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Generation of a conditionally self-eliminating HAC gene delivery vector through incorporation of a tTA^{VP64} expression cassette

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ABSTRACT

Human artificial chromosome (HAC)-based vectors represent an alternative technology for gene delivery and expression with a potential to overcome the problems caused by virus-based vectors. The recently developed alphoid^{tetO}-HAC has an advantage over other HAC vectors because it can be easily eliminated from cells by inactivation of the HAC kinetochore via binding of chromatin modifiers, tTA or tTS , to its centromeric $tetO$ sequences. This provides a unique control for phenotypes induced by genes loaded into the HAC. The alphoid^{tetO}-HAC elimination is highly efficient when a high level of chromatin modifiers as $tetR$ fusion proteins is achieved following transfection of cells by a retrovirus vector. However, such vectors are potentially mutagenic and might want to be avoided under some circumstances. Here, we describe a novel system that allows verification of phenotypic changes attributed to expression of genes from the HAC without a transfection step. We demonstrated that a single copy of tTA^{VP64} carrying four tandem repeats of the VP16 domain constitutively expressed from the HAC is capable to generate chromatin changes in the HAC kinetochore that are not compatible with its function. To adopt the alphoid^{tetO}-HAC for routine gene function studies, we constructed a new TAR-BRV- tTA^{VP64} cloning vector that allows a selective isolation of a gene of interest from genomic DNA in yeast followed by its direct transfer to bacterial cells and subsequent loading into the $loxP$ site of the alphoid^{tetO}-HAC in hamster CHO cells from where the HAC may be MMCT-transferred to the recipient human cells.

INTRODUCTION

Human artificial chromosomes or HAC-based vectors represent a novel system for gene delivery and expression that has several advantages over previous gene and cell therapy strategies (1–4). All HACs by definition contain a functional centromere and, therefore, replicate and segregate like normal chromosomes in human cells without integration into the host genome. HAC vectors are essentially limited only by our ability to generate and handle large DNA fragments, and therefore, may provide long-term expression of complete genetic loci. Several labs succeeded in complementation of gene deficiencies in human recipient cell lines and in creation of the transgenic mice using HAC vectors containing genomic copies of genes with all their regulatory elements, demonstrating their potential as therapeutic gene expression vectors (5–26).

HACs can be engineered by ‘top-down’ or ‘bottom-up’ (*de novo* formation) approaches (1–4,27,28). The top-down approach is based on telomere-associated chromosome fragmentation in the homologous recombination-proficient chicken DT40 cell line. The bottom-up approach includes transfection of human cells with either natural or synthetic alpha-satellite (alphoid) DNA arrays with a size bigger than 30 kb. After transfection of such arrays into human cells, *de novo* HACs are generated that range in size from 1 Mb to 10 Mb due to concatemerization of input constructs and amplification of alphoid DNA.

One of the most advanced HACs is the alphoid^{tetO}-HAC, engineered using a 40 kb synthetic alphoid DNA array (29). This array contains 42-bp tetracycline operator ($tetO$) sequences incorporated into every second alphoid DNA monomer. After multimerization of the input DNA in human cells, the resulting alphoid^{tetO}-HAC contains ~6000 copies of the $tetO$ sequence within the 1.1 mega-base size block of synthetic alphoid DNA (30). Because $tetO$ sequences are bound with high affinity and specificity by the

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tet repressor (tetR), they can be targeted efficiently *in vivo* with tetR fusion proteins. The power of this system is that it allows for specific manipulation of the chromatin composition of a HAC kinetochore *in vivo*, while leaving all kinetochores of the natural chromosomes unperturbed. Targeting of chromatin-modifying proteins into the HAC kinetochore demonstrated that a balance between open and condensed chromatin is critical for kinetochore function (31–34). The frequency of HAC loss was very high when either transcriptionally competent or repressive chromatin was induced in the HAC via the tTA (tet-repressor transcriptional activator containing VP16 domain) (35) or tTS (tet-repressor transcriptional silencer - this contains the KRAB-AB domain of the SD^{kid-1} protein) (36). Tethering these chromatin modifiers caused a block of CENP-A loading and stripping of other proteins of the CCAN complex (the constitutive centromere-associated network) from the HAC kinetochore. As a consequence, the functional HAC kinetochore is inactivated and the HAC is lost during cell division (29,31–34,37).

The alphoid^{tetO}-HAC with a regulated kinetochore represents a powerful system for gene function analysis because it provides a unique possibility to compare the phenotypes of target human cells with and without a functional copy of a gene inserted into the HAC. Such a rigorous control is required for proper interpretation of gene complementation studies. The power of this system was recently demonstrated in experiments for expression of three human full-length genes, *VHL* (~25 kb), *NBS1* (~60 kb), *HPRT* (~60 kb) and *BRC1* (90 kb) from the alphoid^{tetO}-HAC vector transferred in patient-derived cell lines (23,38,39).

The alphoid^{tetO}-HAC elimination by modification of centromeric chromatin is highly efficient using either retrovirus or plasmid-induced expression to provide a high level of chromatin modifiers as tetR fusion proteins. Thus, inactivation of the HAC kinetochore includes a transfection step that is potentially mutagenic. Recently, we described an approach to re-engineering the alphoid^{tetO}-HAC that allows verification of phenotypic changes attributed to expression of genes from the HAC without a transfection step (40). In this alphoid^{tetO}-HAC vector, a *tTS*-containing cassette was inserted into a gene-loading site along with a gene of interest. A relatively low level of expression of the tTS does not inactivate the HAC kinetochore but generates a self-regulating fluctuating heterochromatin on the alphoid^{tetO}-HAC that induces fast silencing of the genes on the HAC without significant effects on HAC segregation. This silencing of the HAC-encoded genes can be recovered by simply adding a ligand (doxycycline). While the alphoid^{tetO}-HAC vector carrying a single copy of *tTS* provides a simple way to verify phenotypic changes attributed to expression of genes from the HAC, persistence of the HAC with a silenced gene in cells might want to be avoided under some circumstances. For example, this is specifically required when the HAC vector is used for generation of iPS cells (3,4,41).

In this paper, we examined whether a single copy of a chromatin modifier, either the transcriptional transactivator *tTA* carrying the VP16 domain or the advanced transcriptional transactivator *tTA*^{VP64} carrying four tandem repeats of the VP16 domain (36), inserted into the alphoid^{tetO}-HAC is enough to inactivate the HAC kinetochore. We

demonstrated that *tTA*^{VP64} constitutively expressed from the HAC induces a fast inactivation of the HAC kinetochore leading to HAC loss. This feature of the alphoid^{tetO}-HAC carrying *tTA*^{VP64} is consistent with its potential broad use in gene function studies and cell reprogramming.

One advantage of HAC-based technologies is the opportunity to work with full-length genes. Transformation-associated recombination (TAR) cloning allows selective isolation of any desired allele of a gene of interest as a predetermined large chromosomal fragment in yeast (42–44). Based on our observation that a single copy transgene encoding a potent chromatin modifier may inactivate a kinetochore, we constructed a new vector, TAR-BRV- tTA^{VP64} that links the gene isolation strategy with the regulated gene expression in the alphoid^{tetO}-HAC.

MATERIALS AND METHODS

Construction of the *tTA*-containing plasmids, tetR-VP64-IRES-DsRed2 and tetR*-VP16-IRES-DsRed2

The transgene cassette tetR-VP64-IRES-DsRed2 was assembled as follows. The plasmid 264-CAG-IRES-DsRed (40) was digested with PacI/NheI, then was gel purified to remove a short (~200 bp) fragment. Next, the tetR-NLS sequence (with complete NLS) was polymerase chain reaction (PCR) amplified from the plasmid 264-CAG-OKS-EF1-tTKRAB-DsRed-pA (kindly provided by Dr. Tomilin, Institute of Cytology, RAS, Russia) with a pair of specific primers PacI-tetR-EYFP-VP16/NheI-NLSrev (Supplementary Table S1). Then the tetR-NLS fragment was gel purified, digested by PacI/NheI and ligated with the 264-CAG-IRES-DsRed plasmid digested by PacI/NheI, producing the intermediate plasmid 264-CAG-tetR-newNLS-completeIRES-DsRed. Next, this plasmid was digested by NheI/AsiSI to insert the VP64 sequence. The VP64 PCR sequence (carrying four copies of a subsection of the VP16 domain) was PCR amplified from the Cas9D10AH840A TagBFP VP64 plasmid (kindly provided by Dr. Kagansky, Edinburgh University, Scotland) with a pair of specific primers NheI-VP64for/ AsiSI-VP64rev (Supplementary Table S1). The VP64 PCR fragment was gel purified, digested with NheI/AsiSI and then ligated with the 264-CAG-tetR-newNLS-completeIRES-DsRed plasmid digested by NheI/AsiSI, producing the final plasmid tetR-VP64-IRES-DsRed2. The transgene cassette tetR*-VP16-IRES-DsRed2 was assembled similarly except it contains only one copy of the VP16 domain. Both plasmids contain the 3'HPRT-loxP cassette that allows their insertion into the single loxP loading site of the alphoid^{tetO}-HAC propagated in HPRT-minus hamster CHO or human HT1080 cells followed by reconstitution of the *HPRT* gene (45,46). Detailed physical maps of the tetR-VP64-IRES-DsRed2 and tetR*-VP16-IRES-DsRed2 plasmids are shown in Supplementary Figures S1 and S2a.

