Supporting Information

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SI Materials and Methods

Lentiviral Stocks. One well of a six-well plate containing ~1×10^6 HEK293T cells was cotransfected with the pEM584 plasmid (see *Materials and Methods*) and the Lenti-X HT packaging mix using Lentiphos HT transfection reagent as recommended (Clontech). Medium was changed 12 to 16 h posttransfection and the plate was incubated for another 48 h before collecting the supernatant containing the lentiviral particles. The supernatant was cleared from the cellular debris by centrifugation at $1,000 \times g$ for 5 min and frozen at −80 °C in small, single-use aliquots. To titer the lentiviral stocks, serial dilutions were added to individual wells of six-well plates containing ~40% confluent HeLa cultures and incubated for 18 h without polybrene. The medium was then changed and the incubation continued for another 18 h before the addition of 5 μg/mL of blasticidin S. The medium was changed every 2 to 3 d until noninfected cells died and the infected cells containing the Bsd gene formed visible colonies. The colonies were stained with 0.1% methylene blue in 50% methanol and counted. The titers were calculated as colony forming units per milliliter by averaging the colony counts multiplied by the corresponding dilution factors. The titers were normally ~0.5 to 1×10^6 cfu/mL.

Genomic DNA Isolation. To isolate genomic DNA, 70% confluent cell cultures grown in 10-cm dishes were trypsinized and pelleted at $500 \times g$ for 5 min in 15-mL falcon tubes. The cell pellets were washed with 1.2 mL PBS and lysed in 0.6 mL of DNA extraction buffer containing 100 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM EDTA, 0.2% SDS, and 0.1 mg/mL proteinase K (Fermentas). The lysates were incubated overnight at 55 °C followed by subsequent phenol, phenol-chloroform (1:1) and chloroform extractions and genomic DNA precipitation with 0.7 volumes of isopropanol at room temperature. DNA pellets were washed with 70% ethanol and rehydrated in 10 mM Tris-HCl, pH 8.0.

Genomic DNA PCR Analysis. Genomic DNAs from the recombination-mediated cassette exchange (RMCE) acceptor cell lines were analyzed by multiplex PCR using Taq DNA polymerase (KAPA Biosystems) and either the 5′ (EF, BR, and PR) (Table S1) or the 3′ junction primer mixtures (GF, BF, and WR) (Table S1). The mixtures were designed to generate distinct PCR products from the acceptor locus before and after inserting the RIPE cassette. The PCR program consisted of a 3-min 95 °C step followed by 37 cycles of melting (94 °C, 20 s), annealing (56 °C, 30 s), and elongation (72 °C, 90 s). DMSO was added to the 5′ junction reactions to the final concentration of 5% to facilitate the amplification of the GC-rich Pur gene. Control PCR reactions were carried out using primers hGAPDH_F1 and hGAPDH_R1 (Table S1) detecting both the bona fide GAPDH gene and a GAPDH pseudogene under the conditions used. The rate of unspecific integration of the pRD-RIPE plasmid into acceptor cell genome was estimated by semiquantitative PCR using Expand Long Template PCR System (Roche) and primers Vector_F1 and Vector_R1 (Table S1). The PCR program consisted of a 3-min 94 °C step followed by 32 cycles of melting (94 °C, 20 s), annealing (56 °C, 30 s), and elongation (68 °C, 45 s). To generate the calibration curve, we programmed PCR reactions with HeLa-A12 genomic DNA spiked with known amounts of the large AgeI-BsrGI fragment of the pRD-RIPE plasmid.

Southern Blot Analysis. Fifteen micrograms of genomic DNA samples were digested with 70 units of the NcoI (New England Biolabs) in 200-μL reactions at 37 °C overnight. The samples were extracted once with phenol-chloroform (1:1), precipitated with ethanol and rehydrated in 20 μL of 10 mM Tris-HCl, pH 8.0. The samples were separated using 0.8% agarose gel electrophoresis in 1×TAE buffer at 5 V/cm and the DNA was transferred to Hybond N^+ membranes (GE Healthcare) as described (1). To prepare the probe, we amplified a ~1-kb fragment from pEM584 using KAPA HiFi DNA polymerase (KAPA Biosystems) and South584_F1 and South584_R1 primers (Table S1). The PCR fragment was labeled using a Megaprime DNA labeling System (GE Healthcare) and [α-32P]-dCTP (Perkin-Elmer) and purified from nonincorporated nucleotides using G-50 spin columns (Geneaid Biotech). Hybridizations were carried out in ExpressHyb hybridization buffer (Clontech) as recommended and the radioactive signal was visualized using a Typhoon Trio imager (GE Healthcare).

RT-PCR and RT-qPCR. Total RNA was purified using TRIzol (Invitrogen) as recommended. RNA samples were treated with 50 units/mL of RQ1 DNase (Promega) at 37 °C for 30 min to remove genomic DNA contamination. Reverse transcription (RT) was carried out using SuperScript III (Invitrogen) and random decamer (N10) primers at 50 °C for 1 h. cDNA samples were analyzed by PCR using Taq DNA polymerase (KAPA Biosystems) or quantitative PCR using Fast SYBR Green Master Mix and a StepOnePlus Real-Time PCR System (Applied Biosystems) as recommended. The corresponding primer sequences are listed in the Table S1. The RT-PCR products were analyzed by electrophoresis in 2% NuSieve 3:1 (Lonza), 1× TAE agarose gels. The qPCR reactions were carried out in triplicate and the data were normalized against the HPRT mRNA levels.

