SUPPLEMENTARY DATA

Non-integrating gamma-retroviral vectors as a versatile tool for transient zinc-finger nuclease delivery

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Supplementary Methods

Cell transfection

For plasmid-based EGFP knockout U2OS-EGFP cells carrying 3 copies of EGFP gene were plated on a 12-well plate and transfected by calcium phosphate precipitation method using 200 and 600 ng of ZFN-expression plasmids, 100 ng of pRK5.mCherry vector as a transfection control, and pUC118 to a total of 1.6 µg/well. At day 2 and 5 post-transfection cells were analysed by flow cytometry (FACS Calibur, Becton Dickinson). ZFN-related toxicity has been determined by the decrease in mCherry positive cell fractions from day 2 to day 5, normalized to cells transfected with empty plasmid [1].

Synthesis and transfection of ZFN encoding mRNA

Synthetic mRNA encoding ZFNs was produced using the mMessage mMachine Kit (Ambion) and ZFN expression vectors in the pVAX backbone, which contains the T7 promoter upstream of the ZFN coding sequences. Nucleofection of 1 x 10^6 K562-EGFP cells with 5 or 10 µg of mRNA was performed using the Amaxa Nucleofector II and Cell Line Nucleofector Kit V (Lonza), according to the manufacturer's recommendations with program T-016. Immediately after nucleofection, cells were transferred to 12-well plates for suspension cells containing pre-warmed RPMI complete medium supplemented with 0.5 µg/ml of interferon inhibitor B18R (recombinant protein carrier free; eBioscience) with or without MG132 (Calbiochem).

Quantification of vector copy number

Retroviral vector copy numbers were determined as previously described [2].

Supplementary references

1 Cornu, T. I. *et al.* DNA-binding specificity is a major determinant of the activity and toxicity of zinc-finger nucleases. *Mol Ther* **16**, 352-358, doi:10.1038/sj.mt.6300357 (2008).

2 Kuehle, J. *et al.* Modified Lentiviral LTRs Allow Flp Recombinase-mediated Cassette Exchange and In Vivo Tracing of "Factor-free" Induced Pluripotent Stem Cells. *Mol Ther*, doi:10.1038/mt.2014.4 (2014).