Construction of the TAR-BRV-tTA^{VP64} vector

The construction of the TAR-BRV- tTA^{VP64} vector was shown in detail in Supplementary Figure S3. Steps 1–4 removed restriction sites XmaI, EcoRI, BglII and XbaI from

the pBeloBAC11 plasmid backbone (New England Biolabs). This was carried out as follows. Step 1: The plasmid pBeloBAC11 was PCR amplified with a pair of primers B161/B196 (Supplementary Table S1). PCR product was then blunt end ligated to form plasmid A096. Step 2: Plasmid A096 was PCR amplified with a pair of primers B244/B245 (Supplementary Table S1). The PCR product was then blunt end ligated to produce the intermediate plasmid A098. Step 3: Plasmid A098 was PCR amplified with a pair of primers B246/B247 (Supplementary Table S1). The PCR product was then blunt end ligated to produce the intermediate plasmid A099. Step 4: Plasmid A099 was PCR amplified with a pair of primers B248/B249 (Supplementary Table S1). The PCR product was then blunt end ligated to produce the intermediate plasmid A102. Step 5: The TAR plasmid pVC604 (22) was PCR amplified with a pair of primers B288/B287 (Supplementary Table S1). The PCR product was then blunt end ligated to produce the intermediate plasmid A106. This step removed restriction sites BglII, NheI, BclI and HindIII sites in the *HIS3* gene of pVC604. Step 6: Then three fragments were ligated as follows. The first fragment, *CEN6-HIS3*, was PCR amplified from A106 with a pair of primers B286/B295 (Supplementary Table S1). This fragment was then digested with XhoI/BamHI. The second fragment, *ARSH4*, was PCR amplified from the plasmid pRS313 (47) using a pair of primers B294/B293 (Supplementary Table S1). The *ARSH4* fragment was then digested with Sall/XbaI. The third fragment was amplified from the plasmid A102 using a pair of primers B022/B280 (Supplementary Table S1) and then digested with NheI/BamHI. All three fragments were then ligated to produce the intermediate plasmid A116. Step 7: The plasmid A116 was PCR amplified using a pair of primers B433/B432 (Supplementary Table S1). The PCR product was then digested with MluI and ligated to produce the intermediate plasmid A152. This step removed the *ARSH4* segment and added MluI and PacI sites to the plasmid. Step 8: This step included ligation of two fragments. The first fragment, 3'*HPRT* (2256 bp in length) was obtained by digestion of the plasmid p264 (37) with XbaI/AscI. The second fragment was obtained by PCR amplification of pBluescript II KS (Stratagene) with a pair of primers B630/B631 (Supplementary Table S1). The B630 primer carries with it the loxP site. The PCR product was subsequently digested with NheI/MluI. Both fragments were then ligated to produce the intermediate plasmid A151. Step 9: The plasmid A153 was then digested with NheI/BamHI, while the plasmid A151 was digested with BglII/SpeI. Both fragments were then ligated to produce the intermediate plasmid A153. Step 10: This step was a single five-way ligation. The first fragment was obtained by PCR amplification of the plasmid pBlueScript II SK (Stratagene) with a pair of primers B489/B688 (Supplementary Table S1). This 2868 bp fragment was then digested with MluI/SacI. The second fragment, *tDNA-CAG* (2097 bp in length), was obtained by digesting the plasmid CAG-EGFP-tDNA (25,48) with SacI/XbaI digest. The third fragment, *CAG* intron (129 bp in length), was obtained by PCR amplification of the p264 plasmid (37) with a pair of primers B510/B509 (Supplementary Table S1). This fragment was then digested with SpeI/EcoRI. The fourth frag-

ment, *tetR-VP64* (842 bp in length), was obtained by PCR amplification of the plasmid tetR-VP64-IRES-DsRed2 (see above) with a pair of primers B487/B486 (Supplementary Table S1). This fragment was digested with EcoRI/BglII. The fifth fragment, *SV40pA* (240 bp in length), was PCR amplified from the plasmid BRV1 (49) with a pair of primers B619/B618 (Supplementary Table S1). This fragment was then digested with BamHI/MluI. All five fragments were ligated together in a single five-way ligation to produce the intermediate plasmid A185. Step 11: This step was a single two-way ligation. The first fragment, *tDNA-CAG-tetR-VP64* (3314 bp in length), was obtained by digesting the plasmid A185 with AsiSI/MluI. The second fragment was obtained by digesting the plasmid A153 with MluI/PacI. Ligation of both fragments produced the final TAR-BRV-tTA^{VP64} vector. A detailed physical map of the TAR-BRV-tTA^{VP64} plasmid is shown in Supplementary Figure S5.

Cell lines and media

The hypoxanthine phosphoribosyltransferase (HPRT)-deficient fibrosarcoma HT1080 cell line was obtained from Dr. Zoia L. Monaco (Wellcome Trust Centre for Human Genetics, University of Oxford, Roosevelt Drive, Oxford OX3 7BN, UK). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% (v/v) tet system-approved fetal bovine serum (Clontech Laboratories, Inc.), 1% (v/v) Penicillin-Streptomycin (10 000 U/mL) (Life Technologies) at 37°C in 5% CO₂. Hypoxanthine phosphoribosyltransferase (HPRT)-deficient Chinese hamster ovary (CHO) cells (JCRB0218) were maintained in Ham's F-12 nutrient mixture (Invitrogen) plus 10% FBS, 1% P/S and 8 µg/ml of BS. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA, USA), 1% penicillin-streptomycin (P/S; Invitrogen, USA) with 2 µg/ml of Blastidicin S (BS; Invitrogen) at 37°C in 5% CO₂. After loading of *tTA*^{VP64}-containing plasmids into the loxP site of the alphoid^{tetO}-HAC, the HT1080 and CHO cells were cultured in 1× HAT medium, 2 µg/ml Blastidicin S and 1 µg/ml doxycycline (Life Technologies).

Loading of the *tTA*-containing plasmids into the loxP site of the alphoid^{tetO}-HAC

A total of 1–3 µg of the *tTA*^{VP64} - or *TA*^{VP16} -containing construct DNA and 0.8–1 µg of the Cre expression pCpG-iCre vector DNA were co-transformed with 6 µl of XtremeGENE 9 DNA transfection reagent (Roche) into HPRT-deficient human HT1080 and hamster CHO cells containing the alphoid^{tetO}-HAC that were grown on a 6 well plate with DMEM, 10% FBS, 1% PenStrep, 2 µg/ml Blastidicin S (Life Technology) and 1 µg/ml doxycycline (Life Technology). HPRT-positive colonies were selected after 2–3 weeks growth in HAT medium. 5–7 clones were then selected in each experiment. Correct loading of the transgene cassette into the HAC was confirmed by genomic PCR with a specific pair of primers that diagnose reconstitution of the *HPRT* gene (Supplementary Table S1).

FISH analysis with a BAC probe

FISH analysis was performed as previously described (29,40). HAC-containing cells were cultured in medium with 10 $\mu\text{g/ml}$ of colcemid (Invitrogen) for 3 h at 37°C. Metaphase cells were trypsinized and collected by centrifugation for 5 min at 483 \times g, treated in hypotonic solution 50 mM KCl for 20 min at 37°C and washed three times in 10 ml of methanol:acetic acid (3:1) fixative solution. Cells were centrifuged at 174 \times g for 5 min between washes. Cells were then diluted to the appropriate density with fixative solution, spread onto precleaned slides (Fisher Scientific) above steam (boiling water), and allowed to age 2 days at room temperature. Metaphase chromosomes on the slide were denatured by 70% formamide/2 \times SSC for 2 min at 72°C. Samples were dehydrated through a 70%, 90% and 100% ethanol series for 4 min each and left to air-dry. Orange 552 dUTP (5-TAMRA-dUTP) (Abbott Molecular) labeled probes were denatured in hybridization solution at 78°C for 10 min and left at 37°C for 30 min. The hybridization mix probe was applied to the sample and incubated at 37°C overnight. Slides were washed with 0.4 \times SSC, 0.3% Tween 20 for 2 min at 72°C, briefly rinsed with 2 \times SSC, 0.1% Tween 20 (5s-1min) and air dried in darkness. The samples were counterstained with VectaShield mounting medium with DAPI (Vector Labs). Slides were analyzed by fluorescence microscopy. Images were captured using a DeltaVision microscopy imaging system in the CRC, LRBGE Fluorescence Imaging Facility (NIH) and analyzed using image J software (NIH). The probe used for FISH was BAC32-2-mer(tetO) DNA containing a 40 kb of alphoid-tetO array cloned into a BAC vector as described previously (29). BAC DNA was labeled using a nick-translation kit with Orange 552 dUTP (5-TAMRA-dUTP) (Abbott Molecular).

