

Efficient conditional and promoter-specific *in vivo* expression of cDNAs of choice by taking advantage of recombinase-mediated cassette exchange using FIEEx gene traps

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ABSTRACT

Recombinase-mediated cassette exchange (RMCE) exploits the possibility to unidirectionally exchange any genetic material flanked by heterotypic recombinase recognition sites (RRS) with target sites in the genome. Due to a limited number of available pre-fabricated target sites, RMCE in mouse embryonic stem (ES) cells has not been tapped to its full potential to date. Here, we introduce a universal system, which allows the targeted insertion of any given transcriptional unit into 85 742 previously annotated retroviral conditional gene trap insertions, representing 7013 independent genes in mouse ES cells, by RMCE. This system can be used to express any given cDNA under the control of endogenous trapped promoters *in vivo*, as well as for the generation of transposon 'launch pads' for chromosomal region-specific 'Sleeping Beauty' insertional mutagenesis. Moreover, transcription of the gene-of-interest is only activated upon Cre-recombinase activity, a feature that adds conditionality to this expression system, which is demonstrated *in vivo*. The use of the RMCE system

presented in this work requires one single-cloning step followed by one overnight gateway clonase reaction and subsequent cassette exchange in ES cells with efficiencies of 40% in average.

INTRODUCTION

A number of different strategies for the modification of the mouse genome have been used, most of which involve homologous recombination (HR) techniques to introduce genetic material into desired genetic locations. However, HR efficiency is often dependent on the nature of the genomic target site itself as well as on the design of the targeting vector. The use of site-specific recombinases (SSR) allows secondary modifications of previously inserted cassettes (1,2). Recognition motifs of SSRs usually consist of two palindromic sequences separated by an asymmetric spacer, which determines their orientation. Engineered target sites with defined base pair changes in the spacer or inverted repeats were also shown to recombine efficiently with each other. Generally, SSRs will recombine identical (homotypic) recognition targets (for a review, see 3,4). Although originally derived from Bacteriophage P1 or *S. cerevisiae* respectively, Cre (causes recombination in the phage P1

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genome) and FLP (flippase; named for its ability to flip DNA segments in *S. cerevisiae*) recombinases have been demonstrated to function efficiently in human cell lines and mouse embryonic stem (ES) cells (2,5–7). Recently, the German Gene Trap Consortium (GGTC) introduced the use of multipurpose cassettes, which are delivered randomly to the genome by retroviral gene trapping and allow post-entrapment modifications (8,9). We used the so-called FIEEx (FLP-excision; 10) technology in order to conditionally inactivate or reactivate gene trap mutations by using two independent FIEEx arrays, which each allow unidirectional inversions of trapping cassettes. The FIEEx method involves two subsequent recombination events, the first of which is a FLP inversion, and the second is a FLP excision. The result is an inverted gene trap vector cassette flanked by two heterotypic FLP recombination target sites (FRT and F3) that can be re-inverted in a cell-specific manner by Cre recombinase but could serve as well as a genomic docking-site for FLP recombinase-mediated cassette exchange (RMCE; 11,12). By representing a decrease in entropy, the reversal of excision (i.e. the integration of DNA cassettes) occurs against thermodynamic and kinetic barriers and is therefore highly inefficient transiently (13). However, as we demonstrate, the combination of an alternative selection marker and a codon-optimized, thermostable FLP recombinase (FLPo; 14) resulted in efficient exchange of DNA cassettes, with 'FIEExed' β -galactosidase/neomycin-resistance (β -geo) cassettes.

The power of this exchange system lies within the size of the GGTC and Eucomm sublibraries, which were established using FIEEx-containing gene trap vectors. With a total number of 7013 independent genes containing FIEEx vector insertions that were characterized molecularly by genomic splinkerette (splk) sequence tags (15; <http://www.genetrap.de>; <http://www.eucomm.org>), the libraries represent catalogues of mouse ES cells, with the potential to establish mouse lines for each clone. RMCE in FIEEx containing gene trap clones facilitates the placement of any cDNA construct under the control of previously trapped endogenous promoters of choice, which will usually recapitulate their expression *in vivo*, if the interaction of promoters with other transcriptional elements is not influenced by the insertion.

Therefore, in order to design a powerful RMCE vector system, compatible with the established GGTC FIEEx library, we developed a simple procedure that involves a single-cloning step into a Gateway Entry vector (Invitrogen) followed by an overnight Gateway reaction to end up with the final RMCE vector. This circular vector is co-transfected along with FLP recombinase into FIEEx clones, followed by hygromycin selection for 9 days. Since exchange efficiencies typically range around 40% on average, few clones need to be isolated and PCR-screened using generic primers in order to distinguish correctly exchanged alleles from randomly inserted cassettes. Even more easily, any cDNA, which is already situated between the attL 1/2 sites of a pEntry vector, can be introduced directly into the exchange vector described here without further cloning steps.

MATERIALS AND METHODS

Vectors

Exchange vectors pEX-Dest and pEx-FLP. The pre-Gateway pEX-FLP backbone, designated as pEx-Dest was synthesized (<http://www.geneart.com>) and consists of an adenoviral splice acceptor sequence (in the following referred to as SA), followed by unique XhoI, SalI and NotI restriction sites, flanked by two identically oriented loxP sites and finally by inversely oriented 5'-FRT and 3'-F3 sites and served as a backbone for further cloning.

The hygromycin-coding sequence followed by a phosphoglyceratekinase (PGK)-polyA signal was PCR-amplified using a set of primers with a 5'-overhang containing the NotI restriction sites and AT-spacer nucleotides: 5'-atcgccgcccgcgccaccatgaaaagcctga-3', 5'-atcgccgcccgaagcttctgatgaattaga-3', using pPGK-Hygromycin-pA as a template (provided by Ralf Kühn). After T/A-cloning in pCR II-Topo vector, the fragment was cloned into the NotI site of pEX-Dest.

Between the 3' loxP and the F3 site, a unique PmeI restriction site was used to insert a blunt-end Gateway cassette A (Invitrogen) containing an attR 1/2 flanked ccdB cassette (16) to finalize pEx-Dest (Figure 1).

Entry vector pENTR-EX. The basis for pENTR-EX was a pENTR4 insert (Invitrogen) which contains attL1/2 sites flanking the ccdB cassette (Figure 1A). For pENTREx-FLP-dsRed, the coding sequence of the red fluorescence protein (dsRed, from *Discosoma sp.*) was PCR-amplified from DsRed2-N1 (Clontech, 17) using the oligonucleotides: 5'-ataagcttaccatggcctcctccgaggac-3' (fwd), 5'-atgagctctacaggaacaggtggtggcg-3' (rev) and cloned into a pCRII Topo plasmid.

