SUPPLEMENTARY MATERIALS AND METHODS AND SUPPLEMENTARY DATA (Holkers et al. 2011).

Construction of the episomal HR assay system

Using PCR-based site-directed mutagenesis, an XmaJI cleavage site was introduced into the GFP-coding sequence of the starting construct that served as basis to generate the acceptor plasmid panel (Figure 1B). This maneuver resulted also in the disruption of the GFP *ORF* by a stop codon contained within the XmaJI recognition sequence. Next, DNA fragments composed of hybridized synthetic oligodeoxyribonucleotides with XmaJI-compatible cohesive termini encompassing the test sequences 1 through 4 (45-mers) or the recognition sequence for the I-SceI meganuclease (42-mers; Figure 1B, orange) were individually inserted at the PCR-created XmaJI site (Figure 1B). The four test sequences consisted of two different DNA segments of 25 bp (Figure 1B, red and cyan) extended at the 3' end with two different 9-bp DNA sequences (Figure 1B, green and violet) and a BcuI recognition sequence (Figure 1B, grey). Subsequently, another copy of each of the four test sequences was added at the BcuI site leading to acceptor plasmids in which the *GFP* ORF is interrupted by the direct repeats DR.1, DR.2, DR.3 or DR.4 or the inverted repeats IR.1, IR.2, IR.3 or IR.4 (Figure 1C). Due to their identical GC content, the four different test sequences were predicted to possess very similar and low folding free energies when in a tandem antiparallel orientation while those corresponding to their arrangement in a direct repeat configuration were calculated to be rather high (Figure 1C). Thus, on theoretical grounds the inverted repeats of the four test sequences have a

higher likelihood to originate secondary structures via intrastrand hybridization than their isogenic counterparts displaying a direct repeat arrangement (Figure 1C).

DNA transfections

HeLa cells were transfected using ExGen 500 *in vitro* Transfection Reagent (Fermentas). Typically, 8×10^4 cells were seeded in a well of a 24-well plate (Greiner Bio-One). The next day, a total of 400 ng DNA (i.e. 200 ng pUC19 DNA + 200 ng donor plasmid or acceptor construct, 200 ng donor plasmid + 200 ng acceptor construct or 133 ng donor plasmid + 133 ng acceptor plasmid + 133 ng pCAG.I-SceI) in 47.2 µl of 150 mM NaCl was mixed with 2.8 µl of transfection agent and added to the culture medium. After overnight incubation at 37°C in 90% humidified air/10% CO_2, the transfection medium was replaced by fresh medium and the cells were placed back in the incubator until further use. All other cell types were transfected with the aid of 25-kDa linear polyethyleneimine (PEI; Polysciences). Typically, 2×10^5 cells were seeded in a well of a 24-well plate. The following day, a total of 600 ng DNA in 50 µl of 150 mM NaCl was mixed with 50 µl of PEI solution consisting of 48.2 µl of 150 mM NaCl and 1.8 µl of 1 mg/ml PEI and incubated for 15 minutes at room temperature before addition to the culture medium. The mass ratios of the input plasmids were the same as for the HeLa cell transfections. After overnight incubation at 37°C in 90% humidified air/10% CO_2, the transfection medium was replaced by fresh medium and the cells were put back in the incubator until further use. For each transfection experiment, DNA delivery efficiencies were determined by the inclusion of a reporter plasmid.

T7 endonuclease I treatment

A total of 1 µg plasmid DNA containing direct- or inverted repeat sequences was treated for the indicated time periods with 1 U of T7 endonuclease I (New England Biolabs) at 37°C in a 20-µl reaction mixture containing 1× NEB buffer 2. The digestion products were analyzed in a 1 % agarose gel in 1×TAE buffer (40 mM Tris-20 mM acetic acid at pH 8.0-8.5; 1 mM EDTA).

DNA topoisomerase I treatment

A total of 5 µg of DNA was treated with 5 U of *E. coli* DNA topoisomerase I (New England Biolabs) for 30 min at 37°C in a reaction mixture containing 1× NEB4 provided by the supplier. The enzyme was heat-inactivated at 65°C for 20 min. DNA supercoiling was assessed by agarose gel electrophoresis in the absence of ethidium bromide. DNA was visualized post-electrophoresis using ethidium bromide at a final concentration of 1.5µg/ml in 1× TAE buffer. Subsequent incubation of topoisomerase I-treated DNA with T7 endonuclease I was performed using 0.5 µg DNA per reaction in 1× NEB4 buffer.