Analysis of HAC mitotic stability by FISH

Fluorescence images of human cells carrying the HAC with the *tTA*^{VP64}-containing cassette were analyzed. Cells were processed for fluorescence *in situ* hybridization (FISH) using the BAC32-2-mer(tetO) DNA probe after 3, 4, 7 or 14 days of culturing without HAT selection in the presence or absence of doxycycline. Metaphase spreads were screened for the presence or absence of the HAC. At least 60–140 metaphases were screened for each clone. The stability of the HAC was calculated as the percentage of alphoid^{tetO}-HAC-containing metaphase spreads in the absence or presence of doxycycline.

Reverse-transcriptase polymerase chain reaction

The alphoid^{tetO} non-coding transcripts were detected in HPRT-deficient HT1080 cells containing alphoid^{tetO}-HAC by RT-PCR using specific primers listed in Supplementary Table S1. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. A measure of 2 μg RNA was subsequently used for RT with random hexamer primers, using the Roche Transcriptor High Fidelity cDNA Synthesis Kit. Real-time PCR analysis of cDNA equivalent to \sim 70 ng (alphoid^{tetO}) input RNA was then performed using a SYBR Green Mastermix (JumpStart, Sigma) on a LightCycler480 system

(Roche). Oligonucleotide primer pairs are tetO-F/tetO-R2 (Supplementary Table S1) for the alphoid^{tetO} non-coding transcripts and actin-F/actin-R (Supplementary Table S1) for control β -actin transcripts. For each oligonucleotide primer pair and every plate, a standard curve was created using genomic DNA derived from the corresponding cell line, thereby calculating the transcript copy numbers relative to the transcript copy number for the genomic locus. Background values (no reverse transcriptase) were subtracted, and all values were normalized to β -actin expression. For time course experiments, the transcript levels were expressed relative to the dox⁺ values of the alphoid^{tetO}, which was arbitrarily set to 1. Similar conditions were used to quantify transcription of the *tTA*^{VP64} from the HAC and from transiently transfected *tTA*^{VP64}-containing plasmid in human HT1080 cells. As a control, the level of transcripts for the human *GAPDH* gene was measured. A list of primers is presented in Supplementary Table S1.

Microcell-mediated chromosome transfer (MMCT)

The alphoid^{tetO} HAC with the *tTA*^{VP64}-containing cassette was transferred from hamster CHO to human epithelium HeLa using a standard MMCT protocol (37,39). Microcells were collected from about 1×10^8 CHO cells containing the alphoid^{tetO}-HAC carrying the *tTA*^{VP64} gene. HAC was transferred into human HeLa cell line (3×10^6) via fusion of microcells with target cells. For fusion we have used Neo EX HVJ Envelope Transfection Kit (Cosmo Bio, Japan). Blasticidin S (BS) was used to select resistant colonies. Blasticidin S selection (4 $\mu\text{g/ml}$) was applied 48 h later and HeLa Blasticidin S resistant clones were picked after 2–3 weeks of growth under selective conditions. Typically 1–3 BS^R colonies were obtained in one MMCT experiment involving HAC transfer from hamster to human cells. Based on FISH analysis, more than 95% of cells in the colony contained a HAC.

ChIP assay and real-time PCR

ChIP with antibodies against trimethyl H3K4me3 (Upstate) and CENP-A was carried out according to a previously described method (40). Briefly, cultured cells were cross-linked in 1% formaldehyde for 10 min at 37°C. After addition of 1/10 volume of 1.25 M glycine and incubation for 5 min, fixed cells were washed twice with cold PBS buffer. Soluble chromatin was prepared by sonication (Bioruptor sonicator; Cosmo Bio) to an average DNA size of 500 bp in sonication buffer and immunoprecipitated in IP buffer (20 mM Tris-HCl, pH 8.0, 600 mM NaCl, 1 mM EDTA, 0.05% SDS, 1.0% Triton X-100, 20% glycerol, 1.5 μM aprotinin, 10 μM leupeptin, 1 mM DTT and 40 μM MG132). Protein G sepharose (Amersham, USA) blocked with BSA was added, and the antibody–chromatin complex was recovered by centrifugation. The recovery ratio of the immunoprecipitated DNA relative to input DNA was measured by real-time PCR using a CFX96 real time PCR detection system (Bio-Rad) and iQ SYBR Green Supermix (Bio-Rad). Primers for alphoid^{tetO} repeat (tetO) as well as for control loci, 5S ribosomal DNA, are listed in Supplementary Table S1. At least three independent ChIP experiments were performed to estimate the level of enrichment.

Statistical analysis

Statistical analysis was made using Prism (GraphPad Software Inc., La Jolla, CA, USA). An unpaired Student *t*-test was conducted to examine HAC stability upon binding of tetR-VP64 to HAC $\text{alphoid}^{\text{tetO}}$ array induced by doxycycline withdrawal. $P < 0.05$ was considered significant.

RESULTS

Construction of the tTA^{VP64} - and tTA^{VP16} -containing HACs in HPRT-minus fibrosarcoma human HT1080 cells

The mega-size $\text{alphoid}^{\text{tetO}}$ -HAC contains a unique loxP site combined with the 5'-end of the *HPRT* sequence fragment such that gene loading events can be selected by reconstitution of a functional *HPRT* gene. The loxP-targeted region in the HAC also contains the drug-resistance marker hygromycin (*Hygro*) and the thymidine kinase gene of herpes simplex virus 1 (*HS-tk*) as well as the *Bsr* gene (repeated ~30–40 times within the mega-size $\text{alphoid}^{\text{tetO}}$ DNA array) (29,30,37).

In this study, two transcriptional transactivator *tTA*-containing vectors, tetR-VP64-IRES-DsRed2 (Figure 1a; Supplementary Figure S1) and tetR*-VP16-IRES-DsRed2 (Supplementary Figure S2a) were constructed (see Materials and Methods for details) and then inserted into the loxP site of the $\text{alphoid}^{\text{tetO}}$ -HAC in HPRT-deficient human HT1080 cells (Figure 1b) in order to determine the consequences of a single copy *tTA* expression on HAC stability. The tetR-VP64-IRES-DsRed2 vector contains the tTA^{VP64} sequence (four tandem repeats of the minimal VP16 domain DALDDFDLML fused with TetR), the color marker *DsRed2* and the 3'-end *HPRT* sequence fragment. The tTA^{VP64} and *DsRed2* sequences are transcribed from the same *CAG* promoter but are separated by an internal ribosome entry site (*IRES*) sequence (Figure 1a). Therefore, while the tetR-VP64-IRES-DsRed2 vector encodes a single $tTA^{\text{VP64}}/DsRed2$ transcript it is translated into two proteins: tTA^{VP64} (which binds to the multiple tetO-sequences in the $\text{alphoid}^{\text{tetO}}$ array of the HAC) and *DsRed2* (which is translated from *IRES* at a lower efficiency). The *chs4* insulator flanks the whole cassette. The tetR*-VP16-IRES-DsRed2 vector is identical to tetR-VP64-IRES-DsRed2 but contains only one copy of the VP16 domain. The tTA^{VP64} or tTA^{VP16} binding to tetO sequences is negatively regulated by doxycycline.

For insertion into the HAC, each vector was co-transformed along with the Cre-recombinase expression vector into HPRT-minus HT1080 cells carrying the $\text{alphoid}^{\text{tetO}}$ -HAC (Figure 1b). After insertion of the vector into the HAC the *HPRT* gene was reconstituted (Figure 1d) and the order and orientation of the transgenes is as shown in Figure 1c. The efficiency of gene loading into the HAC in HPRT-minus HT1080 cells was $1-2 \times 10^{-4}$. From four to six recombinant clones were selected by growth on HAT medium after 12–15 days for each vector. All clones were selected in the presence of doxycycline to prevent *tTA* binding to the $\text{alphoid}^{\text{tetO}}$ array. Fluorescence microscopy revealed expression of the *DsRed2* transgene in all analyzed clones. The presence of the autonomous form of the HACs carrying the tTA^{VP64} or tTA^{VP16} cassette was examined by FISH

analysis. A fluorescence *in situ* hybridization image of one representative clone with the tTA^{VP64} cassette is shown in Figure 1e–g and with the tTA^{VP16} cassette in Supplementary Figure S2b. As seen, the HACs are maintained autonomously. These clones were used for further analysis.