Next, the pCRII Topo-dsRed HindIII/SacI fragment was ligated to HindIII/SalI and SacI/SalI fragments of the β -geo gene trap vector (adenoviral splice acceptor, β -galactosidase/neomycin phosphotransferase fusion gene, bovine growth hormone polyadenylation sequence, a gift of Phil Soriano to T. F.) to yield an SA-dsRed-BGHpA harbouring vector. To generate pENTREx-FLP-dsRed, the pENTR4 insert and a plasmid containing a 'Sleeping Beauty' (SB) transposon (PGK-Neomycin-pA flanked by SB inverted and direct repeats (IR/DR), the transposase recognition sites) were cut each by *EcoRI*/SalI and ligated. Then, the SalI and the NotI restriction sites of the ligation product were removed, by digesting with the respective enzymes, overhangs were blunted by T4 DNA polymerase and the vector was religated.

The insert flanked by the IR/DR sequences was obtained after HindIII digest to ligate the resulting cassette with a HindIII fragment of a pEx-FLP-synthetic backbone (<http://www.geneart.com>), containing a SalI site, followed by two unique oriented lox5171 sites, which flank unique NotI and *AseI* restriction sites.

Next, an SA-dsRed-pA sequence was released by XhoI and ligated to the attL1/2 sites of the pENTR-EX vector, linearized with SalI.

A hyperactive SB transposase expression plasmid (Caggs-SB100X-pA) is under the control of a chicken-beta actin-CMV enhancer (Caggs) promoter (18). A codon-

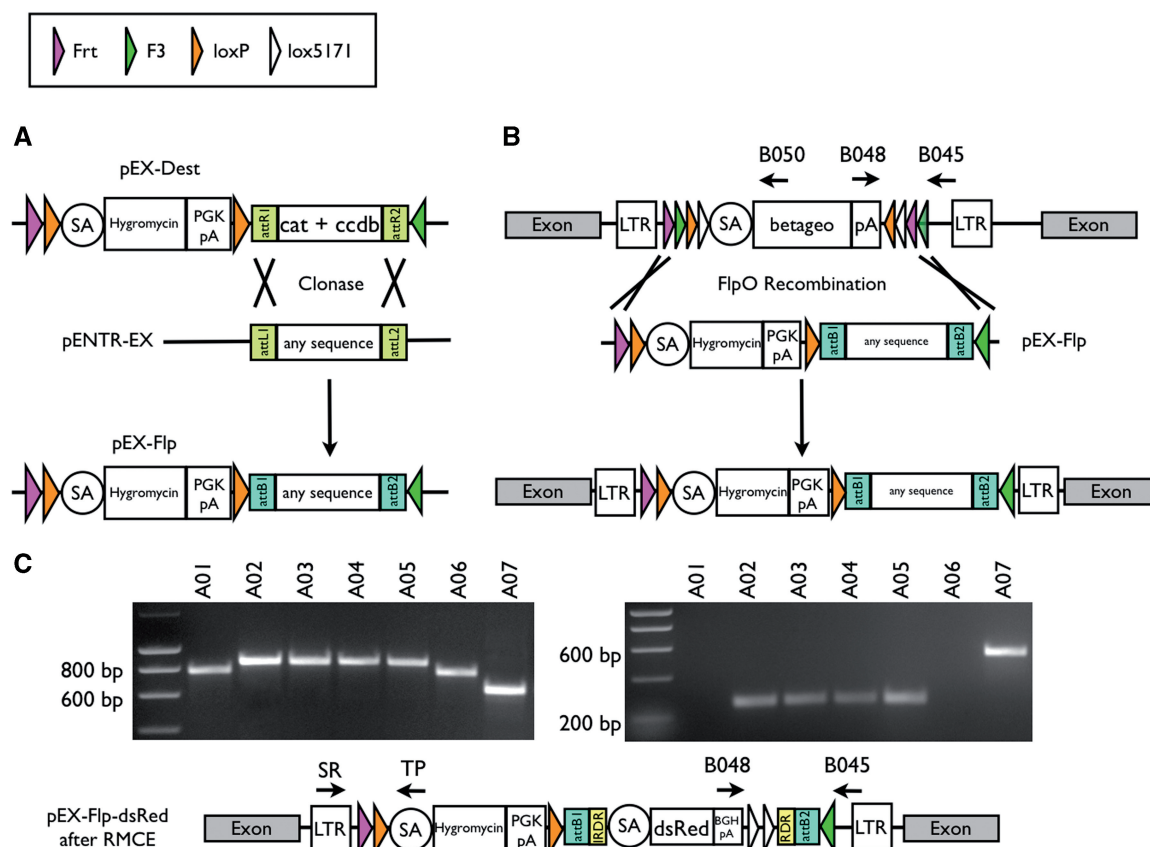


Figure 1. (A) Schematic outline of the procedure for individual modification and use of pEX-FLP. Any DNA sequence, which is cloned between the attL1/2 recombination sites in the pENTR-EX, can be exchanged with an attR1/2 flanked cat ccdB cassette of pEX-Dest *in vitro*. (B) Schematic illustration of the FLPo RMCE in ES cells. pEX-FLP harbors two heterospecific flippase recombination sites, therefore cotransfection of FLPo and pEX-FLP to any FIEs gene trap ES cell clone allows replacement of β -geo cassette. (C) Analysis of successfully exchanged locus with pEX-FLP-dsRed containing a adSA-dsRed-BGHpA sequence flanked by IR/DR of Sleeping Beauty transposase. A representative PCR screen of seven hygromycin resistant clones (E307D01 derivatives, A01–A07) concerning orientation of the genetrap vector and successful exchange (left: primers B045, B048 and B050; right: primers SR, TP). A01 and A06 are inverted β -geo insertions and show an 800-bp band on the left and no band on the right. A02–A05 are successfully exchanged clones and show an 839-bp band on the left and the small 239-bp band on the right. Clone A07 carries the original β -geo insertion and gives rise to the 631-bp band on the left and the 516-bp band on the right.

optimized FLP recombinase (PGK-FLPo-pA; 14) was a gift from Phil Soriano to F.S. Caggs-CRE-IRES-Puro plasmid was described (4).

To generate pENTREx-FLP-hTDP-43(A315T), the SacI fragment of SA-dsRed-BGHpA (see above) was cloned into pBluescriptII-KS followed by the isolation of a BamHI/SalI fragment, which was then cloned to the BamHI/XhoI sites of pENTR4.

A full-length cDNA clone containing TDP-43(A315T) with 5'-HindIII and 3'-SacI overhangs was ligated as a HindIII/SacI fragment to the ade2-SA and BGH-pA of a SA-dsRed-BGHpA cassette in pENTR4, from which the dsRed coding sequence had been removed by HindIII/SacI.

