin phosphotransferase gene lacks an intron). Both sets of co-transfected cells are grown under drug-selection and the resulting drug-resistant colonies (*dark purple circles*) are counted. The parallel pU6iNEO experiment is essential to determine to what extent the effects of A3A overexpression are LINE-1 specific. Note the greater decrease in G418-resistant colonies in "LINE-1 + A3A" than "pU6iNEO + A3A" [79]

2.3 Fixing and Staining

1. Fix: 2 % formaldehyde, 0.2 % glutaraldehyde, in 1× PBS, pH 7.4.
2. Stain (can use any one of the three):
   (a) 0.1 % bromophenol blue, weight to volume (w/vol) in H_2O.
   (b) 0.1 % crystal violet blue, w/vol in H_2O.
   (c) 0.4 % Giemsa, w/vol in H_2O.

3 Methods

Standard practices for handling cultured cells should be applied, such as use of laminar flow biosafety hoods and sterile techniques for all reagents. The transfection efficiency and the concentration of drugs used for genetic selection should first be optimized for each cell type used in this assay. The rationale and a timeline of the assay are presented in Fig. 1. Cultured cell lines are routinely verified/authenticated by short tandem repeat (STR) analyses and checked for mycoplasma to ensure their integrity and lack of contamination, respectively.

3.1 LINE-1 Retrotransposition Assay in HeLa-JVM Cells

1. Day 1—Plate cells: Seed 2×10^4 HeLa-JVM cells in each well of a 6-well tissue culture plate in HeLa-JVM DMEM growth media. Cells are grown in a humidified incubator at 37 °C with 7 % CO_2 (*see* **Note 1**).
2. Day 2—Transfect cells: Cells typically are transfected 14–16 h post-plating, day zero (d0) (Fig. 1b), using the FuGENE® 6 transfection reagent following the manufacturer's instructions. Every retrotransposition assay should include the following transfection conditions: (1) a vector-only (pCEP4) or mock transfection; (2) a wild type LINE-1 retrotransposition plasmid (e.g., pJM101/L1.3, pJJ101/L1.3, or pLRE3-*mE*GFPI), which serves as a positive control; and (3) a mutant LINE-1 plasmid (e.g., pJM105/L1.3 has a mutation in the RT domain of L1 [51]), which serves as a negative control. To assay for retrotransposition, prepare a transfection mix in a 1.5 mL microcentrifuge tube containing 1 μg pCEP4 or a LINE-1 expression plasmid (Fig. 1a) and 3–4 μL of FuGENE® 6 in 100 μL of Opti-MEM® I. Incubate the solution at room temperature for 20 min. Add the transfection mix to the growth medium of one well of cells in a 6-well tissue culture plate. To determine the transfection efficiency, prepare an additional transfection mix(es) in a 1.5 mL microcentrifuge tube containing 1 μg pCEP4 or a LINE-1 expression plasmid, 0.5 μg of an hrGFP expression plasmid, and 4–6 μL of FuGENE® 6 in 100 μL of Opti-MEM® I. Incubate the solution for 20 min at room temperature. Then, add the transfection mix to the growth medium of one well of cells in a 6-well tissue culture plate; these transfections will be used to calculate transfection efficiencies. Transfect three wells in each plate for each transfection condition to yield three technical replicates. It is important to calculate transfection efficiency and adjust retrotransposition assay results for each L1 plasmid individually, as subtle differences in L1 plasmid size and transfection mix composition can affect transfection efficiency. These differences could impact the final result of the retrotransposition assay (*see* **Note 2**).
3. Day 3—Stop the transfection: Approximately 16–24 h post-transfection, 1 day post-transfection (d1) (Fig. 1b), aspirate the medium from the cells and add fresh HeLa-JVM DMEM growth medium to the cells.
4. Day 4—Determine the transfection efficiency: 2 days post-transfection (d2) (Fig. 1b), trypsinize the hrGFP-transfected cells and collect the cells from each well in separate microcentrifuge tubes (*see* **Note 3**). Spin the cells at 2000 × *g* at 4 °C for 5 min and aspirate the medium. Wash the cells in 1× PBS, spin at 2000 × *g* at 4 °C for 5 min, then aspirate the PBS. Resuspend the cell pellet in 250–500 μL 1× PBS. Determine the number (i.e., percentage) of hrGFP-expressing cells, gating for live