***In vitro* mapping of the T7 endonuclease I cleavage site in acceptor DNA**

A total of 4 µg of acceptor DNA molecules containing the ITR sequence were treated with 10 U of T7 endonuclease I for 5 min at 37°C. Next, the reaction mixtures were incubated for 30 minutes at 37°C in the presence or in the absence of the restriction enzymes SalI or HincII in, respectively, 1× O^+ or in 1× Y^+ buffer (both enzymes and respective buffers were purchased from Fermentas). DNA fragment length analysis was carried out through electrophoresis in a 1% agarose gel in 1× TAE buffer.

Flow cytometry and light microscopy

The frequency of GFP-positive cells and the fluorescent signal intensity in the GFP-positive cells were determined using a BD LSR II flow cytometer (BD Biosciences). Data were analyzed with the aid of BD FACSDiva 5.0.3 software (BD Biosciences). Untransfected HeLa cells were used to set the background level of fluorescence. Typically, 10,000 viable single cells were analyzed per sample.

For the light microscopic analysis of cell cultures, an IX51 inverse fluorescence microscope equipped with an XC30 Peltier-cooled digitial color camera (both from Olympus) was used. Images were processed using Cell^{F} 3.4 imaging software (Olympus).

Southern blot analysis. One fifth (i.e. 20 µl) of the extracted extrachromosomal DNA was incubated with XbaI and DpnI. XbaI releases the *GFP* ORF plus downstream SV40 pA from the acceptor plasmids for easy screening, whereas DpnI selectively digests the prokaryotic input DNA. The resulting DNA fragments were separated in a 0.7% agarose gel in 1× TAE buffer. Next, the DNA was transferred by capillary action to an Amersham Hybond-XL membrane (GE Healthcare) using a standard Southern blot technique. The 744-bp *GFP*-specific probe was obtained by digestion of plasmid pA1.GFP.A2 with XbaI and XhoI (both from Fermentas) followed by preparative agarose gel electrophoresis. The DNA probe was labeled with EasyTide (α-^{32}P) dCTP (3000 Ci/mmol; Perkin Elmer) using the DecaLabel DNA Labeling Kit (Fermentas). Prior to their application in hybridization experiments, the radiolabeled DNA fragments were separated from unincorporated dNTPs through size exclusion chromatography using Sephadex-50 (GE Healthcare) columns. A Storm 820 PhosphorImager (Amersham Biosciences) was used

for the detection of labeled DNA. Images were acquired using the Storm scanner control 5.03 software and processed using ImageQuant Tools 3.0 software (both from Amersham Biosciences).

PCR amplification of recombination products

For the detection of two-sided HR events, the PCR mixtures contained 4 µl of template DNA, 0.4 µM of primer 1 (5'-ATGGTGAGCAAGCAGATCCTGAAG-'3) and 0.4 µM of primer 2 (5'-CCGAGAAGGAAGTGCTCC-3'), 0.4 mM of each dNTP (New England Biolabs), 1× Hi-Fi reaction buffer and 1 U of VELOCITY DNA polymerase (both from Bioline) in a final volume of 50 µl. The PCRs were performed in a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad) using the following cycling conditions: 3 min at 98°C, followed by 30 cycles of 30 sec at 98°C, 30 sec at 66°C and 30 sec at 72°C, and a final extension period of 4 minutes at 72°C. Conditions for the detection of one-sided HR events were the same except for the replacement of primer 2 by primer 3 (5'-GACGTTGTAAAACGACGGCCAGT-3'). As internal control for the quantity and quality of the episomal DNA specimens, PCRs were performed using primer 4 (5'-GCCAGATTTTTCCTCCTCTCC-3') and primer 5 (5'-AACTTGTTTATTGCAGC-3'). All PCR parameters were the same as before apart from the annealing temperature, which was 54°C instead of 66°C, and the number of PCR cycles, which was changed to 26.