Gateway reaction. The final exchange vectors pEX-FLP-hTDP-43(A315T) and pEXFLP-dsRed were established from pENTR-Ex vectors and pEX-Dest in a Gateway reaction using clonase and following standard protocols (Invitrogen).

Gene trap insertions. With the exception of Tardbp and Gtf2ird insertions (Figure 2), gene trap clones with

insertions of rFLP^{ros}abeta^{geo} or rsF^{ros}abeta^{geo} were chosen randomly in either 0, +1 or +2 reading frames. rsF^{ros}abeta^{geo} consists of a splice acceptor α -geo polyA cassette, flanked on each side by FRT, F3, loxP and lox5111 or lox511, in head-to-head orientation (FIEs array, Figure 1B). For further details, see <http://www.genetrap.de>.

ES cell culture

Gene trap clones. Transfections were performed using the FIEs conditional GGTC gene trap clones (for further details see <http://www.genetrap.de>):

E079H11, E068C09, E311D09, E224B05, D045A10, E287F07, E326E05, E307D01, E326E12, E288B02, E224H09, E326E04 and E284H06.

Insertions were determined by splinkerette (splk)-PCR (15) and confirmed by genomic PCR using the following primers:

E311D09 (Etl4): 5'-gccggaagagatgctgagtc-3', 5'-taccctgtatccaataaaccc-3'

E068C09 (Etl4): 5'-aaactggtttcattggggatca-3', 5'-taccctgtatccaataaaccc-3'

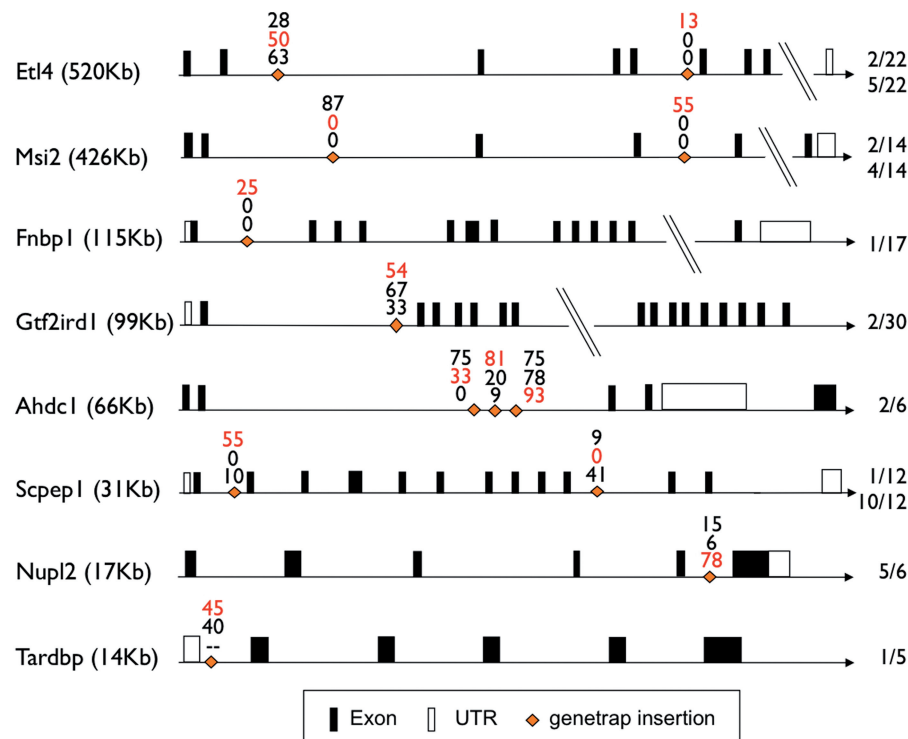


Figure 2. Schematic overview of the efficiency of FLPo-mediated RMCE in FLPex gene trap ES cell clones. Shown are all transfected gene trap clone insertions and the corresponding rounded efficiency of exchange reaction of pEx-FLP-dsRed in three different reading frames (0 upper, +1 middle and +2 lowest values in percent, red values indicating the frame matching the gene trap insertion). For the Tardbp insertion, the exchange vector pEx-FLP-hTDP-43(A315T) was used. All gene trap clones used had insertions in an intron of the respective gene, and the ratio on the right indicates in which intron out of how many total introns the insertion is located. On the left, the size of the genes is indicated in kilobases, and the total size of the genes is not drawn in scale, with omitted DNA indicated. Untranslated and translated exons according to Ensembl. MGI Gene Identifiers and absolute expression levels in ES cells are listed in Supplementary Table S1.

E326E05 (Msi2): 5'-tccccatgtttctgtaattgg-3', 5'-gccaaaccta caggtgggtctttt-3'
 E307D01 (Msi2): see hygromycin excision.
 E287F07 (Fnbp1): β -geo insertion not confirmed.
 E079H11 (Gtf2ird1): 5'-atcgaatgtagcccaggatg-3', 5'-gccaa acctacaggtgggtctttt-3'
 E326C04 (Ahdc1): 5'-catcttgaacctcaagtttgccttt-3', 5'-taccg gtgtatccaataaaccc-3'
 E284H06 (Ahdc1): 5'-ctggtctctctccactgtgtt-3', 5'-taccggt gtatccaataaaccc-3'
 E224H09 (Ahdc1): 5'-agaggtgacctgctggaatg-3', 5'-taccggt gtatccaataaaccc-3'
 E288B02 (Scepl1): 5'-ccaaggtgggaaagatgaggtg-3', 5'-gccaa acctacaggtgggtctttt-3'
 E326E12 (Scepl1): 5'-cacatggtgaccttcagagcag-3', 5'-gccaa acctacaggtgggtctttt-3'
 D045A10 (Tardbp): 5'-ccaagtcccatggtgacaac-3', 5'-cgagtgt tgcttcggagag-3'

Recombinase-mediated cassette exchange. All clones were derived from feeder independent E14 Tg2A.4 cells (19; a gift from Kent Lloyd to T. F.) gene trap lines, with the exception of D045A10, which was derived from a TBV2 cell line (20) and was cultured on mouse embryonic fibroblast feeder layer. ES cell lines were grown under standard culture conditions (<http://www.genetrap.de>). Cells were co-electroporated with 30 μ g supercoiled

plasmid DNA of pEX-FLP and 70 μ g of FLPo and selected for hygromycin resistance after 48 h (150 μ g/ml, Sigma-Aldrich) for at least 9 days.

About 10^7 cells were electroporated per experiment with an EPI 2500 Elektroporations-Impuls generator 0–2500 V (Fischer), cells were pulsed for 2 ms at 300 V in 0.4-cm cuvettes in 700 μ l phosphate-buffered saline (PBS). After electroporation, ES cells were plated on gelatine-coated culture dishes (5×10^6 cells/100 mm).