One copy of the transcriptional transactivator tTA^{VP64} within $\text{alphoid}^{\text{tetO}}$ -HAC is enough to inactivate HAC kinetochore

To evaluate the effect of a single copy of the *tTA* on HAC stability, cells bearing HACs carrying the tetR*-VP16-IRES-DsRed2 or tetR-VP64-IRES-DsRed2 constructs were transferred into non-selective medium (HAT⁻ and BS⁻) either with doxycycline (dox⁺) to inhibit binding of the *tTA* to the $\text{alphoid}^{\text{tetO}}$ array sequence or without doxycycline (dox⁻) to permit *tTA* binding. Cells were cultured in these media for different time intervals. Then a fluorescence image and FISH analysis with the probe specific for the $\text{alphoid}^{\text{tetO}}$ -HAC were carried out (see Materials and Methods). For each HAC-containing clone at least 70–80 metaphases were examined. No significant difference in the mitotic stability of the HAC carrying the tTA^{VP16} cassette was observed when cells were grown in dox⁺ or dox⁻ medium (Supplementary Figure S2c), indicating that the cassette with one copy of the VP16 domain cannot inactivate the HAC kinetochore. Note that it has been previously shown that the same tTA^{VP16} cassette expressed from the virus or plasmid-based multiple copy vector caused loss of the $\text{alphoid}^{\text{tetO}}$ -HAC (29,37,39).

In contrast, a dramatic $\text{alphoid}^{\text{tetO}}$ HAC loss was observed in dox⁻ medium when the tTA^{VP64} cassette was inserted into the HAC. Data on FISH analysis presented as a percentage of HAC-containing metaphases in different media (dox⁺ versus dox⁻) are summarized in Table 1. As seen, the absence or presence of doxycycline had essentially little, if any, effect on stability of the HAC after 3 days of culture. However, after 14 days, expression of the tTA^{VP64} fusion protein caused a dramatic increase in the population of cells lacking the HAC when the cells were grown in the absence of doxycycline (dox⁻). As a consequence, a disappearance of the *DsRed2* fluorescence signal expressed from the HAC was observed when the cells were grown in the absence of doxycycline (Figure 2a and b). Note that expression of a single copy tTA^{VP64} from the HAC is 57-times lower than expression of the same transgene from the multiple copy vector transiently transfected into HT1080 cells (Supplementary Figure S4). Differences between two analyzed *tTA* cassettes is likely explained by a stronger effect of four tandem repeats of the VP16 domain in tTA^{VP64} on chromatin status in the HAC kinetochore compared to tTA^{VP16} . Indeed, tTA^{VP64} was developed to increase the potency of transcriptional activation of tTA^{VP16} (36).

Next, we examined the kinetics of HAC loss in one of HT1080 clones after 3, 4, 7 and 14 days of culture in the medium with and without doxycycline. As seen, the fraction of cells carrying the HAC decreased dramatically in dox⁻ medium after 4 days of culture while remaining relatively stable in dox⁺ medium (Figure 3).

Altogether, these data indicate that the expression of the tTA^{VP64} consisting of four copies of VP16 loaded into the

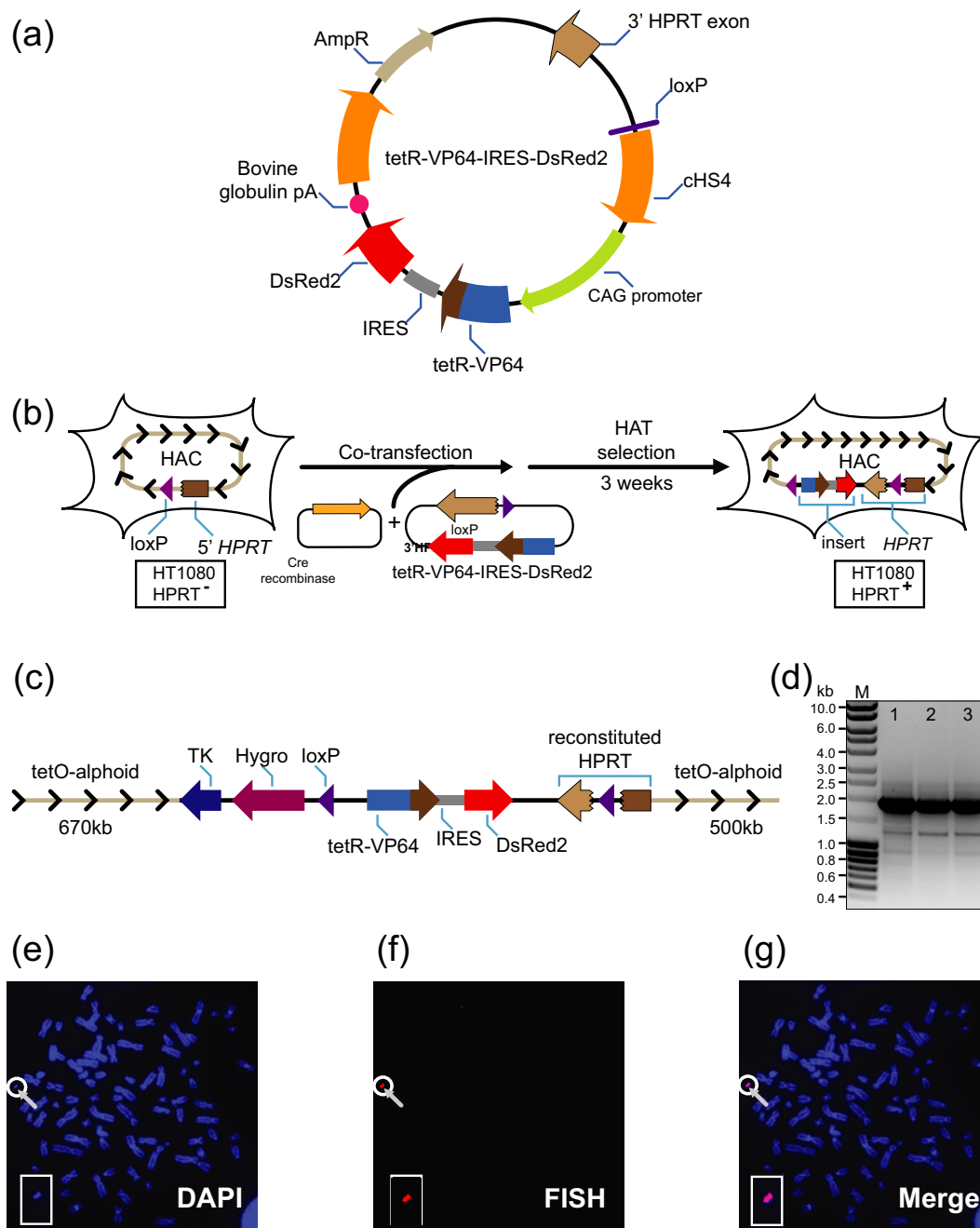


Figure 1. Loading of the tTA^{VP64} -containing construct into the alphoid^{tetO}-HAC propagated in human HPRT-minus HT1080 cells. (a) Diagram of the construct tetR-VP64-IRES-DsRed2 used in this study. The construct contains a 3' HPRT sequence and loxP site, the tTA^{VP64} (tetR with the VP64 carrying four copies of VP16 activation domain) that is co-transcribed with the *DsRed2* transgene under the same CAG promoter. The *cHS4* insulator (54) flanks the expressing cassette from both sides. (A similar construct, tetR*-VP16-IRES-DsRed2, carrying one copy of the VP16 domain is described in Supplementary Figure S2). Both constructs were inserted into the loxP site of the alphoid^{tetO}-HAC propagated in HPRT-deficient HT1080 cells. (b) Loading of the vector into the loxP site of the HAC is accompanied by reconstitution of the *HPRT* gene allowing cell selection on HAT medium. (c) A map of the resulting transgene cluster in the HAC containing TK and *Hygro* genes, the *DsRed2* color marker and the reconstituted *HPRT* gene. Arrows indicate direction of transcription of the transgenes and 30–40 copies of *BS* incorporated in the alphoid^{tetO}-HAC. (d) Lanes 1, 2 and 3 correspond to PCR products obtained with the genomic DNA isolated from HAC-containing clones of HT1080 cells using specific primers for the *HPRT* gene. The PCR products were sequenced and confirmed reconstitution of the *HPRT* gene. M-ladder marker. (e–g) FISH analysis of the HAC-containing HT1080 clone. Chromosomal DNA was counterstained with DAPI (blue) (e). The HAC was visualized using a BAC32–2-mer(tetO) probe (red) (f and g).