Figure S1

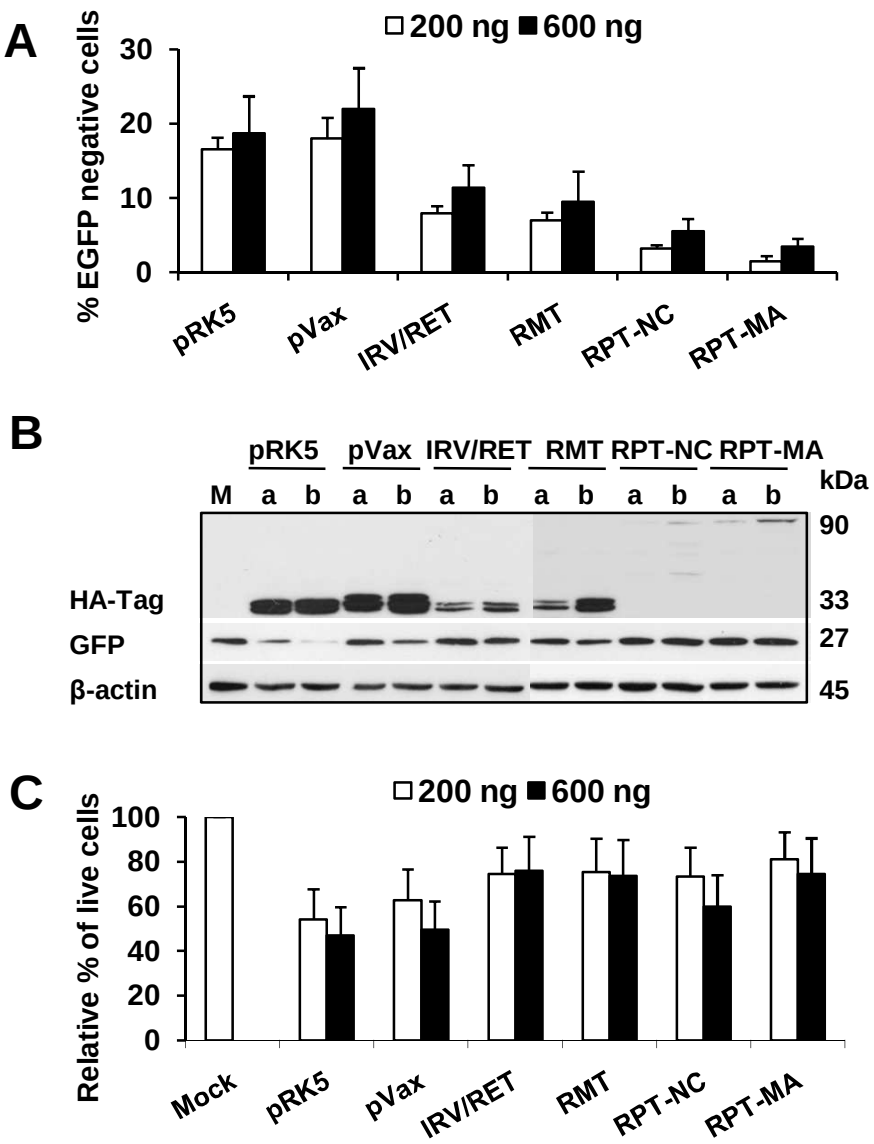

Figure S1. ZFN activity and toxicity in a plasmid-based assay. Human osteosarcoma cell line U-2 OS carrying 3 copies of destabilized EGFP gene was co-transfected with 200 or 600 ng of plasmid DNA expressing or encompassing ZFN sequence in retroviral vector backbones along with 100 ng of mCherry expression vector for transfection control using calcium phosphate method. **A.** EGFP(292)-ZFN activity. The reduction in EGFP expression was measured by flow cytometry at day 5 post-transfection and is shown as percentage of EGFP negative cells after background subtraction from mock-treated cells. **B.** Western blot analysis of ZFN expression and activity. Cells were harvested 48 h after transfection and the cell lysates were probed with antibodies against HA tag, GFP and β-actin. The lines correspond to the vector type shown in A. Small letters a and b represent the plasmid DNA dose, 200 or 600 ng, respectively. M – mock transfected cells. **C.** ZFN-related toxicity. The relative cell survival was determined as the percentage of mCherry-positive cells remaining in the populstion from day 2 to 5 post-transfection, normalized to mock control. Abbreviations: pRK5 – parental vector backbone for expression and cloning ZFN; pVax – expression vector; IRV/RET – pRSF91.ZFN.pre vector for production of integraiting or episomal retroviral vector; RMT – pRSF91.aPBS.ZFN.pre plasmid for retrovirus-mediated mRNA transfer; RPT-NC – pcDNA3.Prot.NC.ZFN plasmid for retrovirus-mediated protein transfer from nucleocapsid position; RPT-MA – pcDNA3.Prot.MA.ZFN plasmid for retrovirus-mediated protein transfer from matrix position. The experiments were performed twice in duplicate.