Hygromycin resistant colonies were transferred to 96-well dishes and expanded to three replicates of 48-wells. One plate was frozen as a stock, one was used to determine β -gal activity and one to isolate genomic DNA for further analysis.

Cre transfection. One exchange clone (E307D01 derivative) was transfected with 50 μ g of supercoiled Caggs-CRE-IRES-Puro plasmid. After electroporation, 50% of electroporated cells were plated on a 5×100 -mm gelatine-coated dishes and cultured for 48 h without selection. Pooled genomic DNA served as a template for PCR analysis. The remaining cells of the electroporation were plated on a 1×100 -mm gelatine-coated dish and puromycin-selected for 5 days (1 μ g/ml, Sigma). After expansion, total RNA was extracted (Trizol) and subsequent RT-PCR was performed.

SB transfection. One exchanged clone (E307D01 derivative) was transfected with variable amounts of supercoiled SB plasmid (0, 10 and 70 µg). After plating each electroporation on 1 × 100-mm gelatine-coated dishes, cells were cultured for 48 h, and genomic DNA was extracted of each pooled dish for PCR analysis.

Analysis of exchange clones

5' FRT site PCR. Before cassette exchanges, the integrity of 5' FRT sites was determined by PCR using the oligonucleotides: SR 5'-gccaaacctacaggtgggtcttt-3' and B034 5'-tgtaaacgacgggatccgcc-3' (9). Loss of a 5' FRT site yields a 548-bp product instead of a 673-bp product.

X-gal staining. Initially β-Gal-positive gene trap clones become β-Gal-negative after successful cassette exchange or gene trap inversion, therefore by β-gal activity false positive clones can be excluded. X-Gal staining was performed as described (21).

Southern blot analysis. To evaluate copy number and possible random insertions after RMCE, genomic DNA of exchanged clones was analysed in a Southern blot analysis using a 800-bp neomycin (Pst I fragment of pPKJ; 22) and a 727-bp hygromycin (EcoR I/Sca I fragment of pEX-FLP) probe. DNA from 151 independent clones (90xE224B05, 12xE288B02 and 10xE326E12 derivatives) was digested overnight at 37°C by HindIII for both probes and run on 0.8% agarose gel in 1xTAE. Gels were blotted on a nylon membrane (Hybond N) and hybridized following standard procedures.

FLP inversion PCR. Conditional gene trap insertions are flanked by FLP cassettes. Therefore, FLPo also inverted the β-geo gene trap vector, which resulted in false negative β-gal activity. To analyse all RMCE clones, a multiplex PCR was performed, which yielded either the inversion band of about 800 bp or the original band of 630 bp using following oligonucleotides: 5'-ctccgctcctcttcctcat-3' (B045), 5'-cctccccgtgcttccttgac-3' (B048) and 5'-tttgaggggacgacagat-3' (B050). Correctly exchanged clones produce a 839-bp fragment in this assay for pExFLP-dsRed (Figure 1C) and 448 bp for pEx-FLP-hTDP-43(A315T; Figure 4C).

Exchange PCR. Clones were screened for successful exchange by using an internal oligonucleotide in the splice acceptor of pEX-FLP-dsRed and one in the 5'-LTR: 5'-gccaaacctacaggtgggtcttt-3' (SR) and 5'-atcaag gaaacctgactactg-3' (TP). Using these primers, positive exchange resulted in a 239-bp band, no exchange yielded a 631-bp PCR product. For pEx-FLP-hTDP-43(A315T), a positive exchange produced a 262-bp product.

Further analysis

Hygromycin excision by Cre. This experiment was first performed *in vitro*, using the exchange clone A03, a derivative from gene trap clone E307D01. To analyse Cre-mediated excision, DNA of five pooled transfected dishes (50 µg Caggs-Cre-IRES-Puro) compared to the original exchange clone was extracted and screened by

PCR for hygromycin excision using following oligonucleotides: 5'-atcaaggaacacctggactactg-3' (TP) and 5'-gccaa acctacaggtgggtcttt-3' (SR). Undeleted hygromycin cassette led to a 239-bp product, after successful deletion a 619-bp product was amplified.

To analyse the resulting dsRed expression after Cre-mediated deletion under control of the endogenous promoter, RNA of three individual clones and the original exchange clone was extracted and analysed in a RT-PCR followed in a triplex PCR. Three oligonucleotides were designed, one situated in Msi2 exon II (5'-aatgtttatcggtggactgac-3'), one in Msi2 exon III (5'-cgttcgttggtggtatctct-3') and an internal primer in the dsRed cassette (5'-gtgcttcacgtacaccttgag-3'). This PCR yielded a 427-bp product for the wild-type genomic sequence, a 550-bp fragment after successful Cre excision and a 94-bp fragment of the wild-type transcript.

In vivo excision of hygromycin by Cre was done using Rosa26Cre transgenic mice (Taconic; 006467-T-F Heterozygous C57BL/6NTac-Gt(ROSA)26Sortm16(cre)Arte). Transgenic Cre mice were mated to pEx-FLP-hTDP-43(A315T) mutants, and embryos were sacrificed at E17.5. Genomic DNA was isolated from tails, and genotyping was done using SR/TP and B048/B045 primer combinations and Cre-specific primers pCre1 5'-atgcccaagaagaaggaaggt-3' and pCre2 5'-gaaatc agtcgcttcgaacgtaga-3'. Undeleted hygromycin produced a band of 262 bp, and deletion of hygromycin led to a slightly larger fragment of 321 bp. The product for Cre-specific primers was 447 bp. For pEx-FLP-hTDP-43(A315T), the SR/TP and B048/B045 PCR product sizes after hygromycin deletion differed in size from all other clones tested. The reason for this was the absence of SB-transposase IR/DR recognition sites from the pEx-FLP-hTDP-43(A315T) vector (Figure 4).

Western blot analysis

Isolated proteins (RIPA buffer) were run on Nu-PAGE 10% Bis-Tris gel (Invitrogen) and transferred onto PVDF membrane (Pall Corporation). ES cell protein was probed with a polyclonal rabbit anti RFP antibody (Abcam, 1:5000 dilution) or a monoclonal mouse anti-beta-actin (Biozol, 1:5000). The secondary antibody was peroxidase-conjugated goat anti-rabbit (Jackson Immuno Research Laboratories, Inc., 1:10 000 dilution) or goat anti-mouse (Jackson Immuno Research Laboratories, Inc., 1:10 000). Mouse tissue protein was probed with TARDBP polyclonal antibody: 10782-2-AP; Proteintech Group, Inc.: Purified rabbit anti human TARDBP polyclonal Antibody (dilution: 1:1500) and TARDBP monoclonal antibody: anti-human TARDBP antibody (ab57105; ABCAM; 1.25 µg/ml). For signal detection, ECL Detection Reagents I + II (GE Healthcare UK Limited) was used in conjunction with Amersham Hyperfilm ECL.