Immunoblotting. Proteins were extracted from PBS-washed cells with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Nonidet P-40, 1 mM PMSF and 1× Complete EDTA-free protease inhibitor mixture (Roche), separated by 4% to 20% gradient SDS/PAGE (Bio-Rad), and immunoblotted using the following primary antibodies: mouse monoclonal anti-PTBP1 (Zymed; 1:1,000 dilution), rabbit monoclonal anti-Ago2 (Cell Signaling Technology; 1:1,000), goat polyclonal anti-TUT4/ZCCHC11 (Imgenex; 1:500), or mouse monoclonal anti-GAPDH (Ambion; 1:10,000). The protein bands were visualized using corresponding horseradish peroxidase-conjugated secondary antibodies (mouse and rabbit, GE Healthcare; goat, Santa Cruz Biotechnology) and ECL detection reagents from Millipore or Thermo Scientific.

Luciferase Assays. HEK293T-A2 cells encoding RIPE-shRNAs against firefly luciferase or LacZ were pretreated with 2 μg/mL doxycycline (Dox) for 36 h and seeded into 96-well plates (Costar, #3610) at 4×10^4 cells/well in antibiotic-free medim. The suspended cells were immediately cotransfected with 160 ng of the *Photinus pyralis* firefly luciferase reporter plasmid pGL3-control (Promega) and 40 ng of the *Renilla reniformis* luciferase plasmid pTK-Renilla (a modified version of pGL4.74; Promega) per well using Lipofectamine 2000 as recommended (Invitrogen). The medium was changed 6 h posttransfection to include penicillin, streptomycin and Dox and the incubation continued for another 18 h. The activities of the two luciferases were measured 24 h posttransfection using Dual-Glo Luciferase

Assay System (Promega) and a Fluoroskan Ascent FL microplate fluorometer and luminometer (Thermo Scientific).

Microscopy and FACS. Cells cultured in six-well plates were incubated with 2 μg/mL Dox for 48 h or left untreated. The expression of fluorescent proteins was then analyzed by either epifluorescence microscopy using an Eclipse Ti microscope (Nikon) equipped with a CoolSNAP HQ2 CCD camera (Photometrics) or FACS using a LSR II Flow Cytometer (BD Biosciences). To prepare the FACS samples, the cells were trypsinized and resuspended in buffer containing 89% PBS, 10% FBS and 1 mM sodium pyruvate.

1. Sambrook J, Russell RW (2001) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY), 3rd Ed.

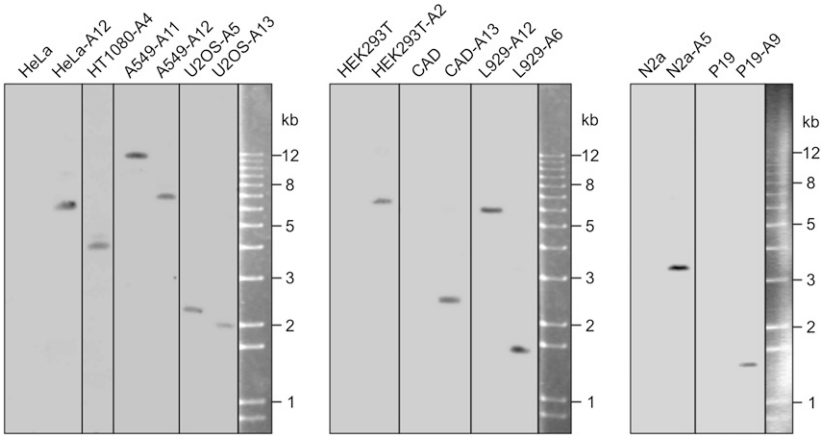

Fig. S1. Single-copy insertion of the high-efficiency and low-background (HILO)-RMCE acceptor site-containing provirus. Southern blot analysis of the human and mouse acceptor cell lines generated by lentiviral transduction of the HILO-RMCE acceptor locus (lanes marked with "A" and the clone number) were carried out using NcoI-cut genomic DNAs and a lentivirus vector-specific ^{32}P-labeled probe. The presence of a single band in the acceptor line samples corresponds to a single lentiviral integration site within the cellular genome. The parental cells contain no vector-specific sequences, as expected.