Figure S2

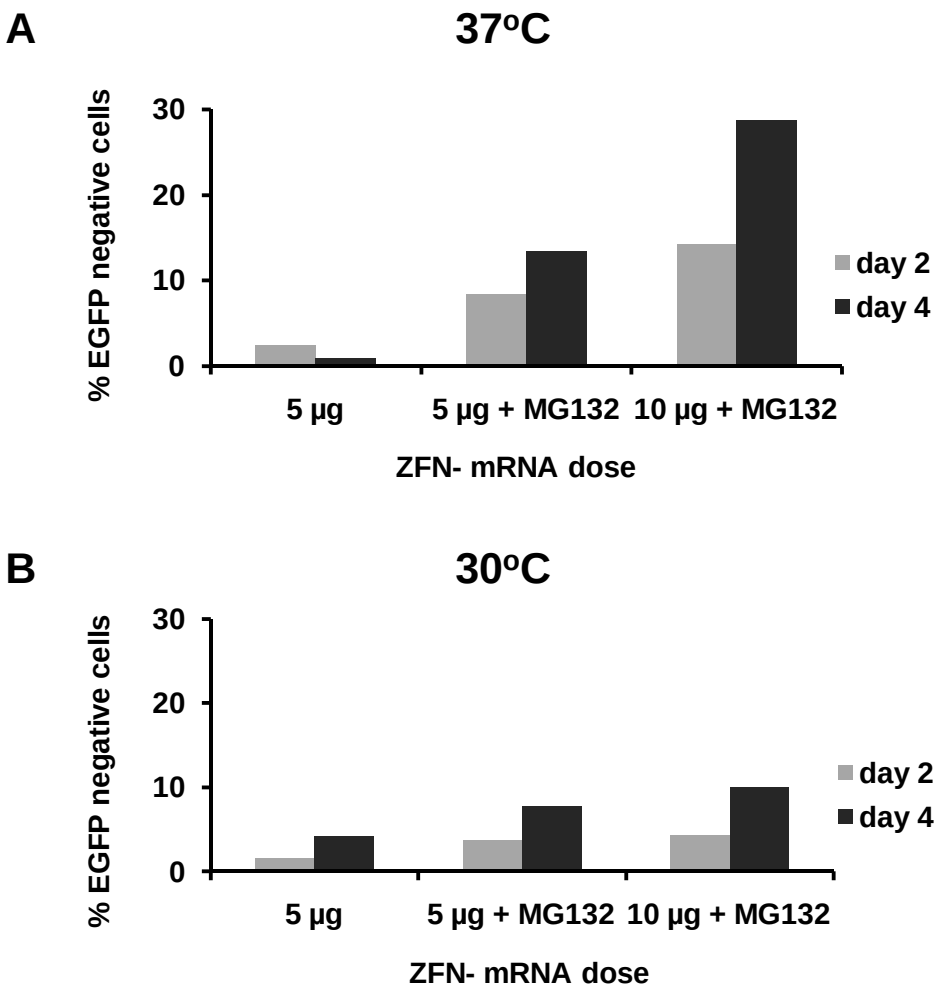

Figure S2. mRNA-ZFN delivery to K562-EGFP cells via nucleofection. The mRNA for ZFN translation was produced based on the pVax vectors containing ZFN sequence under T7 promoter using the mMessage mMachine kit (Ambion) and following the vendor's recommendations. The mRNA was purified using lithium chloride precipitation method and was denatured for 10 min at 50°C to remove secondary structures. For nucleofection 1 x 10^6 K562-EGFP cells were mixed with 5 or 10 µg of ZFN-mRNA and pulsed using Nucleofector program T-016 on Amaxa Nucleofector II. The Cell Line Nucleofector kit V (Amaxa/Lonza) was utilized according to the manufacturer's instructions. After nucleofection cells were plated on a 12-well plate for suspension cells in 2 ml RPMI complete medium supplemented with 0.5 µg/ml interferon inhibitor B18R and 0.5 µM MG132. After 1 h incubation at 37°C cells were divided (1:1) onto two plates and cultured either at 37 or 30°C. 12 h post-nucleofection cells were washed with PBS and medium was completely changed to remove the MG132. The decline in EGFP signal was measured 2 and 4 days after nucleofection by flow cytometry on FACS Calibur (Becton Dickinson). Mock values are subtracted from samples. **A.** ZFN-mediated EGFP knockout at 37°C. **B.** ZFN-mediated EGFP knockout at 30°C.