Transposase remobilization by SB100. This experiment was performed using exchange clone A03 (E307D01 derivative), which was transfected with different amounts of SB plasmid (0, 10 and 70 µg). To analyse mobilization of the

dsRed cassette by the SB100 transposase, two PCRs with one 3' external reverse oligonucleotide (B045) and two different forward primers, either in the BGHpA of the dsRed cassette (B048) or in the hygromycin coding sequence (H) were performed. Mobilization events led to a 1009-bp product using the H/B045 primer combination in addition to the 839-bp product (B048/B045). Oligonucleotide sequences are as follows: 5'-caagctctgatagagttggtaag-3' (H), 5'-ctccccctgccttcttgac-3' (B048) and 5'-ctcgcctctcttctccat-3' (B045).

RESULTS

The vectors

Any given DNA can be introduced into pEX-Dest via the Gateway (Invitrogen) system; therefore, the RMCE system introduced here can be applied universally. For this purpose, it was designed as a two-component vector system, consisting of pEX-Dest, harboring an *in vitro* selection marker and all necessary recombinase recognition sites (RRS) and the shuttle vector pENTR-EX. Both components harbour the corresponding attL/R sites to insert any sequence of interest to establish the final pEX-FLP in a Gateway reaction. Instead of pENTR-EX, any Gateway-compatible entry vector could be used (Figure 1A).

pENTR-EX. The vector pENTR4 (Invitrogen) served as a source for attL1/2 recombination sites in pEX-FLP. The cat/ccdb cassette was replaced by the IR/DR flanked adSA-dsRed-BGHPA-lox5171-lox5171 fragment (Figure 1C). The features of the inserted cassette are (i) inverted and direct repeats (IR/DR), which are recognized by the SB transposase; (ii) an adenoviral splice acceptor sequence (SA) followed by the promoterless coding sequence of the red fluorescence protein (dsRed); (iii) the Bovine Growth Hormone poly(A) sequence (BGHPA); (iv) two identically oriented lox5171 sites which flank unique cloning sites, for later removal of an additional cassette, if needed. Only in the case of D045A10 (insertion in Tardbp), the cat/ccdb cassette was replaced by an ade-2 SA-flanked human cDNA (kindly provided by Manuela Neumann) carrying an A315T mutation (23) followed by BGHPA (Figure 4).

pEX-Dest. The vector was designed for FLP-mediated RMCE with FLEX gene trap vectors (Figure 1A; 8). The features of the pEX-Dest are (i) the face-to-face oriented 5' FRT and 3' F3 recombination sites, which flank all other functional parts; (ii) the adSA followed by the hygromycin resistance gene; (iii) two head-to-tail oriented loxP sites flanking the hygromycin selection cassette for Cre-mediated excision *in vitro* or *in vivo*; (iv) the PGK poly(A) signal; (v) the clonase recombination sites attR1/2 to enable the insertion of the pENTR-EX insert.

RMCE using FLEX gene trap clones. All gene trap clones presented in this study had insertions of a retroviral SA- β -geo-pA vector, flanked by RRS in FLEX configuration (8; see Figure 1B). In brief, the cassette consists of a

combination of inversely oriented original and mutant SSRs for FLP and Cre recombinases. This configuration allows unidirectional inversion of the β -geo cassette by the respective recombinase. Since the pEX-FLP insert was flanked with one set of heterotypic oppositely oriented SSRs for FLP recombinase, transient co-transfection of pEX-FLP and FLPo into FLEX gene trap clones allows recombination between the homotypic RRS. The RMCE strategy is outlined in Figure 1B.

Different outcomes after co-transfection of pEX-FLP and FLPo and hygromycin selection are possible: (i) recombination of pEX-FLP and β -geo in identical orientation in the event of recombination between the outer FRT/F3 sites; (ii) recombination of pEX-FLP in inverse orientation, resulting in hygromycin sensitivity in the event of recombination between the inner FRT/F3 sites. Inverse orientation was not selected, since it may be followed by another FLEX inversion and excision event, leading to correctly exchanged clones; (iii) inversion of the β -geo cassette combined with a random insertion of the pEX-FLP within a transcriptionally active gene, leading to false positive clones (see Supplementary Figure S1 for a comprehensive description of theoretical events before and after RMCE).

Different PCR strategies were used to identify successfully exchanged clones. Hygromycin resistant clones were first analysed for β -gal activity. β -Gal-negative clones were further screened by 'exchange PCR' using a 5' primer in the LTR (SR) and a nested primer in the splice acceptor (TP), which yielded either the hygromycin band of 239 bp (A02–A05), the β -geo band of 516 bp (A07) or no band after β -geo inversion (A01; Figure 1C right). To distinguish clones with inverted β -geo cassettes and random insertions of pEX-FLP and to identify false positive clones as described earlier, a triplex PCR using primers B045, B048 and B050 was performed, which yielded different product sizes depending on the orientation of the gene trap vector (8). DNA from RMCE clones yielded a slightly larger product (839 bp; A02–A05) as compared to the β -geo inversion (800 bp; A01, A06), whereas original β -geo insertions led to a 631-bp product (A07; Figure 1C, left). An assortment of clones was additionally screened in Southern blot analyses using lacZ, neomycin and hygromycin probes to detect multiple or possible random insertions. More than 90% of successfully exchanged clones showed an expected loss of lacZ and neomycin resistance (data not shown).

A total of 13 different conditional gene trap clones with insertions in eight independent genes were co-transfected with pEX-FLP in three reading frames (see Figure 2). The Scep1 clones were electroporated twice and results were averaged.