Cloning and sequencing of recombination products

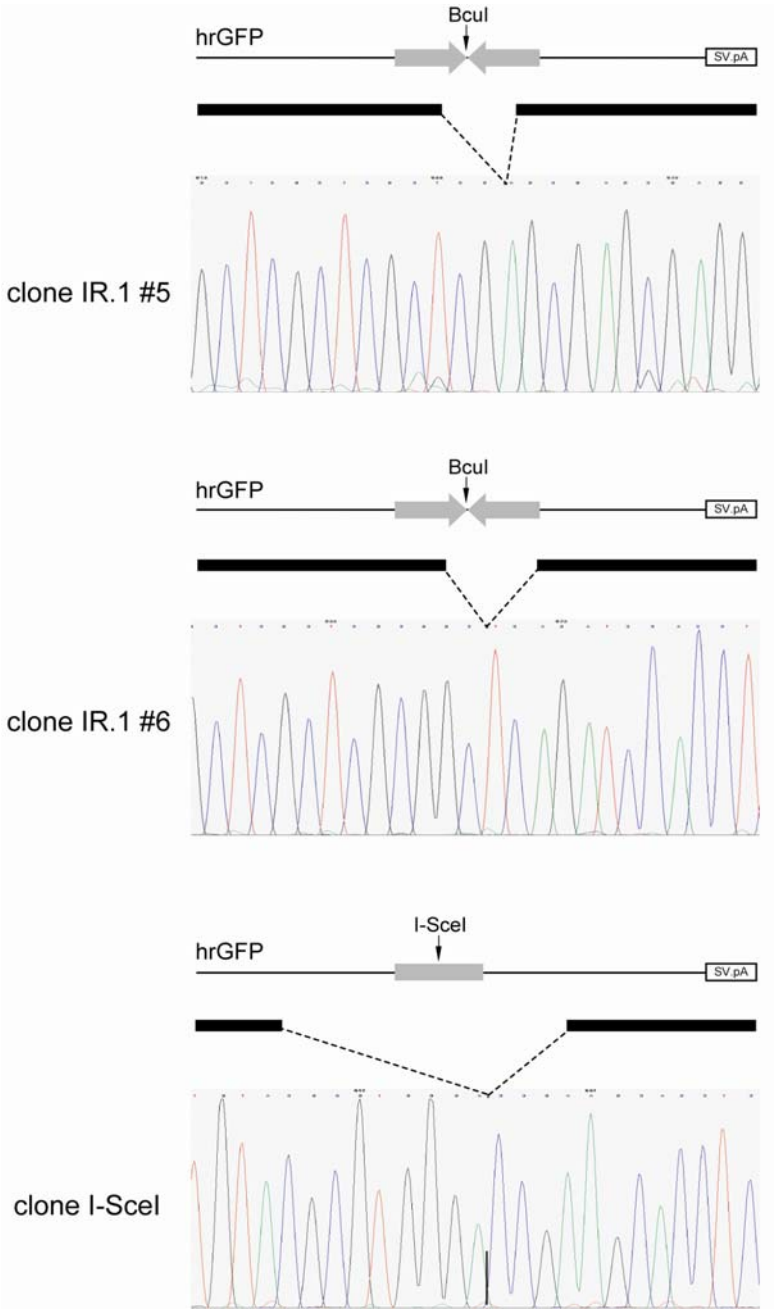
Ten µl (i.e. $1/5^{th}$) of the PCR products obtained with primers 1 and 2 was run on a 1% agarose gel, the ± 1.2-kb DNA fragments were excised from gel and purified using the

QIAEX II Gel Extraction Kit (Qiagen). Individual amplicons were then inserted into the cloning vector pJET1.2 using the CloneJET PCR Cloning Kit (Fermentas) and introduced into chemocompetent *Escherichia coli* strain DH5α.MCR cells. Individual clones were picked for small-scale DNA isolation and the resulting samples were subjected to nucleotide sequence analysis with the previously described primer 5 using the 96-capillary 3730XL DNA Analyzer and BigDye Terminator v3.1 Cycle Sequencing Kit (both from Applied Biosystems).