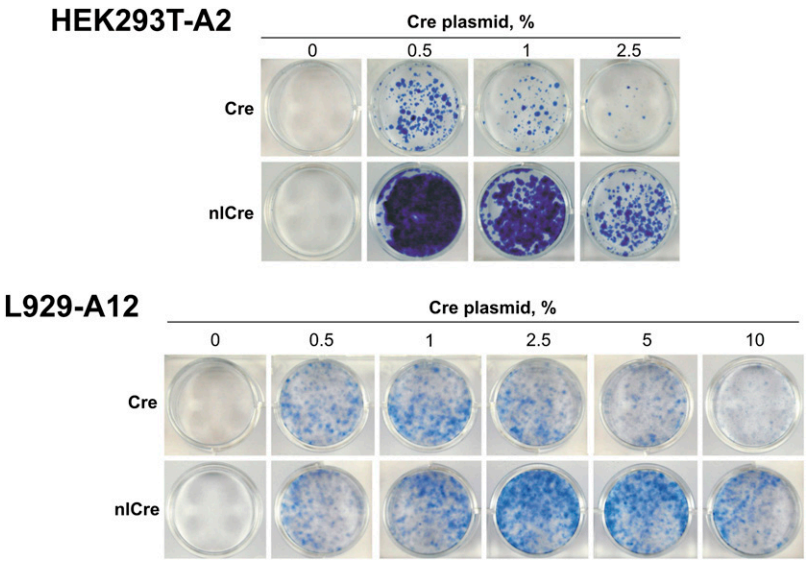

Fig. S2. Nuclear localization signal-containing Cre is a more efficient HILO-RMCE recombinase than the wild-type Cre. HEK293T-A2 and L929-A12 cells containing the RMCE acceptor locus were cotransfected in a 12-well format with the pRD-RIPE plasmid and the indicated amounts of the pCAGGS-Cre or pCAGGS-nlCre plasmids. Puromycin-resistant colonies were stained with methylene blue and photographed. Note that the wells with the optimal pCAGGS-nlCre concentrations contain more colonies than the wells with the optimal pCAGGS-Cre concentrations.

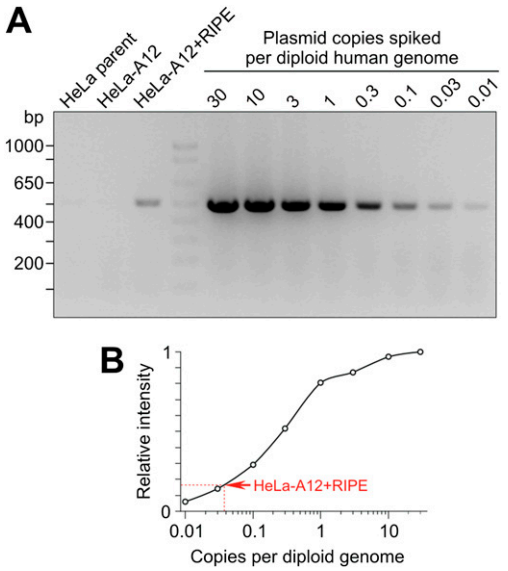

Fig. S3. HILO-RMCE generated cell populations show a low rate of unspecific integration events. Because plasmid DNA can integrate into cellular genome through a nonhomologous end-joining mechanism (1), we estimated the frequency of such unspecific events during the HILO-RMCE procedure. For this purpose, we designed PCR primers specific to the vector part of the pRD-RIPE plasmid that could be inherited by the recombinant cells only in the case of unspecific integration. We then used these primers to analyze 0.45 μg (corresponding to ∼60,000 copies of diploid human genome) of genomic DNA from the HeLa-A12+RIPE cells by semiquantitative PCR. Similar amounts of genomic DNAs from the HeLa and HeLa-A12 cells were used as negative controls. A calibration curve was obtained by spiking HeLa-A12 genomic DNA samples with known amounts of linearized pRD-RIPE plasmid. (*A*) Agarose gel analysis of the PCR reactions showing the presence of the pRD-RIPE vector-derived PCR fragment in the HeLa-A12+RIPE sample but not in the HeLa and HeLa-A12 controls. (*B*) Quantification of the results in *A* demonstrating that the apparent load of unspecific integration in HeLa-A12+RIPE cell population is <0.04 pRD-RIPE copies per diploid genome. This reasonably low value is possibly an overestimation because a fraction of the original circular pRD-RIPE plasmid used in the HILO-RMCE procedure may theoretically survive the relatively brief selection procedure.

1. Lieber MR (2010) The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu Rev Biochem* 79:181–211.

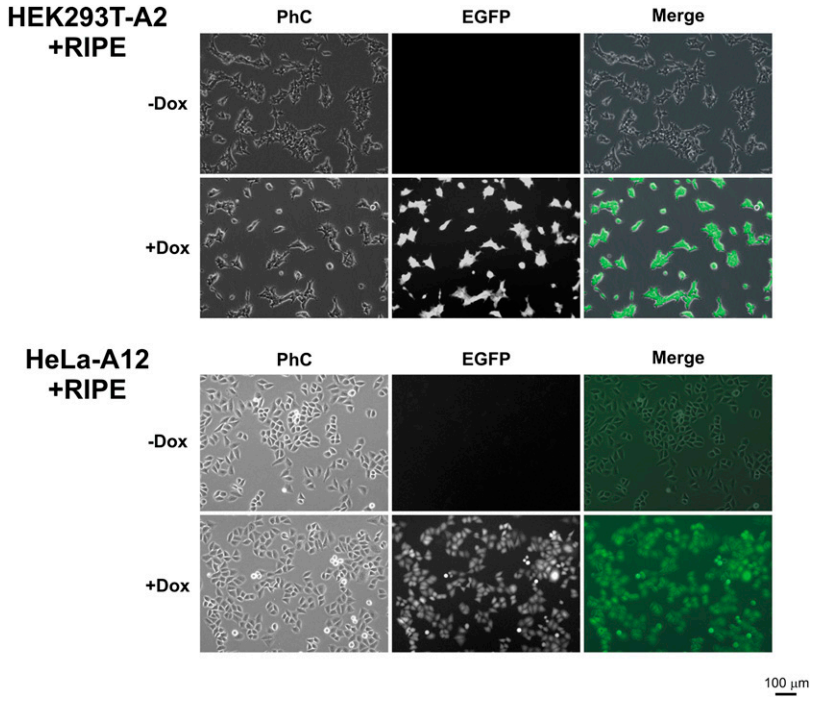

Fig. S4. Uniform induction of EGFP expression in HILO-RMCE cell pools. HEK293T-A2 and HeLa-A12 cell pools containing the HILO-RMCE-integrated RIPE cassette were incubated with 2 μg/mL Dox for 48 h or alternatively left untreated. The EGFP expression was then studied using epifluorescence and phase contrast (PhC) microscopy. Note that all viable cells express EGFP in Dox-treated samples, whereas EGFP is not detected in the corresponding Dox-negative controls.