Insertions in the same gene were either chosen for different gene trap vector reading frames or different introns (Ahdc1, Msi2, Etl4 and Scep1) to determine possible differences in exchange efficiencies between 5' and 3' insertions in the same gene and between different reading frames of pEX-FLP. Cell number and DNA amount per electroporation were identical in all cases to obtain comparable results. The efficiencies of RMCE were calculated by relating the numbers of correctly exchanged clones

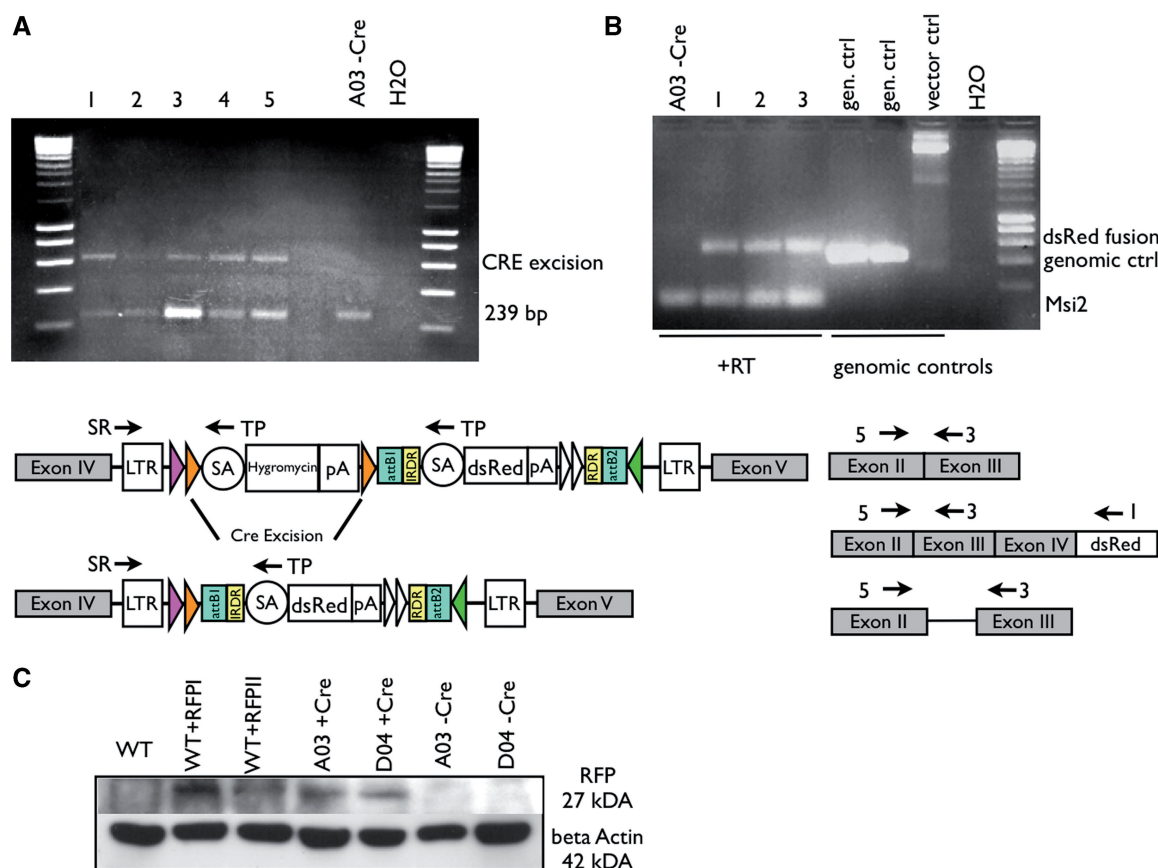


Figure 3. (A) Removal of hygromycin resistance upon Cre activity. Cre-transfected exchange clone A03 (E307D01/pExFLP-dsRed derivative) was analysed using primers located within both the hygromycin/dsRed cassettes (TP) and the LTR of the original gene trap vector (SR). PCR on total genomic DNA of five pooled transfected dishes (lanes 1–5) compared to genomic DNA of clone A03 before Cre transformation (A03 –Cre). The internal primer (TP) gives rise to the larger product (619 bp) after successful excision of the hygromycin cassette. Before hygromycin excision, primer TP amplifies a smaller product of 239 bp. (B) RT-PCR and triple PCR analysis to determine splicing to the dsRed after hygromycin excision by using external primers in exon II (5) of the insertion locus (Msi2) and an internal dsRed primer (I) and as internal wild-type control a primer located in exon III (3) of the Msi2 gene. cDNA of individual clones derived from Cre-transfected clone A03 after puromycin selection (lanes labelled 1–3) compared to cDNA of clone A03 before Cre transformation (lane A03 –Cre). As control genomic DNA of two gene trap clones (E307D01 and E326E12) and pEX-FLP-dsRed plasmid was used. (C) Western blot analysis of protein extracted from ES cells probed with a polyclonal RFP antibody. As controls wild-type ES cells either untransfected (WT) or transiently transfected with two different dsRed expression plasmids (WT + RFPI + II) were used. Exchange clones A03 and D04 were analysed prior (–CRE) and after (+CRE) transient CRE transfection. DsRed protein positive samples show a 27-kDa band. Lower blot shows loading control with beta actin monoclonal antibody (42 kDa).

to the total number clones isolated per electroporation. Of the gene trap insertions used for exchange reactions, seven were in frame 0, four in frame +1 and two in frame +2, ten in more 5' introns and three in more 3' introns of the respective gene (Figure 2).

Total clone number of all 38 electroporations varied from less than 10 clones in 17 cases up to several hundred clones per plate, the average clone number per electroporation was 124. The exchange efficiency of the matching frame varied from 0 to 93% and was 40% on average. The efficiency of both nonmatching frames per clone varied between 0 and 100%, and the average efficiency was 25%. In 8 of the 13 different electroporated gene trap clones, the matching frame was most efficient, while in five cases a nonmatching frame led to successful exchange.

In five cases (Etl4 3', Fnbp1, Scep1 5' and both Msi2 insertions), only one electroporated pEX-FLP frame led to hygromycin resistant clones, and in four out of these, it represented a frame matching the β -geo insertion.

The only exception was the Msi2 insertion in intron 2, where only frame 0 yielded positive clones, while the β -geo insertion was in frame +1 (Figure 2).

In vitro excision of selection marker. Further screens were performed using the successfully exchanged clone A03 (Figure 1C) derived from the E307D01 (Msi2, intron 4) gene trap clone.

To minimize secondary effects, the selection marker of pEX-FLP vector was designed to be removable by Cre recombinase *in vitro* or *in vivo*. To demonstrate excision *in vitro*, clone A03 was transiently transfected with Caggs-Cre-IRES-Puro plasmid with and without subsequent puromycin selection. Genomic DNA of plates without selection were pooled and screened by PCR with primers located within the splice acceptor sequence (hygromycin and dsRed) and in the LTR. This PCR yielded different-sized products before (239 bp) or after (619 bp) excision of the selection marker (Figure 3A). Lanes marked 1–5 (pooled transfected plates) show both

band sizes, whereas untransfected A03 only shows the smaller product. Without puromycin selection, Cre excision occurred only partially. To ensure the splicing to the dsRed cassette after Cre excision, the same transfection was performed followed by puromycin selection and cDNA of individual clones was screened by PCR with primers located in Msi2 exon II and an internal primer in the dsRed coding sequence (Figure 3B). This primer set yielded a 550-bp product in three independent clones (lanes 1–3), which was sequence-verified. As an internal control, a third primer located in Msi2 exon III was added, which yielded a 94-bp product of the wild-type transcript. As further controls, genomic DNA of two gene trap clones (E307D01 and E326E12) and the plasmid DNA of pEX-FLP were used. Gene trap genomic DNA templates yielded the wild-type genomic product (427 bp) including the intron 3/4.