cells, on a flow cytometer (Accuri Flow Cytometer or similar; *see* **Note 4**). The number of live cells that express hrGFP serves as an indication of the percentage of cells successfully transfected with plasmids (i.e., transfection efficiency).

5. Days 5–16—Select cells for retrotransposition events: Begin drug selection 3 days post-transfection (d3) and continue until 14 days post-transfection (d14) (Fig. 1b).
   (a) For *mneoI*-based assays, add 400 μg/mL G418 to the HeLa-JVM DMEM growth medium. Change the G418-containing medium every day until 14 days post-transfection (d14).
   (b) For *mblastI*-based assays, add 10 μg/mL blasticidin S-HCl to HeLa-JVM DMEM growth medium and change the blasticidin S-HCl-containing medium once at 8 days post-transfection (d8) (*see* **Note 5**).
   (c) For *mEGFPI*-based assays, add 5 μg/mL puromycin to HeLa-JVM DMEM growth medium. Change the puromycin-containing medium every other day until 9 days post-transfection (d9) to select for cells that have the L1 expression plasmid.
6. Quantification of the LINE-1 retrotransposition assay.
   (a) For *mneoI* and *mblastI* assays, 14 days post-transfection (d14) rinse the cells with 1× PBS and fix the cells (using Fix solution, Subheading 2.3, **item 1**) for 30 min to 1 h at room temperature or longer at 4 °C. Rinse the cells in water and stain (using one of the three Stain solutions, Subheading 2.3, **item 2**) at room temperature for 1 h. Rinse the cells with water and let dry (*see* **Note 5**). Count the stained foci in each well.
   (b) For *mEGFPI* assays, 9 days post-transfection (d9), trypsinize the cells and collect the cells from each well in separate microcentrifuge tubes. Collect the cells by centrifugation at 2000 × *g* at 4 °C for 5 min. Aspirate the medium. Rinse the cells with 1× PBS and spin the cells again at 2000 × *g* at 4 °C for 5 min. Aspirate the PBS and resuspend the cell pellet in 250–500 μL 1× PBS. Analyze the number of EGFP-expressing cells, gating for live cells, on a flow cytometer. The number of live cells that express EGFP serves as an indication of the number of cells that have successfully undergone a round of de novo retrotransposition.
7. To calculate the retrotransposition efficiency, drug-resistant colonies or EGFP-expressing cells are counted and adjusted for transfection efficiency (*see* **Note 6**).
   (a) For G418- or blasticidin S-resistant colonies, calculate the mean colony counts (from **step 6a**) for the three wells of

the same transfection condition (three technical replicates). To calculate the adjusted retrotransposition mean, divide the mean colony counts and the standard deviation by the transfection efficiency (calculated in **step 4**) (*see* **Note 6**). To express the adjusted retrotransposition values as a percentage of the wild type control (i.e., the retrotransposition efficiency), divide the adjusted retrotransposition mean of an experimental sample (e.g., the number of retrotransposition events generated from a mutant L1 expression construct) by the adjusted retrotransposition mean of wild type L1 (e.g., pJM101/L1.3) and then multiply by 100 (*see* **Note 7**).

(b) For EGFP-expressing cells, divide the mean percentage of EGFP-expressing cells (calculated in **step 6b**) and the standard deviation from three wells per transfection condition (three technical replicates) by the transfection efficiency (calculated in **step 4**) to calculate the adjusted retrotransposition mean (*see* **Note 6**). The adjusted retrotransposition mean for *mEGFPI* retrotransposition assays represents the percentage of puromycin-resistant cells that express EGFP. Again, at least three biological replicates, each containing three technical replicates, should be done.