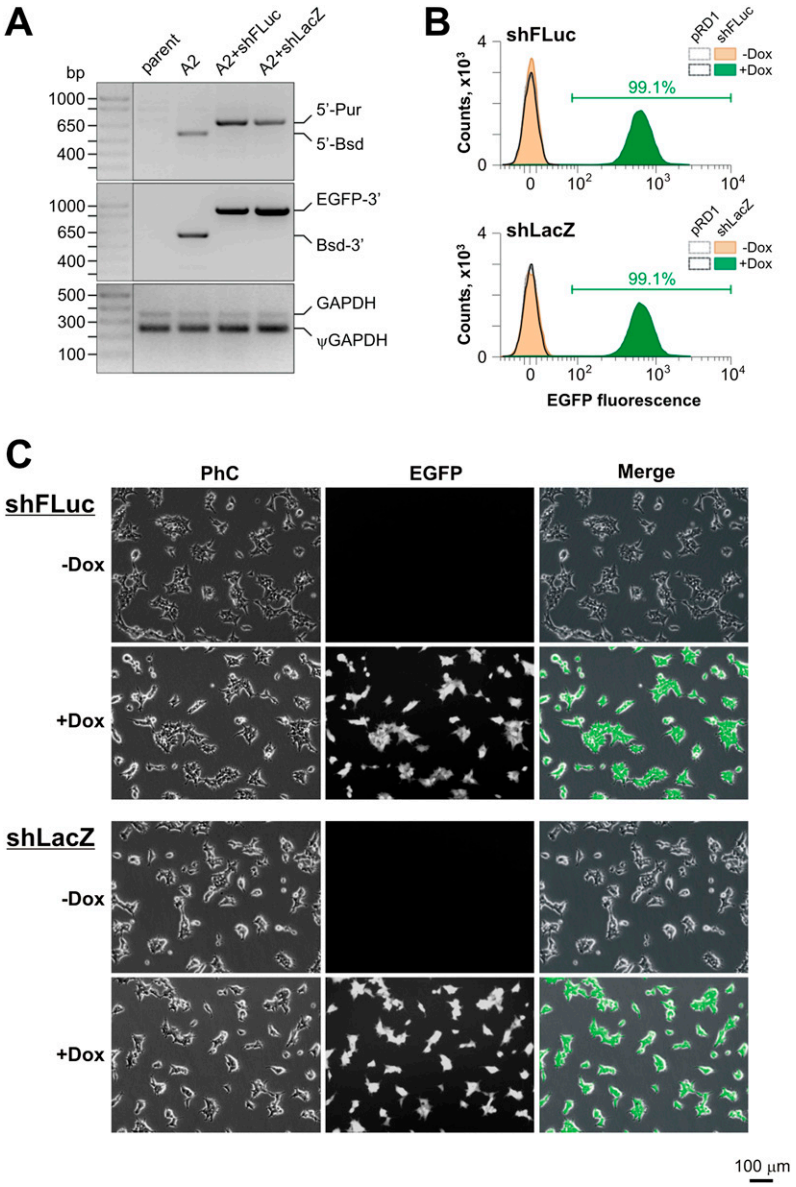

Fig. S5. HILO-RMCE generates virtually homogeneous cell populations expressing Dox-inducible shRNAs. (*A*) Genomic DNAs from the parental HEK293T line, the acceptor HEK293T-A2 line, and pooled HILO-RMCE clones containing RIPE-encoded shFLuc or shLacZ shRNAs were analyzed by multiplex PCR to detect the changes at the 5′ (primers EF, BR, and PR; Fig. 2*A* and Table S1) and the 3′ boundaries (GF, BF, and WR; Fig. 2*A* and Table S1) of the acceptor locus following the integration of the RIPE cassettes. Note that the DNA from the original acceptor line generates the 5′-Bsd and Bsd-3′ PCR products, whereas the HILO-RMCE-targeted cell pools give rise to the 5′-Pur and EGFP-3′ products. No PCR products are formed in the reactions containing the parental HEK293T DNA, as expected. GAPDH, control amplifications with primers detecting the bona fide GAPDH gene and a pseudogene (ψGAPDH). (*B* and *C*) HILO-RMCE colonies produced by cotransfecting HEK293T-A2 with pCAGGS-nlCre and pRD1 or modified pRD-RIPE plasmids encoding either shFLuc or shLacZ shRNAs were pooled and incubated with 2 μg/mL Dox for 48 h or left untreated. The EGFP expression was then studied using either (*B*) FACS or (*C*) epifluorescence combined with the phase contrast (PhC) microscopy. Note that virtually all cells become EGFP-positive in the pRD-RIPE samples treated with Dox but not in the corresponding Dox-negative controls.