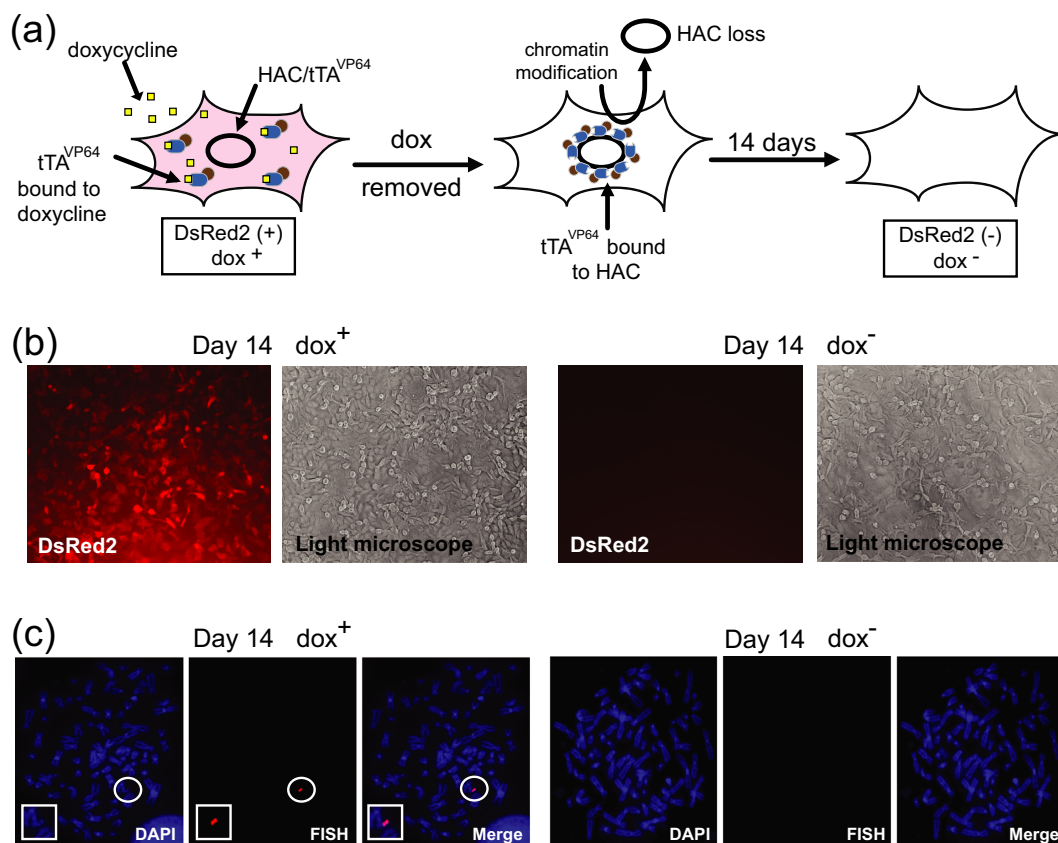


Figure 2. (a) A scheme representing binding of tTA^{VP64} protein with the HAC followed by HAC loss from the cells as result of doxycycline removal. (b) Bright and fluorescent images of one representative clone with the HAC carrying the tTA^{VP64}-DsRed2 cassette cultured in dox⁺ medium and after removal doxycycline followed by 14 days of culture. Cells were cultured without HAC selection (in HAT⁻ and BS⁻ medium). (c) Representative images of the metaphase spread after 14 days of culture in the presence and absence of doxycycline. Chromosomal DNA was counterstained with DAPI (blue). The HAC was visualized using a BAC32-2-mer(tetO) probe (red).

Table 1. Stability of the alphoid^{tetO}-HAC/tTA^{VP64} in dox⁺ and dox⁻ medium in human fibrosarcoma HT1080 cells

# clone ^a	Medium	3 days	14 days
1	dox ⁺	99%	93%
	dox ⁻	90%	0%
2	dox ⁺	99%	92%
	dox ⁻	99%	0%
3	dox ⁺	80%	85%
	dox ⁻	75%	5%
4	dox ⁺	80%	72%
	dox ⁻	70%	1%

^aFor each HAC-containing clone at least 70–80 metaphases were examined.

alphoid^{tetO}-HAC induces HAC loss, presumably as a result of changes in the HAC kinetochore chromatin.

Self-elimination of the alphoid^{tetO}-HAC carrying the tTA^{VP64} cassette is not cell line specific

Similar results on HAC self-elimination in dox⁻ medium were obtained in hamster ovarian CHO cells and in human epithelial HeLa cells. For these experiments, the tetR-VP64-IRES-DsRed2 construct was inserted into a single loxP site

of the alphoid^{tetO}-HAC propagated in HPRT-minus hamster CHO cells by Cre/loxP-mediated recombination (see Materials and Methods) (Figure 4a). Several clones were selected on HAT medium. Insertion into the HAC was confirmed by PCR using specific primers (Supplementary Table S1). These primers were diagnostic for reconstitution of the *HPRT* gene, which accompanies Cre/loxP targeting. A FISH image of one representative clone of the HAC in CHO cells in dox⁺ medium after 14 days of culture is

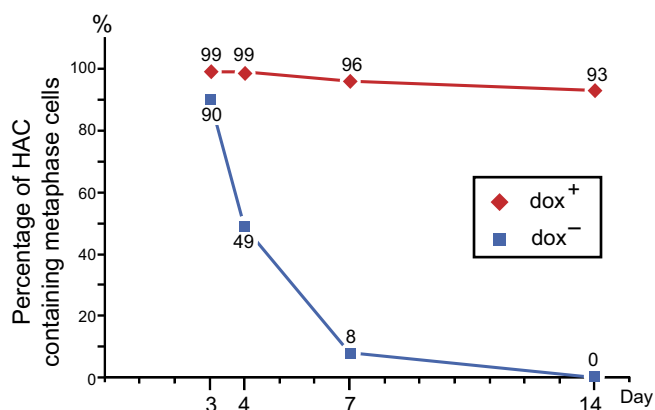


Figure 3. Kinetics of mitotic stability of the HAC carrying the construct tetR-VP64-IRES-DsRed2. The cells were cultured under following conditions: dox⁺ 3 days and dox⁻ 3 days, dox⁺ 4 days and dox⁻ 4 days, dox⁺ 7 days and dox⁻ 7 days, dox⁺ 14 days and dox⁻ 14 days. To quantify the HAC retention, the cells were analyzed by FISH. FISH analysis included detection of the HAC on metaphase spreads. At least 70–80 metaphase spreads were analyzed for each point. Addition of doxycycline, which prevents fusion of the tetracycline repressor from binding to tetO sequences of the HAC, blocks the inactivation of HAC kinetochore. Targeting the transcriptional transactivator *tTA*^{VP64} into the HAC kinetochore in the absence of doxycycline induces HAC loss.

shown in Figure 4b. This FISH confirmed that the HAC is maintained autonomously. One CHO clone containing alphoid^{tetO}-HAC/*tTA*^{VP64} was chosen for transfer to human HeLa cells via MMCT (Figure 4c). FISH analysis confirmed that the HAC propagates autonomously without detectable integration into chromosomes (Figure 4d). The results on the HAC stability in two HAC-containing clones in CHO and HeLa cell lines in dox⁺ versus dox⁻ medium are summarized in Table 2. As seen, expression of the *tTA*^{VP64} cassette from the HAC induces a dramatic HAC loss in the absence of doxycycline in the analyzed cell lines after 14 days of culture (Table 2; Figure 4b and d).

Chromatin changes in the alphoid^{tetO}-HAC kinetochore induced by *tTA*^{VP64} tethering

Previously we have demonstrated that expression of the transcriptional activator (*tTA*) containing only one copy of the minimal VP16 domain DALDDFDLDMML from the viral vectors produced ‘open’ chromatin in the alphoid^{tetO}-HAC allied with a substantial elevation in transcripts specific for synthetic alphoid DNA array (29,31,34). Such a high level of transcription of the alphoid^{tetO} array resulted in a rapid inactivation of the HAC kinetochore due not only to a complete block of CENP-A loading, but also due to stripping of pre-assembled CENP-A and other CCAN (constitutive centromere-associated network) proteins from the HAC kinetochore (32).