3.2 Alu Retrotransposition Assay in HeLa-HA Cells (in trans)

Retrotransposition *in trans* occurs at a lower frequency than *in cis* [43, 51]. Therefore, the retrotransposition assay detailed above is scaled-up. Please note that transfection conditions need to be optimized when using larger tissue culture plates or flasks (*see* Fig. 2 for rationale).

1. Day 1—Plate cells: Seed 5×10^5 HeLa-HA cells in 10 cm tissue culture dish or T-75 flask in HeLa-HA MEM growth medium.
2. Day 2—Transfect cells: Cells typically are transfected 14–16 h post-plating, day zero (d0) (Fig. 1b), using the FuGENE® 6 transfection reagent following the manufacturer's instructions. To assay for Alu retrotransposition, prepare a transfection mix containing 4 μg of a reporter plasmid (e.g., pAlu-neo*Tet*), 4 μg driver plasmid (e.g., pCEP5′UTR-ORF2pΔneo; Fig. 2), and 24–32 μL of FuGENE® 6 in 500 μL of Opti-MEM® I. Incubate the solution at room temperature for 20 min. Add the transfection mix to the growth medium of the cells in the tissue culture dish or flask. To determine transfection efficiencies, prepare a transfection mix containing 0.5 μg each of the reporter plasmid, the driver plasmid, and hrGFP plasmid and 4–6 μL of FuGENE® 6 in 100 μL of Opti-MEM® I. Incubate the transfection mix at room temperature for 20 min. Transfect cells with the hrGFP-containing transfection mix in 6-well plates (as stated in Subheading 3.1, **step 2**).

3. Day 3—Stop the transfection: Approximately 24 h post-transfection, 1 day post-transfection (d1) (Fig. 1b), aspirate the medium from the cells and add fresh HeLa-HA MEM growth medium to the cells.
4. Day 4—Determine the transfection efficiency: same as Subheading 3.1, **step 4**.
5. Days 5–16—Select the cells for retrotransposition events: same as Subheading 3.1, **step 5a**.
6. Quantitate the LINE-1 retrotransposition assay: same as Subheading 3.1, **step 6a**.
7. Calculate the retrotransposition efficiency: same as Subheading 3.1, **step 7a**.

3.3 LINE-1 Retrotransposition Assay Co-transfected with cDNAs

The experimental design is similar to the assay above except cells are transfected with an L1 and a cDNA expression construct. When co-transfecting an L1 plasmid with a cDNA expression construct, it is essential to monitor side effects of cDNA overexpression. In order to monitor potential cDNA off-target effects, HeLa cells should also be co-transfected with the cDNA expression plasmid and a control reporter plasmid that expresses an intact copy of the same selectable marker (i.e., no intron disrupting the reporter) as the LINE-1 retrotransposition plasmid. The LINE-1 retrotransposition assay and control reporter assay must be done in parallel (i.e., at the same time) [79, 80]. For example, the effect of APOBEC3A (A3A) on LINE-1 retrotransposition (*mneoI* reporter cassette) and on a control reporter plasmid (pU6iNEO), as described in Richardson et al., is described below [79]. Retrotransposition efficiency is corrected to the reporter control assay (*see* Fig. 3 and **Note 8**).

1. Day 1—Plate cells: Seed 2×10^4 HeLa-JVM cells in each well of a 6-well tissue culture plate in HeLa-JVM DMEM growth medium (as stated above in Subheading 3.1, **step 1**) (*see* **Note 1**).
2. Day 2—Transfect cells: Cells typically are transfected 14–16 h post-plating, day zero (d0) (Fig. 1b), using the FuGENE® 6 transfection reagent following the manufacturer's instructions. Experimental conditions for L1 transfection include: (1) pCEP4 plus pK_β-arrestin; (2) pJM101/L1.3 plus pK_β-arrestin, (which has no significant effect on LINE-1 retrotransposition and is a control for the co-transfected cDNA) [78]; (3) pJM101/L1.3 plus pK_A3A; (4) pJM105/L1.3 plus pK_β-arrestin or pK_A3A; and (5) mock transfected cells or cells transfected only with pCEP4. In parallel, experimental conditions for the reporter control plasmid include: (1) pU6iNEO plus pK_β-arrestin and (2) pU6iNEO plus pK_A3A. Prepare a transfection mix in a 1.5 mL microcentrifuge tube containing either an L1 expression or control vector