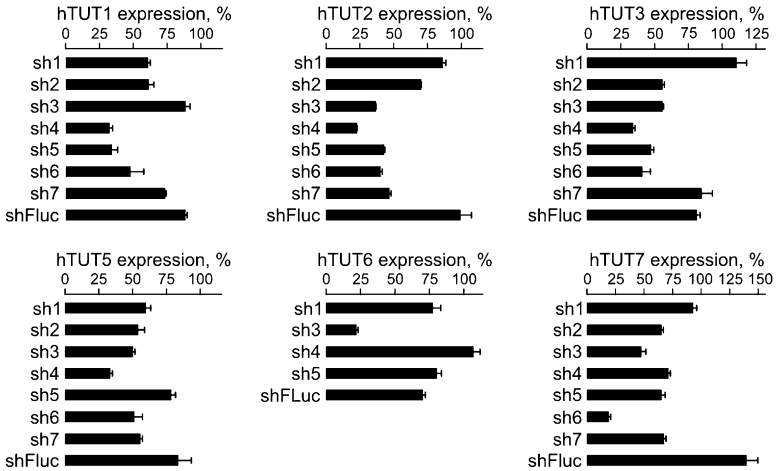

Fig. S6. Optimizing the human *TUT* (terminal uridyl transferases) family RNAi efficiency with HILO-RMCE. An shRNA library against the human *TUT* family was integrated into HEK293T-A2 cells in an arrayed format. The cells were induced with Dox for 72 h or left untreated and the corresponding knockdown efficiencies determined by RT-qPCR. The *TUT* expression levels in the Dox-treated samples are normalized to the corresponding Dox-negative controls. Data are averaged from three amplifications experiments ± SD.