Figure S3

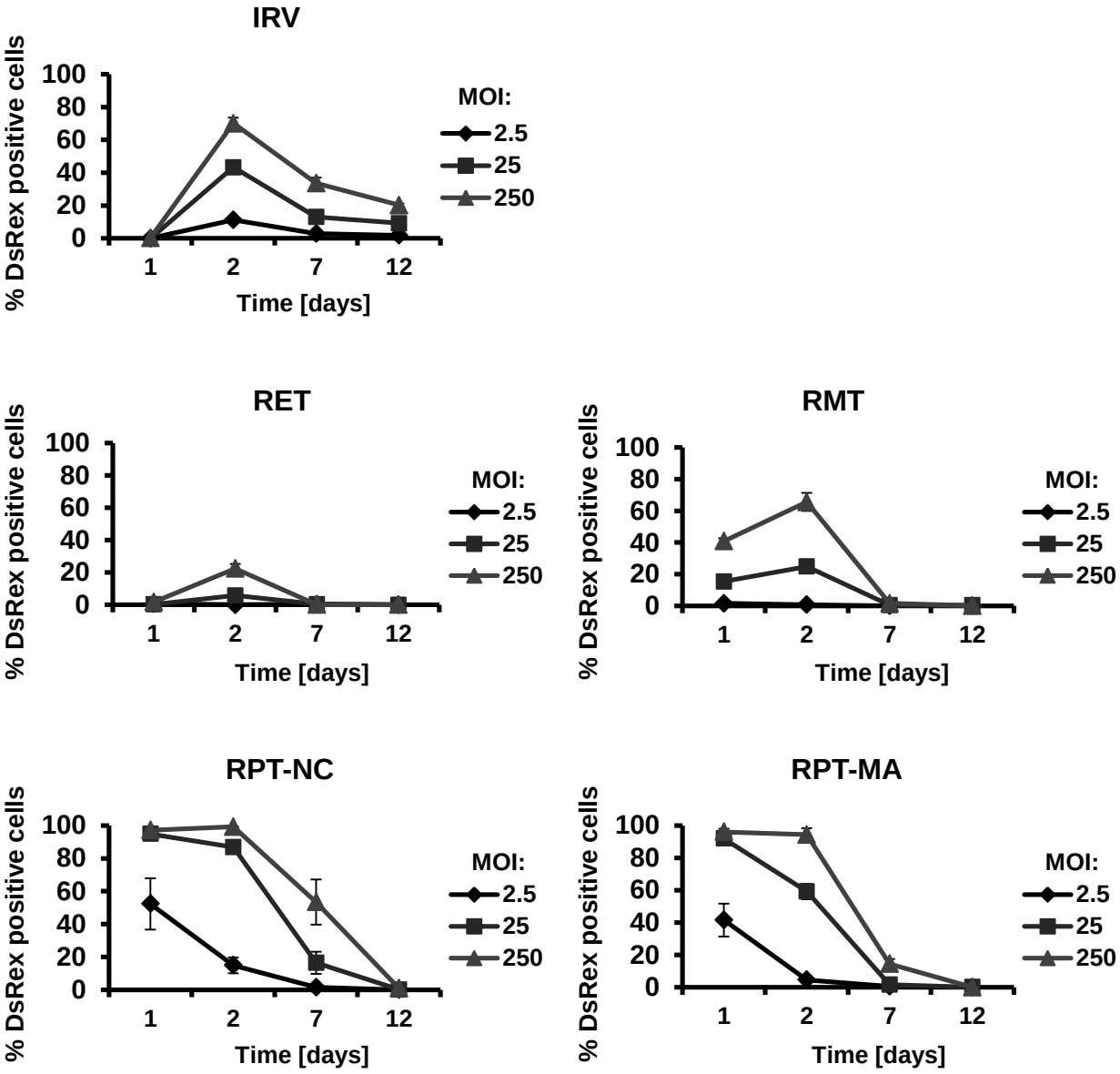

Figure S3. Kinetics and dose-dependent expression of DsRex protein in mES-EGFP cells. mES-EGFP cells containing one copy of EGFP gene at the HPRT locus were transduced with increasing doses of VSV-g-pseudotyped retroviral vectors (MOI 2.5, 25, 250) expressing a marker protein DsRed Express. After transduction cells were kept at 30^{o}C for 5 days then at 37^{o}C for the rest of the experiment. Expression of DsRed Express was measured by flow cytometry at indicated time points. The experiment was repeated twice in duplicate. Mock values are subtracted from samples. Abbreviations: IRV – integrating retroviral vector, RET – retrovirus-mediated episomal transfer, RMT – retrovirus-mediated mRNA transfer, RPT – retrovirus-mediated protein transfer, NC – nucleocapsid position of a transgene, MA – matrix position of a transgene.