(0.5 μg of the pCEP4 vector, 0.5 μg of an L1 expression plasmid, or 0.5 μg pU6iNEO) and a cDNA expression vector (0.5 μg pK_β-arrestin or 0.5 μg of an pK_A3A expression plasmid) with 3–4 μL of FuGENE® 6 in 100 μL of Opti-MEM® I. Incubate the solution at room temperature for 20 min. Add the transfection mix to the growth medium of one well of cells in a 6-well tissue culture plate. To calculate the transfection efficiency, prepare a transfection mix in a 1.5 mL microcentrifuge tube containing the above reagents and 0.5 μg of an hrGFP expression plasmid (as described above in Subheading 3.1, **step 2**).

3. Day 3—Stop the transfection: Approximately 24 h post-transfection, 1 day post-transfection (d1) (Fig. 1b), aspirate the medium from the cells and add fresh HeLa-JVM DMEM growth medium to the cells.
4. Day 4—Determine the transfection efficiency: same as Subheading 3.1, **step 4**.
5. Days 5–16—Select the cells for retrotransposition events: same as Subheading 3.1, **step 5a**.
6. Determine the LINE-1 retrotransposition efficiency and the effect of cDNA expression on the ability of the reporter construct (pU6iNEO) to form drug resistant foci: same as Subheading 3.1, **step 6a**.
7. To calculate the corrected retrotransposition mean, the adjusted retrotransposition mean of L1 plus pK_β-arrestin is divided by the adjusted retrotransposition mean of pU6iNEO plus pK_β-arrestin (*see* **Note 8**). Similarly, the adjusted retrotransposition mean of L1 plus pK_A3A is divided by the adjusted retrotransposition mean of pU6iNEO plus pK_A3A. This corrected calculation accounts for any cytotoxic and/or off-target effects pK_A3A may have on the cell.
8. To calculate the corrected retrotransposition efficiency, divide the corrected retrotransposition mean of L1 plus pK_A3A expression vector by the corrected retrotransposition mean of L1 plus pK_β-arrestin and multiply by 100 (*see* **Note 9**). The retrotransposition efficiency reported reflects the specific inhibition of pK_A3A on LINE-1 retrotransposition (*see* **Note 10**).

4 Notes

1. Each cell line must be optimized for cell plating density, plasmid concentrations, transfection reagents, and the drug concentration needed for selection. The experiment also can be scaled up into flasks:
   (a) For 75 cm^2 flasks: transfect 2×10^6 cells with 8 μg DNA, 32 μL FuGENE® 6, and 500 μL of Opti-MEM® I.

(b) For 175 cm^2 flasks: transfect 6×10^6 cells with 10–25 μg DNA, 40–100 μL FuGENE® 6, and 1 mL of Opti-MEM® I.