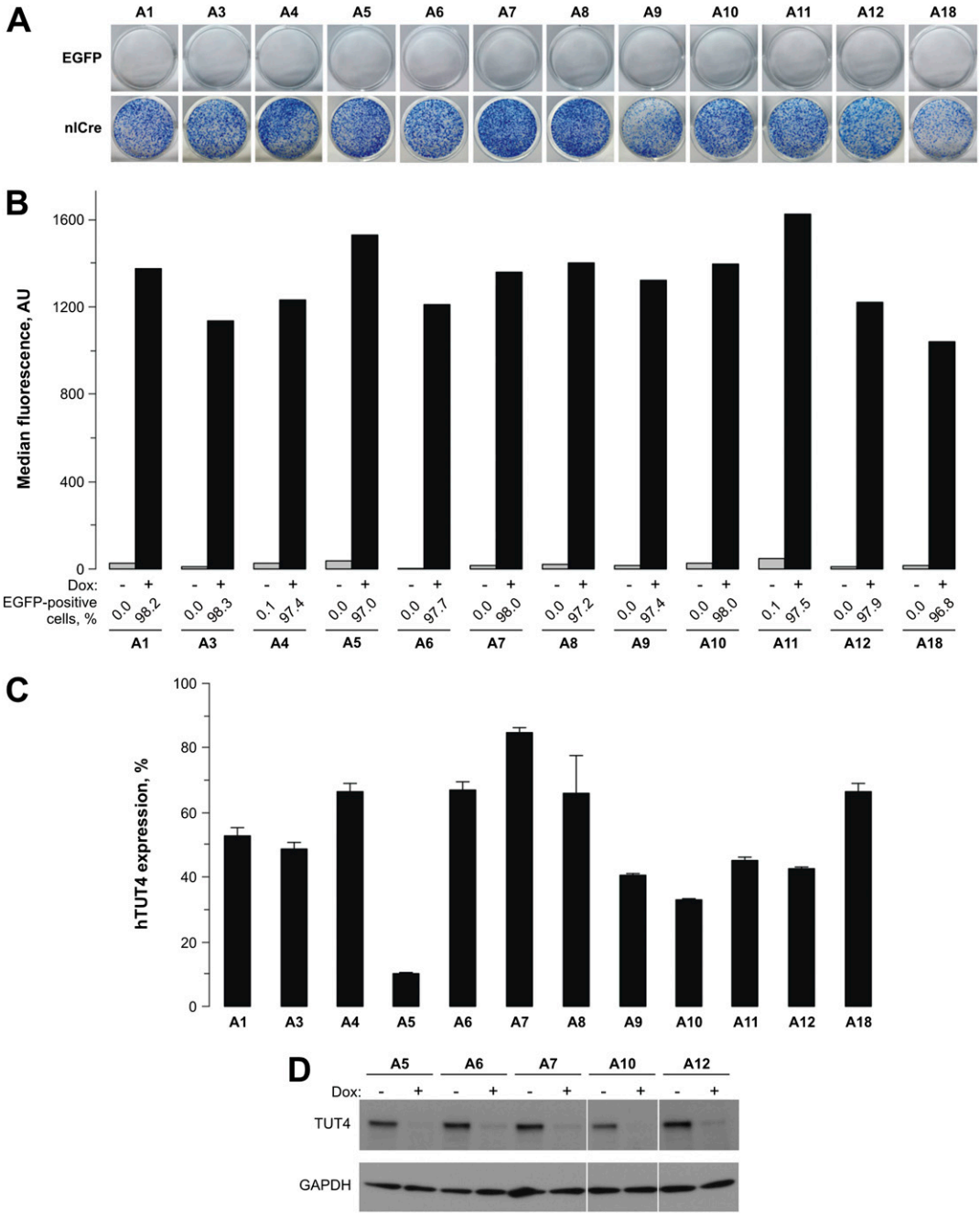

Fig. S7. Identification of HeLa RMCE acceptor clones exhibiting efficient RNAi. (*A*) Twelve independent HeLa acceptor clones were cotransfected in a 12-well format with a mixture containing 99% of pRD-RIPE plasmid and 1% of either pCAGGS-nlCre plasmid (nlCre) or the EGFP-encoding control plasmid pCIG (EGFP). Following the puromycin selection, multiple colonies formed in the presence of pCAGGS-nlCre but not in the presence of pCIG. Note that all 12 clones showed adequate HILO-RMCE efficiencies. (*B*) The experiment in *A* was repeated and the 12 RIPE cassette-containing cell populations were postcultured for 72 h with or without Dox. The cellular EGFP expression levels were then assayed by FACS and the results were plotted as median fluorescence intensities. Percents of EGFP-positive cells in each population calculated as outlined in Fig. 2*E* and Fig. S5*B* are indicated at the bottom of the graph. (*C* and *D*) A RIPE-encoded shRNA against the human *TUT4*/*ZCCHC11* gene was integrated into the 12 HeLa-A clones, induced with Dox for 72 h and the efficiency of the PTBP1 knockdown was analyzed by (*C*) RT-qPCR and (*D*) immunoblotting with TUT4-specific antibodies. The RT-qPCR graph in *C* shows relative TUT4 expression levels normalized to the corresponding Dox-negative controls.

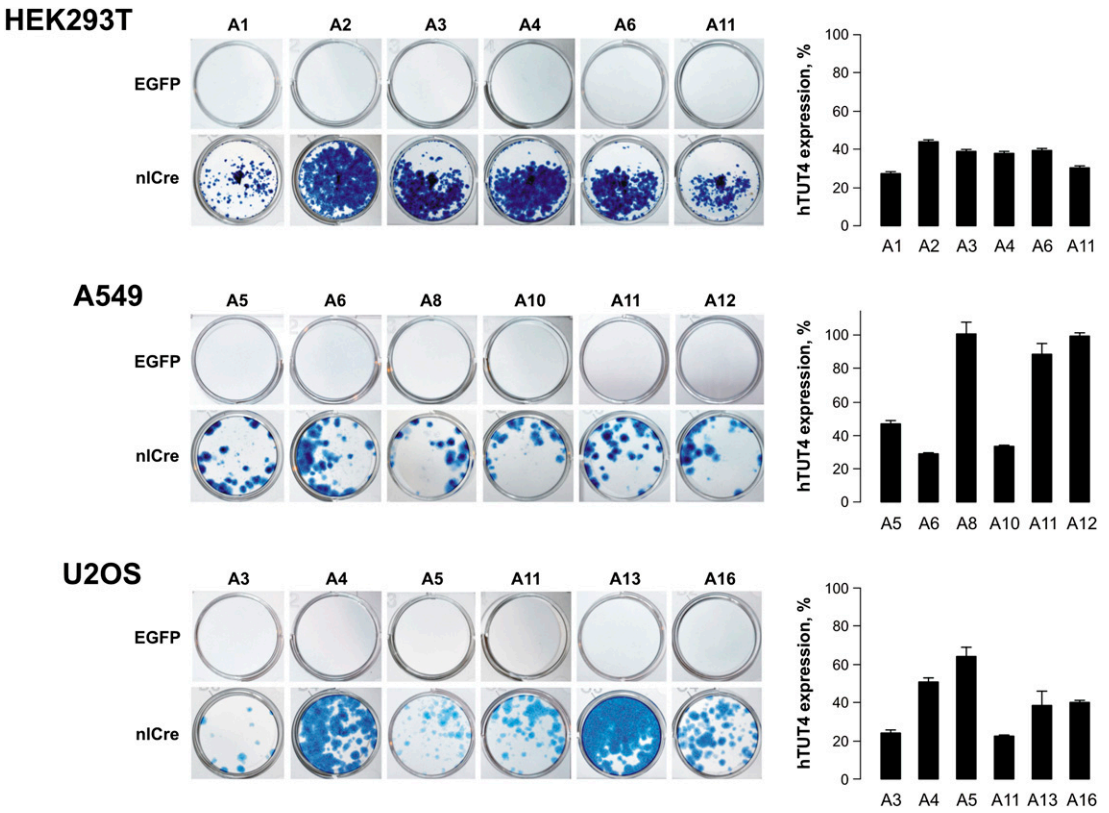

Fig. S8. Identification of RNAi-proficient RMCE acceptor clones for three human cell lines. The experiments described in Fig S7 were repeated with six independent acceptor clones for each of the HEK293T, A549, and U2OS cell lines. (*Left*) Cells were cotransfected in a 12-well format with 99% of pRD-RIPE plasmid and 1% of either pCAGGS-nlCre plasmid (nlCre) or the EGFP-encoding control plasmid pCIG (EGFP) and the RMCE-derived colonies were allowed to form in the presence of puromycin. (*Right*) A RIPE-encoded human TUT4/ZCCHC11-specific shRNA was integrated into the corresponding acceptor cell genomes, the cells were treated with Dox for 72 h and the TUT4 knockdown efficiencies assayed by RT-qPCR. The TUT4 mRNA expression levels are normalized to the corresponding Dox-negative controls. Data are averaged from three amplifications experiments ± SD.