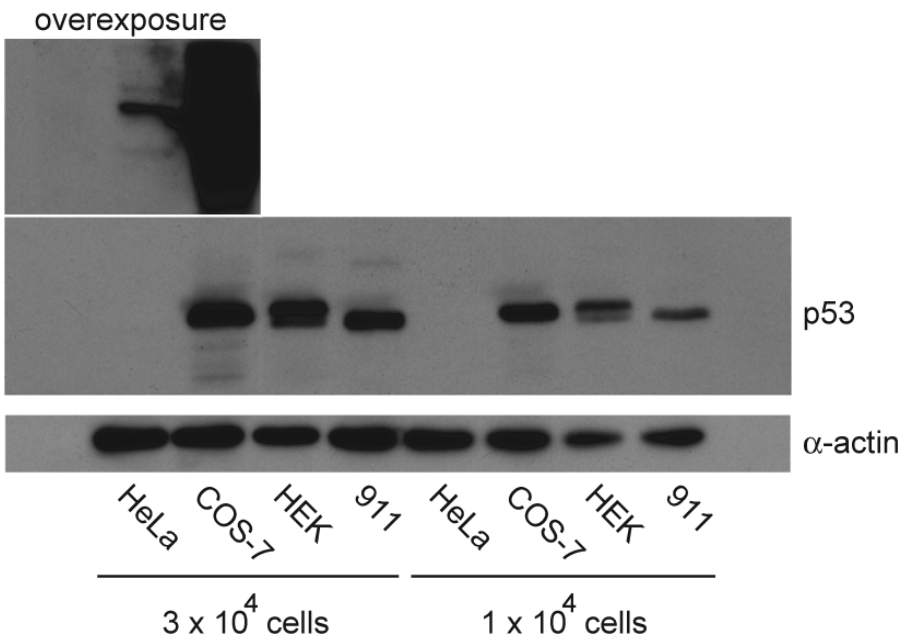
Western blot analysis

To detect p53 in the various cell line used, we performed a Western blot as follows. One-hundred thousand cells were exposed to 50 µl of RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate and 1% Nonidet P-40) containing a cocktail of protease inhibitors (Complete Mini, Roche Applied Science) and were incubated for 5 min at 4°C. The lysates were spun for 5 min at 4°C at 20.000×g after which the aqueous phase was collected. Protein extracts were pretreated for 5 min at 100°C in the presence of sample buffer (2% SDS, 50 mM Tris-Cl pH 6.8, 10% Glycerol with bromophenol blue) containing freshly-added 4% β-mercaptoethanol. The samples were loaded on a 10% SDS-PAGE gel. Electrophoresis was performed using a Bio-Rad Mini-Protean 3 system. After electrophoresis, proteins were transferred onto an Immobilon-P membrane (Millipore) using a Bio-Rad Mini Trans-Blot system. The p53 antigen was detected by using an anti-p53 primary antibody (clone DO-1 [Santa Cruz]) and a horseradish peroxidase-conjugated goat-anti-mouse secondary antibody (Santa Cruz) in TBST (0.2% Tween 20, 150 mM NaCl, 10 mM Tris) supplemented with

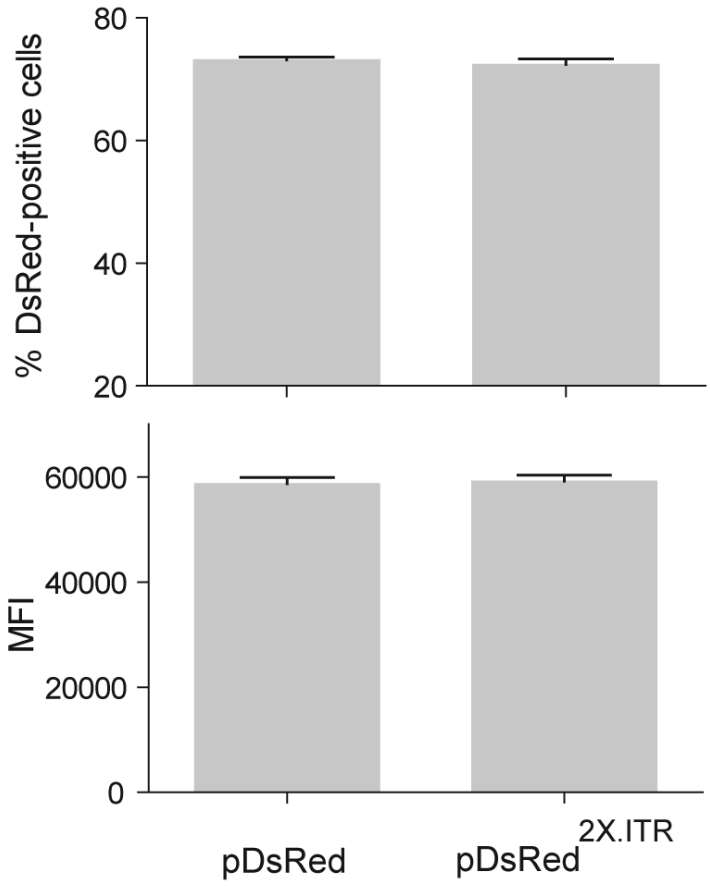
5% Elk milk powder (Campina). An anti-actin antibody (clone C4 [Millipore]) served to control for protein loading. An ECL detection method using luminol (1.25 mM 5-amino-2,3-dihydro-1,4-phtalzinedion, 100 mM Tris-Cl pH 6.8), enhancer (33 mg p-coumaric acid in 10 ml DMSO) and 0.00024% H_2O_2 was deployed for protein detection. Both primary as well as secondary antibodies were applied at a 1:1000 dilution.