In this work, we examined whether a single copy of the *tTA*^{VP64} incorporated into the alphoid^{tetO}-HAC had a similar effect on the HAC centrochromatin as multiple copies of the *tTA* expressed from a viral vector when a large amount of the transcriptional activator is produced.

In our first experiments, the transcription of satellite DNA in alphoid^{tetO} array was found to be significantly in-

creased (3-fold) after 3 days of growth in the medium lacking doxycycline in clone #1 [*t*-test, *t*(4) = 12.3554, *P* = 0.0002]. In clone #2, there was a significant increase (6-fold) after 2 days [*t*-test, *t*(4) = 53.9038, *P* < 0.0001] that further increased by 9-fold after 3 days [*t*-test, *t*(4) = 146.6633, *P* < 0.0001] (Figure 5a and b). These experiments demonstrate that targeting of HAC kinetochore chromatin by a single expressed copy of the *tTA*^{VP64} leads to a significant increase of transcription of non-coding alphoid^{tetO} array after 3 days of cell growth (Figure 5a and b). These results correlate with ChIP data on enrichment of H3K4me3, a marker for the 5' end of actively transcribed genes (28,29,50,51), on alphoid^{tetO} array in the HAC. In two independent clones, the HAC chromatin following *tTA*^{VP64} expression failed to reveal significant structural differences after one day of culture in dox⁻ medium but was significantly (3–4 fold) increased on the alphoid^{tetO} array of the HAC after 3 days of culture in the medium lacking doxycycline [*t*(4) = 31.2421, *P* < 0.0001 for clone #1 and *t*(4) = 49.8532, *P* < 0.0001 for clone #2] (Figure 5c and d). Thus, elevation of satellite transcription of the alphoid^{tetO} array after 3 days was accompanied by enrichment in H3K4me3 chromatin. No significant difference in the level of CENP-A on the alphoid^{tetO} array was observed when cells were grown in the presence or absence of doxycycline after 3 days of culture [*t*(4) = 2.1625, *P* = 0.0966] (Figure 5e). However, a longer culturing of the cells in dox⁻ medium (4, 8 and 12 days) resulted in a dramatic decrease of the level of CENP-A (Figure 5e) followed by kinetochore inactivation and HAC loss (Figure 3) that is in agreement with our previous results on inactivation of the HAC kinetochore by *tTA* using viral vectors (29,31,32).

Development of a novel alphoid^{tetO}-HAC/ *tTA*^{VP64}-based system to verify phenotypic changes attributed to expression of HAC-encoded genes

The phenomenon of HAC loss caused by expression of the *tTA*^{VP64}-containing chromatin modifier from the HAC can be used to develop a powerful system to verify phenotypes induced by a gene(s) loaded into the single loxP site of the alphoid^{tetO}-HAC. This could be achieved by combining the TAR-cloning vector (43), used to selectively isolate any full-length gene from multiple human genomes, with the *tTA*^{VP64}-containing modifier cassette followed by loading of this final vector along with a gene of interest into the HAC. Figure 6a shows a diagram of a newly constructed TAR-BRV- *tTA*^{VP64} cloning vector (see details in Supplementary Figure S5). This vector contains restriction sites for insertion of two unique targeting sequences (hooks) (red dots) homologous to the 5' and 3' ends of a gene of interest and restriction sites for insertion of chromatin insulators (green dots) (48), such as *tRNA* (52,53) or *cHS4* (54) or γ -satellite DNA (55) to protect the captured gene from silencing. The vector also contains a YAC cassette that includes the *HIS3* marker for selection in yeast and the *CEN6* centromere for proper segregation in yeast cells as well as a BAC cassette that includes the *F'* factor origin of replication and the chloramphenicol acetyltransferase (*CmR*) gene for selection in bacterial cells. This allows the TAR-isolated gene to be isolated either directly from yeast (56) or moved to bacterial cells without a step of YAC retrofitting

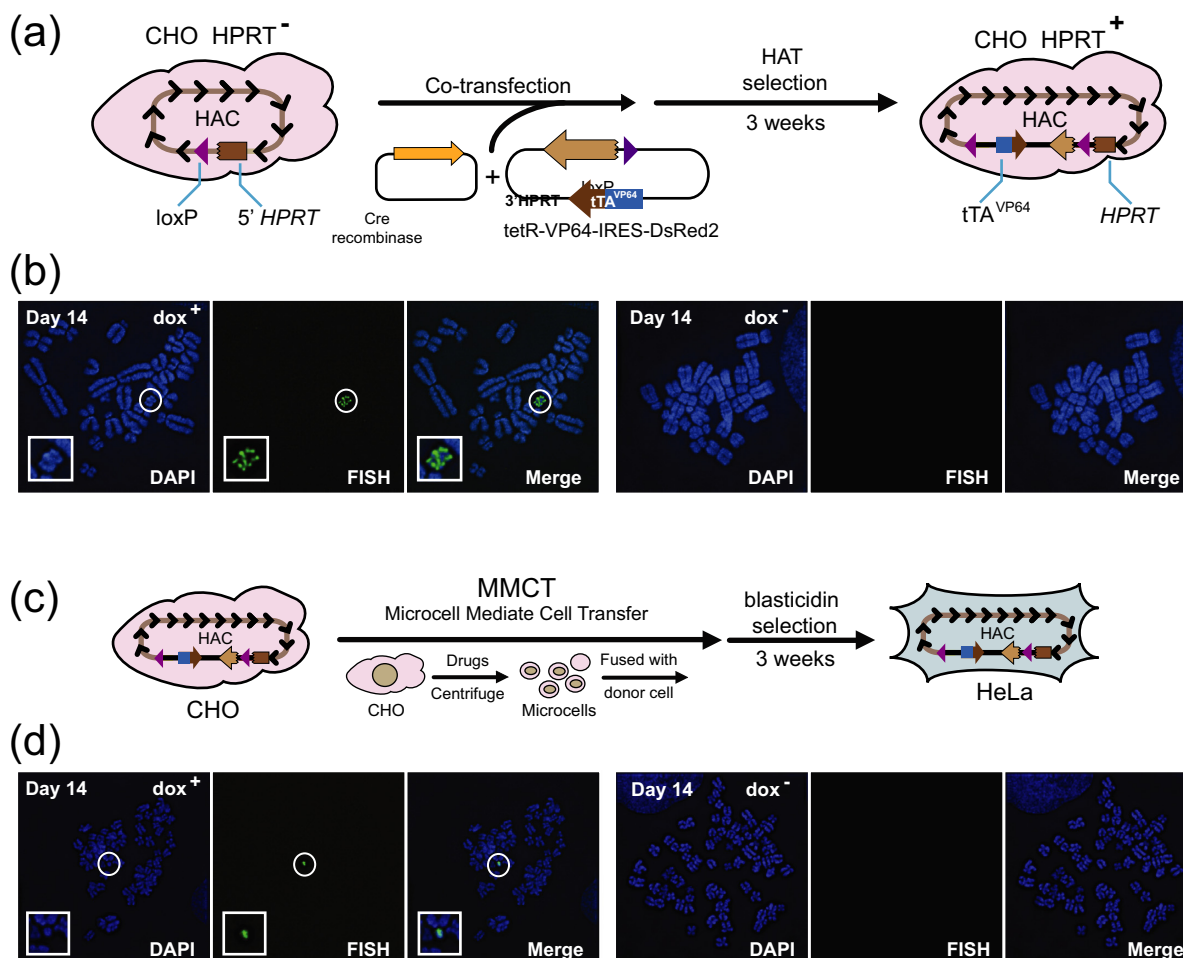


Figure 4. The $\alpha\text{HAC}^{\text{tetO}}$ -HAC/tTA^{VP64} in different cell lines. (a) Insertion of the construct tetR-VP64-IRES-DsRed2 into the loxP site of the $\alpha\text{HAC}^{\text{tetO}}$ -HAC propagated in HPRT-deficient hamster CHO cells by Cre/loxP mediated recombination. (b) FISH analysis of the hamster clone carrying the autonomously propagated $\alpha\text{HAC}^{\text{tetO}}$ -HAC/tTA^{VP64} in dox⁺ medium and after removal doxycycline followed by 14 days of culture. (c) From CHO cells the $\alpha\text{HAC}^{\text{tetO}}$ -HAC/tTA^{VP64} was MMCT-transferred to human HeLa cells. (d) FISH analysis of the HeLa human clone carrying the autonomously propagated $\alpha\text{HAC}^{\text{tetO}}$ -HAC/tTA^{VP64} in dox⁺ medium and after removal doxycycline followed by 14 days of culture. Chromosomal DNA was counterstained with DAPI (blue). The HAC was visualized using a BAC32-2-mer(tetO) probe (green).