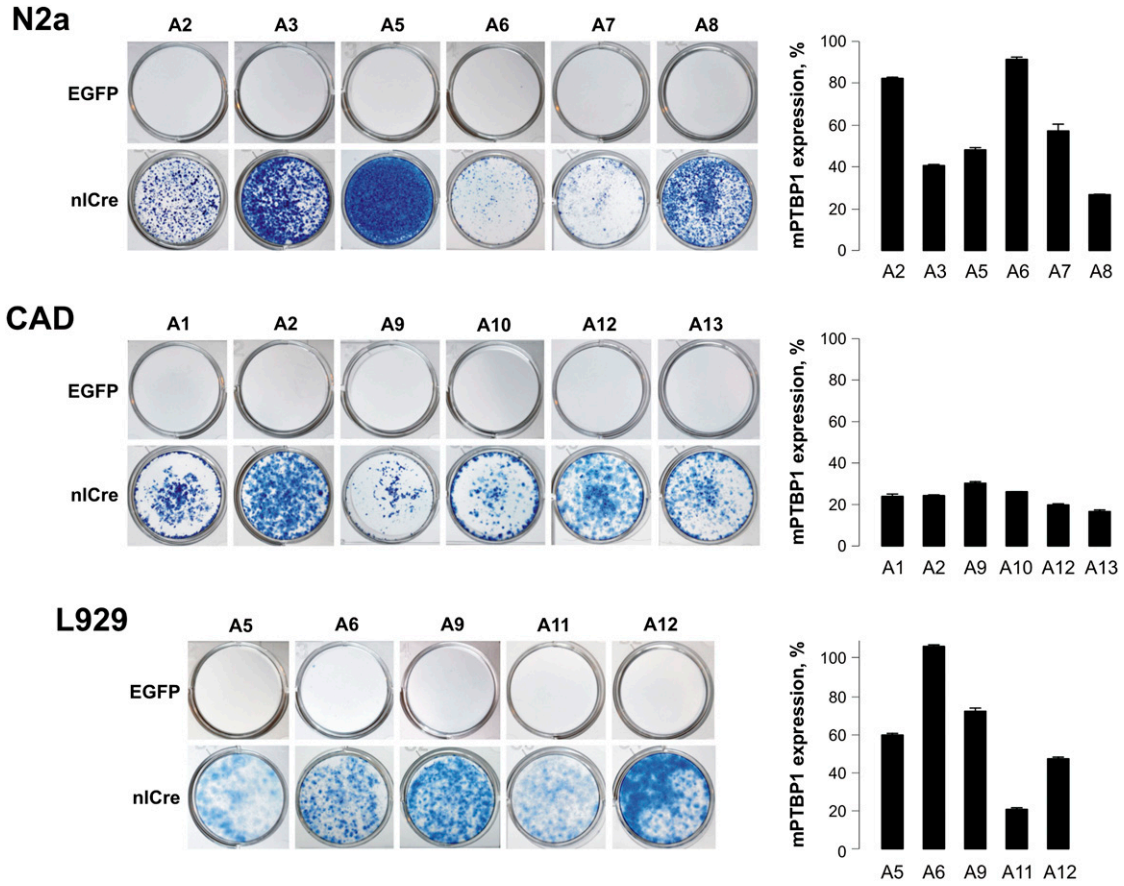

Fig. S9. Identification of RNAi-proficient RMCE acceptor clones for three mouse cell lines. Similar to the optimization experiments presented in Figs. S7 and S8, five to six independent acceptor clones for each of the N2a, CAD, and L929 cell lines were tested for their RNAi performance. (*Left*) Cells were cotransfected in a 12-well format with 99% of pRD-RIPE plasmid and 1% of either pCAGGS-nlCre plasmid (nlCre) or the EGFP-encoding control plasmid pCIG (EGFP) and the RMCE integration events selected with puromycin. (*Right*) The acceptor cells containing a RIPE-encoded mouse Ptbp1-specific shRNA were induced with Dox for 60 h (CAD-A clones) or 72 h (N2a-A and L929-A clones) and the Ptbp1 knockdown efficiency was assayed by RT-qPCR. The Ptbp1 mRNA expression levels are normalized to the corresponding Dox-negative controls and averaged from three amplifications experiments ± SD.