Figure S4

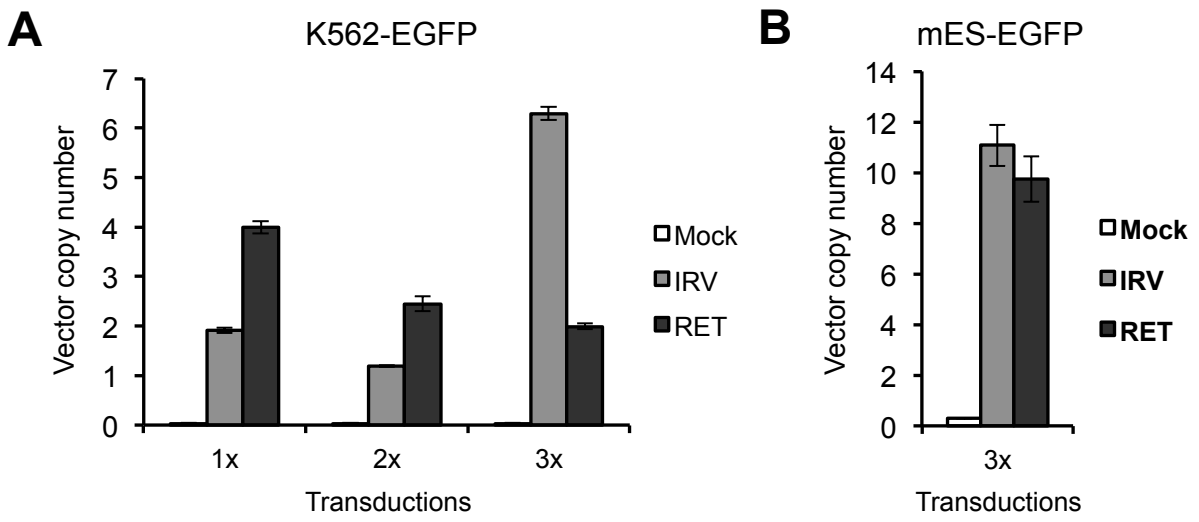

Figure S4. Vector copy numbers in K562-EGFP and mES-EGFP cells after serial transductions. (**A**) K562-EGFP cells. Cells were transduced 1x, 2x or 3x with integrating (IRV) or episomal (RET) retroviral vectors at an MOI of 2 and harvested at day 5 after transduction. (**B**) mES-EGFP cells. Cells were transduced 3x with IRV and RET vectors and harvested at day 3 after the last transduction. Total DNA from both cell lines was isolated and subjected to quantitative PCR. The vector copy number was calculated as the ratio between copy number of the post-transcriptional regulatory element (PRE) contained in the retroviral vector and the endogenous PTBPT2 gene.