Table 2. Stability of the $\alpha\text{HAC}^{\text{tetO}}$ -HAC/tTA^{VP64} in dox⁺ and dox⁻ medium in hamster CHO and human HeLa cells

Hamster ovary CHO cells			
# clone ^a	Medium	3 days	14 days
1	dox ⁺	99%	77%
	dox ⁻	99%	4%
2	dox ⁺	99%	85%
	dox ⁻	99%	5%
Human epithelium HeLa cells			
1	dox ⁺	99%	93%
	dox ⁻	99%	2%
2	dox ⁺	99%	92%
	dox ⁻	99%	3%

^aFor each HAC-containing clone at least 130–140 metaphases were examined.

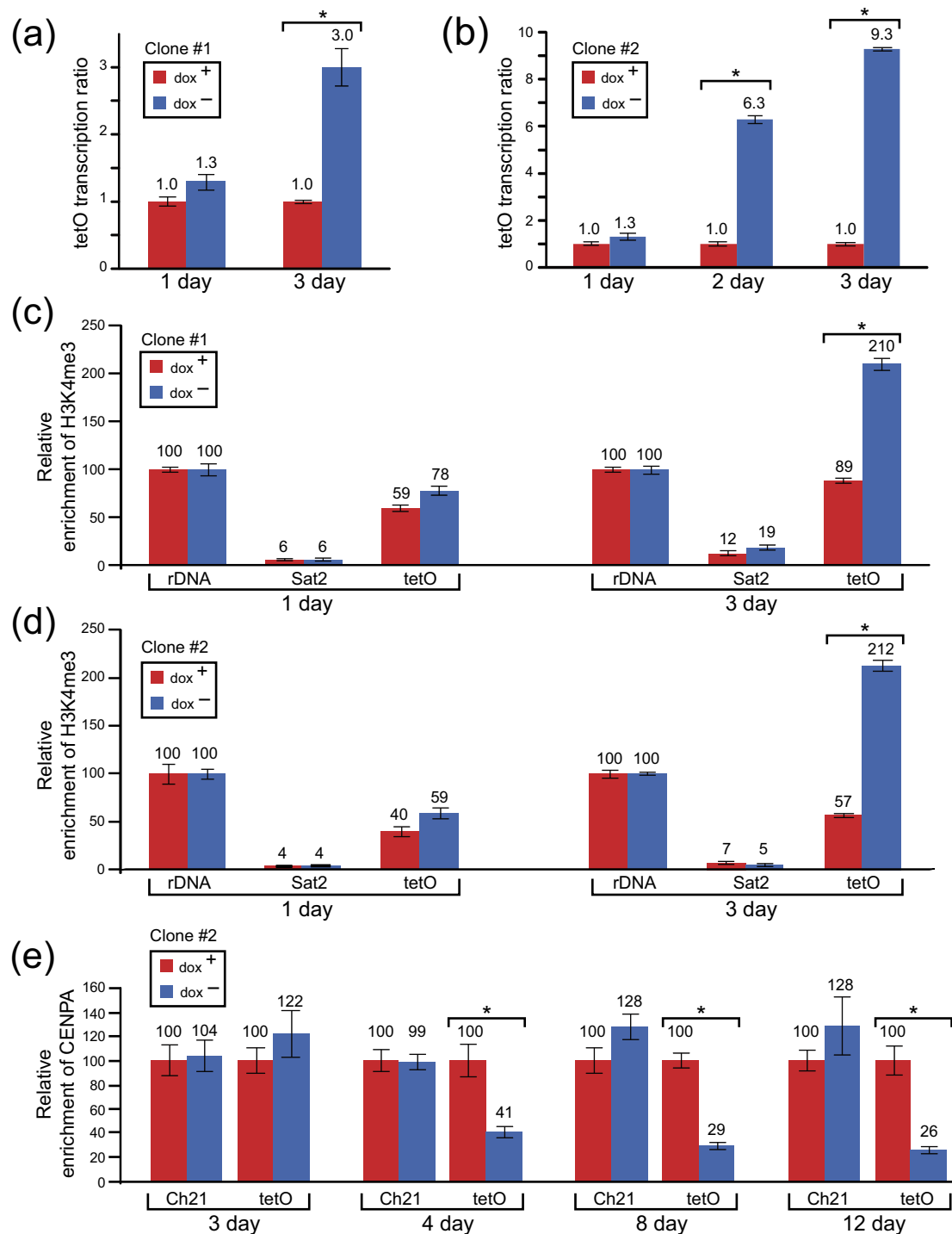


Figure 5. Chromatin/transcription changes in the alphoid^{tetO}-HAC kinetochore induced by tTA^{VP64} tethering. **(a and b)** De-repression of tetO-alpha-satellite DNA transcription in the HAC when cells are grown in the medium lacking doxycycline. Quantitative RT-PCR experiments for two clones, clone #1 and clone #2, showing that the transcripts of non-coding tetO-alpha-satellite DNA repeats in the alphoid^{tetO}-HAC are significantly repressed in the cells growing in doxycycline-containing medium compared to that of the cells that have been grown in the medium lacking doxycycline. **(c and d)** Chromatin immunoprecipitation (ChIP) analysis of non-coding tetO-alpha-satellite DNA repeats in the alphoid^{tetO}-HAC in two clones using antibodies against H3K4me3 in the cells growing in doxycycline-containing medium compared to that of the cells that have been grown in the medium lacking doxycycline after 1 and 3 days of culture. Data were normalized to the internal 5S rDNA controls. **(e)** ChIP analysis of CENP-A chromatin in the alphoid^{tetO}-HAC in the clone #2 cultured in the presence and absence of doxycycline for different time intervals (3, 4, 8 and 12 days of culture). Enrichment is shown relative to the chromosome 21 centromere. Error bars indicate s.d. The significant differences were calculated using Student's two-tailed *t*-test.

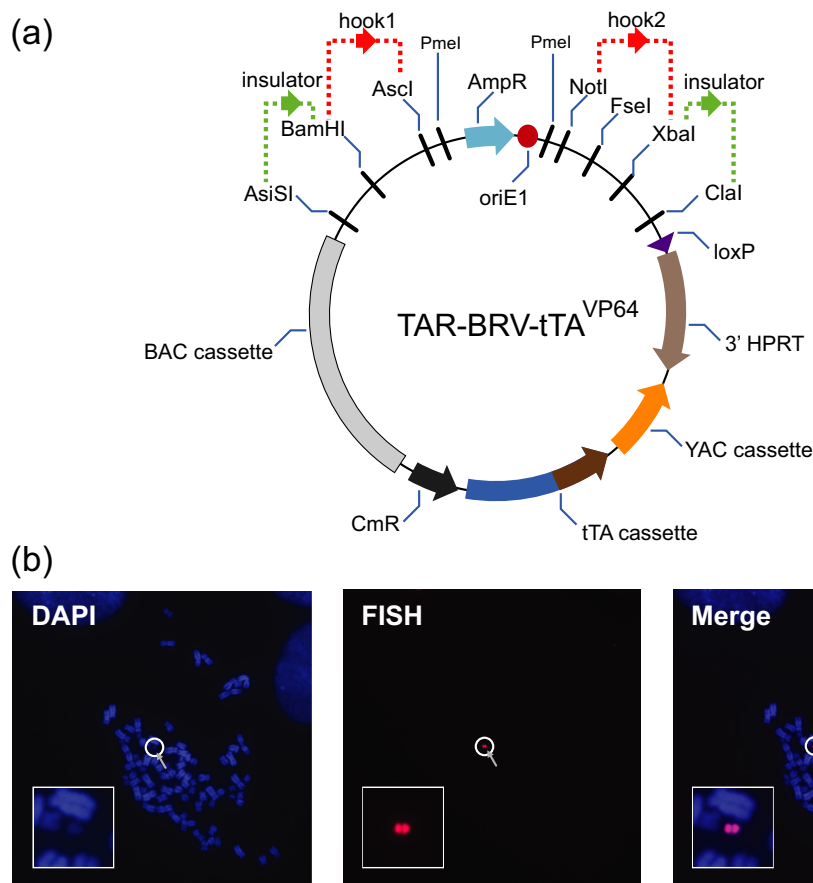


Figure 6. (a) A scheme of the TAR-BRV- tTA^{VP64} vector. This vector contains restriction sites for insertion of two unique targeting sequences (hooks) (dot red) homologous to 5' and 3' ends of a gene of interest and restriction sites for insertion of chromatin insulators to protect a gene from silencing (dot green). The vector also contains a YAC cassette that includes a yeast selectable marker *HIS3* for selection in yeast and a yeast centromere *CEN6* for proper segregation in yeast cells. The TAR-BRV- tTA^{VP64} vector also contains a BAC cassette that includes a *F'* factor origin of replication and the chloramphenicol acetyltransferase (*CmR*) gene for selection in bacterial cells. For gene TAR cloning experiments, the TAR-BRV- tTA^{VP64} vector DNA should be linearized by a unique endonuclease located between the hooks to expose targeting sequences (hooks). The TAR-BRV- tTA^{VP64} vector also contains a 3' *HPRT*-loxP cassette allowing a further loading of a TAR-gene isolate into the unique loxP site of the alphoid^{tetO}-HAC in hamster CHO cells by Cre/loxP mediated recombination, from where the HAC may be MMCT-transferred to the recipient human cells for further gene function analysis. The TAR-BRV- tTA^{VP64} vector also contains the tTA^{VP64} chromatin modifier that is constitutively expressed from the HAC. In the presence of doxycycline the tTA^{VP64} cannot bind to the tetO-sequences in the alphoid^{tetO}-HAC. (b) FISH analysis of the HAC containing the TAR-BRV- tTA^{VP64} vector in HT1080 cells. Chromosomal DNA was counterstained with DAPI (blue). The HAC was visualized using a vector probe (red).