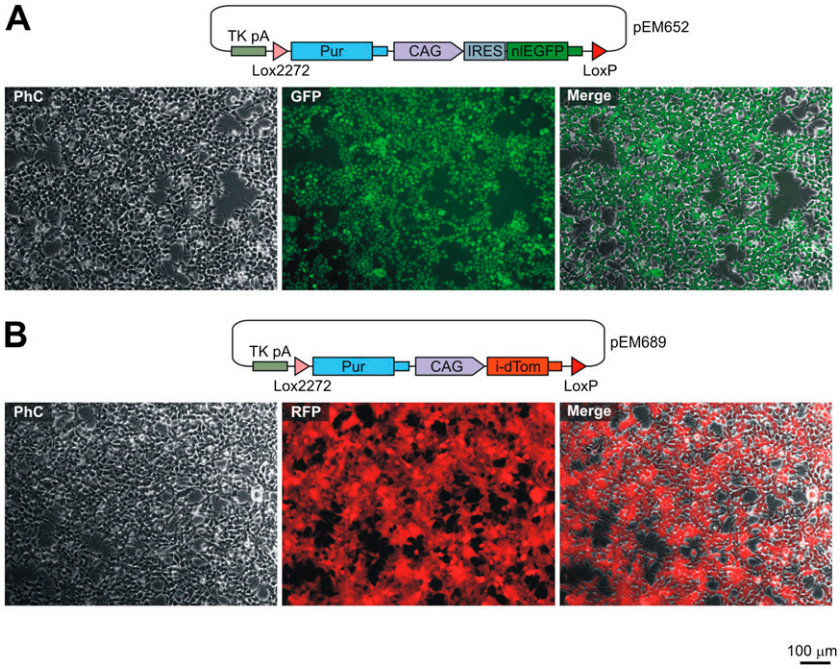

Fig. S10. Using HILO-RMCE for engineering of transgenic cell populations. HEK293T-A2 cells were cotransfected with pCAGGS-nlCre and either of the two pRD vector-based donor plasmids encoding (*A*) CAG-driven EGFP gene containing a nuclear localization sequence and preceded by an internal ribosomal entry site (IRES-nlEGFP) or (*B*) CAG promoter-driven intron-containing dTomato gene (i-dTom). Recombinant cells were selected with puromycin for 7 d, pooled and propagated for another 4 d. Images were taken using phase contrast and epifluorescence microscopy. Maps of the corresponding donor constructs (pEM652 and pEM689) are shown on the upper part of the panels.

Table S1. Oligonucleotides used in this work

Name	Sequence, 5′ to 3′
Cloning, genomic DNA analyses, probe amplification	
shRNAdimer_F1	GAGAAGACAATTGCGTTTCGGTCGACTT
shRNAdimer_R1	*AT*CTCGA*GAATTC*GGCCATTTGTTCCAT
EF	CCAGCTTGGCACTTGATGT
BR	TAGCCCTCCCACACATAACC
PR	TCGTAGAAGGGGAGGTTGC
GF	CGACAACCACTACCTGAGCA
BF	GCAACGGCTACAATCAACAG
WR	GGGCCACAACTCCTCATAAA
Vector_F1	AAGCCATACCAAACGACGAG
Vector_R1	ACGCTCAGTGGAACGAAAAC
hGAPDH_F1	CCTGACCTGCCGTCTAGAAA
hGAPDH_R1	CCCTGTTGCTGTAGCCAAAT
South584_F1	CCAAGCCTTTGTCTCAAGAA
South584_R1	GAGATCCGACTCGTCTGAGG
RT-PCR and RT-qPCR	
hHPRT1_F1	GTTTGTTGTAGGATATGCCCTTGA
hHPRT1_R1	ACTAAGCAGATGGCCACAGAACTA
hPTBP1_F1	TGACCAAGGACTACGGCAAC
hPTBP1_R1	CCATTGCTGGAAAACAGGAC
hTUT1_F1	TCGTGAGGTTCTCACACCAG
hTUT1_R1	GCACCGTACACTGAACACCA
hTUT2_F1	CGAAGATACTCAATGCCACCA
hTUT2_R1	CTCTCTGCAGCTGTGTTCGAC
hTUT3_F1	ACCGAGTAGGGTCGCAAGAT
hTUT3_R1	TGCTGGAAGAACGAAAGAGC
hTUT4_F1	GATGTGACATTGGGGATGCT
hTUT4_R1	AGCATTCCATCCATCAACCA
hTUT5_F1	TAGGGGTTGCTCCTGTTCCT
hTUT5_R1	GGACAGTTTCATGCCGTTGT
hTUT6_F1	CTTTCCCAGGGATGTGGATT
hTUT6_R1	TGTGCTGGGACTGTGACAAG
hTUT7_F1	GCTGGCCCCAAATGATAGAT
hTUT7_R1	AGGGCATCTTCCTGATCTCG
mAgo2_F1	TGCACACGCTCTGTGTCAAT
mAgo2_R1	CAGTGTGTCCTGGTGGACCT
mCltb_F1	GAAAGACCTGGAGGAGTGGA
mCltb_R1	CTAGCGGGACAGTGGTGTTT
mGapdh_F1	AAATGGGGTGAGGCCGGTGC
mGapdh_R1	ATCGGCAGAAGGGGCGGAGA
mHprt1_F1	CCAGACAAGTTTGTTGTTGGA
mHprt1_R1	TTTACTAGGCAGATGGCCACA
mPtbp1_F1	AGTGCGCATTACACTGTCCA
mPtbp1_R1	CTTGAGGTCGTCCTCTGACA
mPtbp2_F1	TGGCTATTCCAAATGCTGCT
mPtbp2_R1	TCCCATCAGCCATCTGTATC
mSrc_F1	ACCTTTGTGGCCCTCTATGA
mSrc_R1	CACATAGTTGCTGGGGATGT

To improve the mAgo2 knockdown efficiency, the mAgo2 sh4 shRNA element was amplified using the RIPE-specific primers shRNAdimer_F1 and shRNAdimer_R1; the PCR fragment was treated with MfeI (New England Biolabs; underlined sequence) and inserted into the mAgo2 sh1-containing RIPE cassette at the EcoRI-EcoRV sites located downstream of the miR-155 element to create an intron-encoded sh1-sh4 tandem. Because the shRNAdimer_R1 primer restores both the EcoRI and the EcoRV sites (italicized sequences), this procedure can theoretically be repeated to generate tandem shRNA arrays of any desired length and complexity.