2. Assays are done with three technical replicates in order to calculate a standard deviation for each experimental variable. Each assay is repeated at least three independent times, yielding three biological replicates.
3. Typically, co-transfect 1 μg L1 plasmid and 0.5 μg hrGFP plasmid for each well of a 6-well plate to calculate transfection efficiency. A mock-transfected cell sample should always be included to properly gate true GFP-expressing and non-expressing cells. Using *mEGFPI* does not interfere with detection of hrGFP expression because GFP expression from *mEGFPI* resulting from retrotransposition is detectable between 7 and 9 days post-transfection (d7–d9) [66]. GFP expression from hrGFP is detectable 1–3 days post transfection (d1–d3). Importantly, GFP expression from the retrotransposed *mEGFPI* reporter cassette is analyzed in cells transfected without hrGFP, as these transfections are done in parallel. Alternatively, a plasmid expressing mCherry can be used to determine transfection efficiency.
4. Retrotransposition efficiency can only be calculated from cells that are transfected with an L1 construct. It is important to account for slight variations in transfection efficiencies when calculating retrotransposition efficiencies. Use of Lipofectamine® (Life Technologies) also has been reported for L1 studies, following manufacturer's directions [24]. Transfection efficiencies will vary among cell lines.
5. Blasticidin S selection was previously reported as starting 5 days post-transfection (d5) in Chinese hamster ovary cells [36]. Selection with blasticidin S should be optimized when using different cell lines. HeLa-JVM cells die sooner under blasticidin S-selection than G418-selection. Blasticidin S-resistant colonies can be fixed and stained 10–12 days post-transfection (d10–d12).
6. Adjusted retrotransposition mean = (average number of drug-resistant colonies)/(fraction of hrGFP-positive cells).
7. Retrotransposition efficiency = 100 × (adjusted retrotransposition mean)/(adjusted retrotransposition mean of the wild type L1). For example, if the adjusted retrotransposition mean of wild type L1 is 88, then the retrotransposition efficiency is 100 × (88/88) = 100 %. Similarly, if the adjusted retrotransposition efficiency of an EN mutant L1 from the same experiment is 1, then the retrotransposition efficiency of the EN mutant L1 is 100 × (1/88) = 1.1 %.

8. Corrected retrotransposition mean = (adjusted L1 plus expression plasmid retrotransposition mean)/(mean number of colonies from the reporter control plus the same expression plasmid). For example, the corrected retrotransposition mean of L1 + pK_β-arrestin is calculated by dividing the adjusted retrotransposition mean of L1 + pK_β-arrestin by the mean number of colonies from pU6iNEO+ pK_β-arrestin. Similarly, the corrected retrotransposition mean of L1 + pK_A3A is calculated by dividing the adjusted retrotransposition mean of L1 + pK_A3A by the mean number of colonies from pU6iNEO+ pK_A3A.
9. Corrected retrotransposition efficiency = 100 × (corrected retrotransposition mean for L1 plus cDNA expression plasmid)/(corrected retrotransposition mean of the wild type L1 plus an empty vector or pK_β-arrestin expression plasmid control).
10. Co-transfections involving cDNAs expressing host factors must be assayed for off-target effects (e.g., cell toxicity) before interpreting the effects on retrotransposition. For example, overexpression of A3A decreases the number of G418-resistant colonies in cells co-transfected with a control G418-resistance plasmid (pU6iNEO), but dramatically decreases the number of G418-resistant colonies in cells co-transfected with an L1/*mneoI* plasmid [79] (Fig. 3). A possible interpretation of these data is that the decrease in LINE-1 retrotransposition is due to the cytotoxicity of A3A overexpression. However, the cytotoxic effects of A3A overexpression are not solely responsible for the reduction in LINE-1 retrotransposition (LINE-1/*mneoI* plus A3A) because the retrotransposition efficiency is corrected to the control plasmid (pU6iNEO plus A3A) to account for the A3A cytotoxic contribution. This correction of the data reveals a clearer representation of the specific effect of A3A on LINE-1 retrotransposition by accounting for the non-specific, off-target effects of A3A overexpression. Testing the same conditions on a non-L1 plasmid containing the same selectable or screenable marker (i.e., drug-resistance or GFP, respectively) would account for any indirect, off-target effects from data interpretation.

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Conflict of Interest

J.V.M. is an inventor on the patent: "Kazazian, H.H., Boeke, J.D., Moran, J.V., and Dombrowski, B.A. Compositions and methods of use of mammalian retrotransposons. Application No. 60/006,831; Patent No. 6,150,160; Issued November 21, 2000." J.V.M. has not made any money from this patent and voluntarily discloses this information.

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