with BAC sequence (39) for further gene DNA isolation. The TAR-BRV- tTA^{VP64} vector also contains a 3' *HPRT*-loxP cassette allowing further loading of the TAR-gene isolate into the unique loxP site of the alphoid^{tetO}-HAC gene delivery vector in hamster *HPRT*-deficient CHO cells by Cre/loxP mediated recombination. From there the HAC may be MMCT-transferred (37,57) to the recipient human cells for further gene function analysis (23,38,39). Importantly, because the TAR-BRV- tTA^{VP64} vector contains the chromatin modifier that is constitutively expressed from the HAC, integrity of the HAC kinetochore and, as a consequence, expression of a gene of interest in the cells can be regulated. In the presence of doxycycline when the tTA^{VP64} does not bind to the tetO-sequences in the alphoid^{tetO}-HAC, the HAC is mitotically stable and each cell contains a product of a gene loaded into the HAC. After removal of the ligand doxycycline, the tTA^{VP64} binds to tetO-sequences of the HAC resulting in HAC loss along with a gene of interest.

As a proof of principle, the TAR-BRV- tTA^{VP64} vector was inserted into the loxP site of the alphoid^{tetO}-HAC in human *HPRT*-deficient HT1080 cells. FISH analysis proved that the HAC is maintained in the autonomous form (Figure 6b). Three randomly chosen clones were analyzed in *dox*⁺ and *dox*⁻ media with the following FISH analysis to confirm HAC loss in the medium lacking doxycycline. To protect the tTA^{VP64} expression from silencing, the *tRNA* insulator (52) was inserted upstream of the *CAG* promoter. Significant HAC loss was observed in three out of three selected clones cultured in the absence of doxycycline after 16 days of culture (Table 3). The greater HAC loss observed for the original tetR-VP64-IRES-DsRed2 vector may be due to the difference either in the structure of the cassettes or the insulator type used (*cHS4* vs *tRNA*) (52–54). It is known that different insulator types have a different level of gene protection (48).

To summarize, the TAR-BRV- tTA^{VP64} gene cloning vector allows to control expression of genes loaded into the

Table 3. Stability of the alphoid^{tetO}-HAC/TAR-BRV- *tTA*^{VP64} in dox⁺ and dox⁻ medium in HT1080 cells

# clone ^a	Medium	2 days	16 days
1	dox ⁺	96%	73%
	dox ⁻	80%	8%
2	dox ⁺	98%	75%
	dox ⁻	84%	8%
3	dox ⁺	98%	72%
	dox ⁻	79%	7%

^aFor each HAC-containing clone at least 70–90 metaphases were examined.

alphoid^{tetO}-HAC by simple removal of a ligand (doxycycline) that results in the kinetochore disassembly followed by the HAC loss.

DISCUSSION

The alphoid^{tetO}-HAC is an advanced HAC-based vector for gene delivery and regulated gene expression (23,29,37,39). This HAC consists of a megabase-size synthetic, higher order alpha-satellite repeat array with a tet operator sequence (tetO) in every other alphoid monomer. This provides a significant advantage for this HAC compared to other HAC-based vectors because its kinetochore can be inactivated by tethering the *tTS* silencer or the *tTA* transcriptional transactivator or other chromatin modifiers to the tetO sequences leading to HAC loss (29,31,32,34,37). This HAC feature provides a control for phenotypes induced by test genes loaded into the alphoid^{tetO}-HAC.

In our previous studies, induction of alphoid^{tetO}-HAC loss was initiated by transfection of cells by viral or plasmid vectors expressing multiple copies of a chromatin modifier, a step that is potentially mutagenic (23,29,37,39). Recently we described a re-engineered alphoid^{tetO}-HAC that allows verification of phenotypic changes attributed to expression of genes from the HAC without the potentially mutagenic transfection step (40). In that HAC, a single copy of the *tTS* was inserted into a unique gene-loading site along with a gene of interest. Expression of the *tTS* generated a self-regulating fluctuating heterochromatin on the alphoid^{tetO}-HAC that induced a fast silencing of the gene on the HAC without significant effects on HAC segregation (40). Importantly, inactivation of a gene on the alphoid^{tetO}-HAC/*tTS* was reversible by addition of doxycycline, presumably because the *tTS* was silencing its own expression. Thus, expression of a single auto-regulated copy of the *tTS* from the alphoid^{tetO}-HAC provides an efficient method to switch off or switch on expression of the gene(s) loaded onto the HAC.

In this work, we engineered the novel alphoid^{tetO}-HAC/*tTA*^{VP64} and demonstrated that a single copy of the transcriptional transactivator *tTA*^{VP64} constitutively expressed from the HAC also allows a stable expression of the HAC gene(s) in the presence of doxycycline. In contrast to the alphoid^{tetO}-HAC/*tTS*, culturing of the alphoid^{tetO}-HAC/*tTA*^{VP64} in medium without doxycycline induces a fast inactivation of the HAC kinetochore leading to HAC loss along with a loaded gene. The different effect of *tTS* and *tTA*^{VP64} on the HAC kinetochore function is presumably due to

that the *tTA*^{VP64} does not self-silence. This provides a pure control for phenotypic changes attributed to expression of a HAC-encoded gene by returning the mutant cell line to its original state following removal of the HAC from the cell. The alphoid^{tetO}-HAC/*tTA*^{VP64} with a regulated kinetochore may have a specific application for generation of induced pluripotent stem (iPS) cells for regenerative medicine. It is worth noting that recent publications demonstrate the use of other HAC vectors for reprogramming mouse embryonic fibroblasts (MEFs) into induced iPS cells (5,6).

In addition, in this work we combined the alphoid^{tetO}-HAC-based gene delivery vector with TAR gene-cloning technology and demonstrated a potential to carry out a complete cycle starting with selective gene isolation using a newly constructed TAR cloning vector, TAR-BRV-*tTA*^{VP64}, followed by *tTA*^{VP64} and a gene of interest expression from the HAC vector and ended by the HAC loss along with a gene. This new vector contains a BAC cassette and, therefore, allows the direct transfer of TAR-isolated genes from yeast to bacterial cells for further BAC DNA isolation. This vector also contains a 3'*HPRT-loxP* cassette allowing direct gene loading into the alphoid^{tetO}-HAC for the following complementation of gene deficiencies in human cells. The gene expression from the alphoid^{tetO}-HAC can be eliminated by a simple removal of doxycycline from the medium because *tTA*^{VP64} is inserted along with a gene of interest into the HAC. A hypothetical scheme illustrating the entire process, starting with TAR isolation of a gene from genomic DNA in yeast using a TAR-BRV-*tTA*^{VP64} vector then proceeding to its direct transfer to bacterial cells and subsequent insertion into the alphoid^{tetO}-HAC and ending with the verification of gene-induced phenotypes by inducing HAC loss, is diagrammed in Supplementary Figure S6. It is worth noting that a YAC/BAC/TAR gene-containing construct could also possibly be introduced into HAC-containing human cells through yeast spheroplast fusion to human cells (58).

Thus, we have designed two approaches to verify gene-induced phenotypes using HAC vectors. In the first case, when a single copy of *tTS* is expressed, a self-regulating fluctuating heterochromatin is generated on the alphoid^{tetO}-HAC. This can induce a fast silencing of the gene(s) on the HAC without significant effects on HAC stability and such gene silencing is reversible by addition of doxycycline (40). In the second case, expression of a single copy of the *tTA*^{VP64} from the HAC induces a fast inactivation of the HAC kinetochore leading to irreversible HAC loss along

with the loaded gene. Both approaches are useful for studies of gene function and have a potential for cell and gene therapy.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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