Figure S5

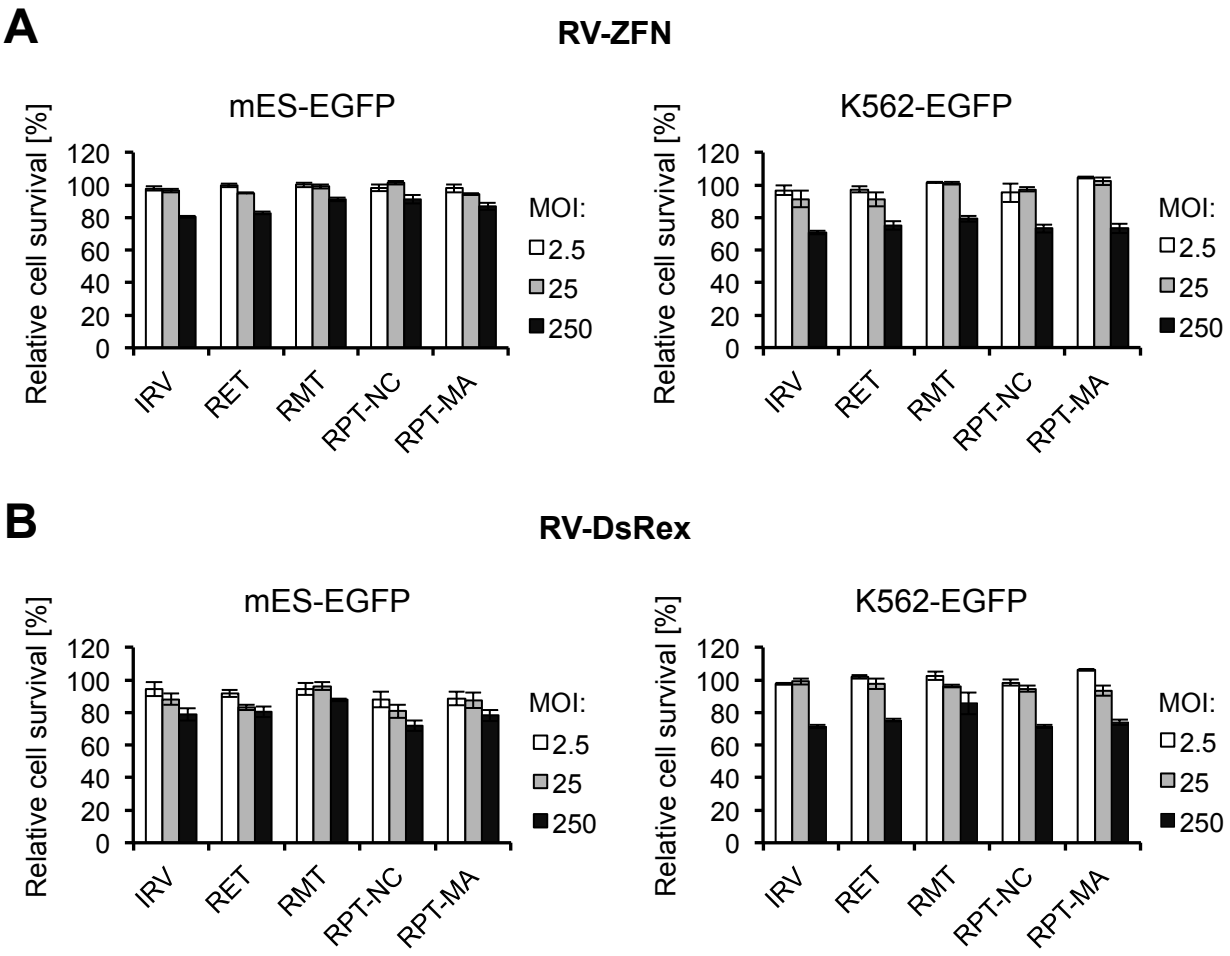

Figure S5. Relative cell survival upon transduction with retroviral vectors expressing ZFNs or DsRex. Cell lines mES-EGFP and K562-EGFP were transduced with increasing doses of retroviral vectors (MOI 2.5, 25, 250) expressing either ZFNs (**A**) or the marker protein DsRex (**B**). The relative cell survival is indicated as the percentage of live cells detected at day 2 after transduction and normalized to mock-transduced cells. IRV, integrating retroviral vector; RET, retrovirus-mediated episomal transfer; RMT, retrovirus-mediated mRNA transfer; RPT, retrovirus-mediated protein transfer; -NC, nucleocapsid position of the transgene; -MA, matrix position of the transgene.