After Cre excision of the selection marker, subsequent expression of the dsRed gene was validated in a western blot analysis. Wild-type ES cells, two different positive exchange clones (A03 and D04, E307D01-derived) before Cre transfection and two puromycin resistant clones after transient Cre transfection (A03 and D04 derived) were screened. As positive control, wild-type ES cells were transiently transfected with two different dsRed expression plasmids (WC150-DsRed2N1 and WC156-DsRed2N1), expanded for 3 days and total protein was extracted. No RFP was detectable before Cre transfection (A03 –Cre and D04 –Cre) or in wild-type protein (Figure 3C).

Germline transmission and excision of hygromycin in vivo. In order to analyse the germline potential of exchange clones, one successfully exchanged clone (Tardbp; clone 5/10) was injected into 73 C57Bl/6 host blastocysts after superovulation. Injected embryos were transferred to four pseudopregnant CD1 females and yielded 16 pups of which five were high-percentage agouti colour male chimeras and one was a low-percentage female. Three of the five male chimeras transmitted the mutation into the germline.

In the exchange vector for the Tardbp insertion, the gene encoding TDP-43 (24), the cDNA of interest was a human mutant form of TDP-43 carrying an A315T mutation, which was recently found in familial ALS patients (23). In order to activate the expression of the human isoform, mice were bred to Rosa26-Cre expressing animals (Tac-^{Gt(ROSA)26Sortm16(cre)Arte}; Taconic), and total protein was isolated from embryos at E17.5. As we have shown in Figure 4, embryos carrying both the Cre recombinase and the exchange vector pEx-FLP-TDP-43 (A315T) exhibit an excision of the hygromycin cassette and initiate the expression of the human mutant isoform. The human isoform was detected by a monoclonal antibody (ABCAM), which does not crossreact with the mouse protein. A polyclonal antibody (Proteintech) which recognizes both the mouse and human TDP-43, served as a control along with an antibody for β -actin.

Overall, seven RMCE clones were injected into host blastocysts of which two went germline and four are in

the chimera stage with a chimaerism over 80%, as based on agouti colour. One injected clone did not yield chimeras.

Mobilization of dsRed by SB transposase. Clone A03 (E307D01 derivative, Msi2 insertion in intron 4) was transiently transfected with different amounts (0, 10 and 70 μ g) of a SB expression vector. Pooled genomic DNA of transfected plates was screened by PCR. SB100-mediated excision was analysed by using two primer combinations. Primers were located either in the hygromycin coding sequence (H) or in the BGHpA of the dsRed (B048) cassette (which would be mobilized by SB100) and with an external primer located 5' of the F3 site (B045). Templates of plates transfected with 10 and 70 μ g SB100 showed both bands, the SB-excision band with primers H/B048 of 1009 bp and the unexcised band of 839 bp with primers B048/B045, whereas the controls 0 μ g and A03 without transfection (A03-SB) only yielded the 839-bp product. As further control, the gene trap clone E307D01 was used as template and yielded only a 631-bp band with primers B048/B045 (Figure 5). All PCR products were sequence-verified.

DISCUSSION

A number of studies have recently demonstrated the advantages of RMCE systems in order to generate mice carrying different knock-in and even 'humanized' alleles. However, these were entirely based on one-gene-at-a-time approaches including all the tedious vector design and gene-targeting efforts (25–29).

As we demonstrate in this work, RMCE with existing FLEEx promoter traps allows the modification of an entire ES cell library in a highly efficient and straightforward manner. Typical exchange efficiencies ranged around 40% in average with a preference towards the first introns of genes, which is in agreement with the overall insertion preferences of splice-acceptor containing gene trap vectors in large-scale screens (30,31).

As we determined, overall exchange efficiencies were directly related to the amount of FLPO recombinase plasmid. At otherwise identical conditions, amounts of FLPO plasmid below 50 μ g resulted in few correct exchange events. Only FLPO plasmid amounts of 70 μ g resulted in significant exchange efficiencies (data not shown). The required high-FLPO amounts may also reflect the fact that a number of different events are possible when exchanging for FLEEx gene traps, namely FLP-mediated inversions and excisions before the exchange reaction (Supplementary Figure S1). Nevertheless, the relatively high-FLPO recombinase plasmid amounts applied here raised some concern about unspecific secondary integrations of pEx-FLP. In three cases (two *Ahdcl*—clones; Figure 2), 500–2080 clones were selected as hygromycin-resistant after electroporation at identical conditions, as compared to only 50–100 clones in the majority of experiments, underlining initially the theoretical possibility of secondary pEx-FLP insertions in transcriptionally active genes. Therefore, 96 hygromycin-resistant *Ahdcl* exchange clones were