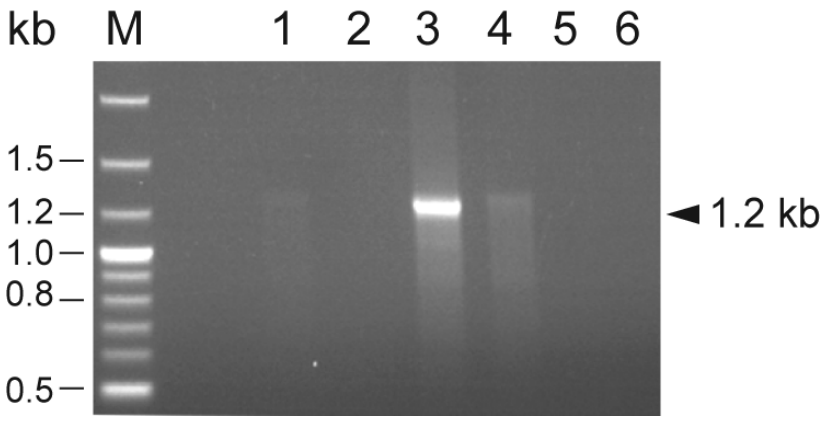
Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3



Supplementary Figure S4

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. DNA sequencing data of pCR4-TOPO-based clones 5 and 6 corresponding to PCR products amplified from extrachromosomal DNA isolated from HeLa cells transfected with acceptor$^{IR.1}$ and treared with BcuI. For comparison, the nucleotide sequence of pCR4-TOPO clone I-SceI corresponding to an amplicon obtained from episomal DNA of HeLa cells co-transfected with acceptor^{ScR} and pCAG.I-SceI and incubated with I-SceI, is also presented. Rearranged DNA templates are depicted as solid lines and are drawn in relation to their respective parental species. Breakpoints are identified by broken lines. For an explanation of the other symbols see legend of Figure 1A.

Figure S2. Western blot analysis of the relative *p53* expression levels in four cell types deployed in the current study.

Figure S3. Testing the impact of secondary structure-forming DNA sequences on plasmid DNA transfection efficiency. HeLa cells were transfected with pDsRed or with pDsRed$^{2X.ITR}$. These constructs differ in that the former lacks AAV ITRs whereas the latter has two AAV ITRs flanking the DsRed.T4 expression unit. Three days post-transfection, gene transfer activities as measured in terms of the percentage of reporter-positive cells and of mean fluorescence intensity (MFI) values were assessed by flow cytometry (upper and lower graphs, respectively).

Figure S4. PCR analysis according to the scheme shown in Figure 6B of extrachromosomal DNA isolated from HeLa cells transfected with acceptor^{ITR} or with donor GFP$^{\Delta ATG}$ (lanes 1 and 2, respectively) or from HeLa cells co-transfected with acceptor^{ITR} and GFP$^{\Delta ATG}$ (lane 3). The same PCR conditions were also applied to extrachromosomal DNA isolated from HeLa cells transfected with acceptor^{ITR} and mixed, prior to PCR, 1:1 (vol/vol) with episomal DNA extracted from HeLa cells transfected with donor GFP$^{\Delta ATG}$ (lane 4). Extrachromosomal DNA from mock-transfected cells and water provided for extra negative PCR controls (lanes 5 and 6, respectively). Lane M, GeneRuler DNA Ladder Mix molecular weight marker.