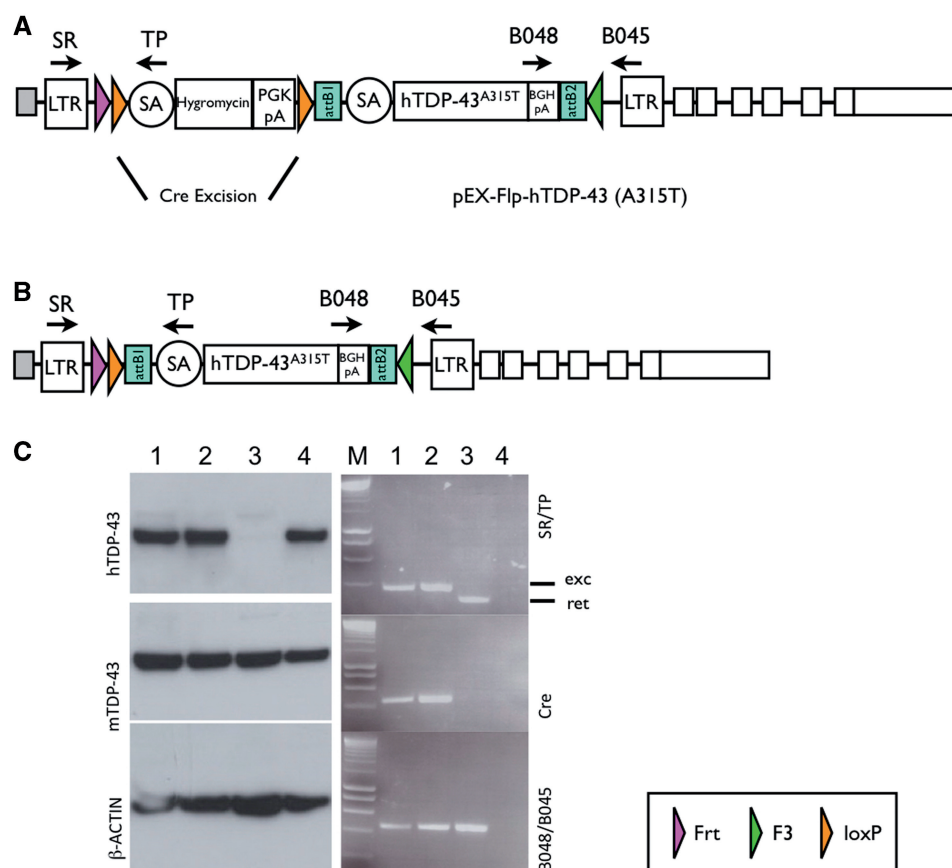


Figure 4. *In vivo* excision of the hygromycin-resistance cassette. (A) The gene trap vector rFLP-RosabetaGeo, which was inserted in intron 1 of the mouse Tardbp gene (clone D045A10 in Fig. 2), was replaced by pEX-FLP-hTDP-43(A315T). A mutant mouse line with this genomic modification was established and crossed with Rosa26-Cre deleter mice. (B) The RMCE allele in the mouse Tardbp intron 1 after Cre-mediated excision of the hygromycin-resistance cassette. (C) Left: total protein was extracted from embryonic heads (lanes 1–3), after mating to Cre-deleter mice at E17.5 or HEK293 cells (positive control, lane 4). Three western blots were done in parallel using antibodies against human Tdp-43 (hTDP-43), human and mouse TDP-43 (mTDP-43) and β-actin. TDP-43 runs at ~45 kDa, β-actin runs at 42 kDa. Right: DNA for genotyping was obtained from tails. PCR was performed using the primers SR/TP or B048/B045 as depicted in (A). The presence of Cre was detected using primers specific for Cre-recombinase. Exc: excision (of the hygromycin-resistance cassette); Ret: retention (of the hygromycin-resistance cassette). LTR: long terminal repeat, SA: splicing acceptor, PGK-pA: phosphoglycerate kinase poly A signal; BGHpA: bovine growth hormone polyA signal; attB1/2: gateway clonase recognition sites.

analysed by Southern blotting using a hygromycin probe. We found that only 2% of successfully exchanged clones carried secondary hygromycin insertions (not shown), and the overall success rate was comparable to other clones. We therefore suspected that differences in endogenous gene expression levels may account for the differing total clone numbers after RMCE. Therefore, expression levels of the trapped genes in Figure 2 were determined according to the absolute gene expression values using a recently published Affymetrix Chip Array data set, providing quantitative information on the expression levels of 7435 ENSEMBL genes in an undifferentiated E14 ESCs (32). However, no correlation with exchange efficiencies was determined (Supplementary Table 1).

Correctly exchanged clones are identified in a generic PCR using primers located within the hygromycin resistance cassette as well as in the LTR of the original gene trap vector, which is retained in the locus after exchange (Figure 1B). By Southern blot, we found more than 90% of successfully exchanged clones with an expected loss of both lacZ and neomycin (data not shown).

In order to express the cDNA of interest, the loxP-flanked hygromycin resistance cassette needs to be removed. As we show for the clones E307D01 and D045A10 (Figure 3), hygromycin was excised upon Cre activity *in vitro*. As we demonstrate for DsRed in Figure 3C, the gene-of-interest becomes active only 'after' Cre activity, a feature that adds conditionality to this RMCE system if tissue-specific Cre recombinases are used *in vivo*.

Cre removal may be performed *in vivo* in order to avoid higher passages of ES cell clones, which may result in reduced germline rates. As we demonstrate, mating of a mouse line carrying a human mutant form of Tardbp to a ubiquitously expressing Cre deleter line resulted in solid expression of the human isoform in mouse embryos after hygromycin excision (Figure 4). These mutants represent potential mouse models for familial amyotrophic lateral sclerosis and will be published elsewhere.

Although a successful exchange reaction is expected to occur in a frame-dependent manner, we show that matching frames become less important towards the 5'

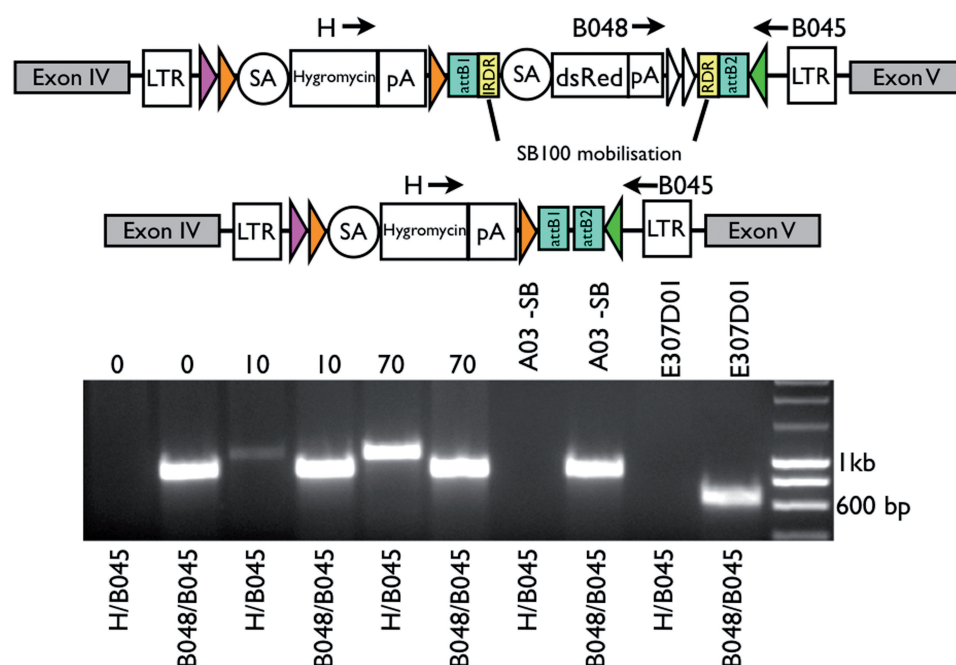


Figure 5. PCR analysis of SB100 transfection exchange clone to determine mobilization of IR/DR flanked cassette. Genomic DNA of pooled SB100 transfection dishes (0, 10 and 70- μ g SB100 plasmid) of exchange clone A03 (E307D01/pExFLP-dsRed derivative) was screened with primers located 5' of the F3 site (B045), in the pA of dsRed (B048) and in the hygromycin-resistance cassette (H). DNA was screened with either primer combination H/B045 or B048/B045, as controls genomic DNA of clone A03 (A03 -SB) and E307D01 was used. Primers H/B045 yielded a 1-kb product after successful excision of the IR/DR flanked cassette. Without mobilization, a 839-bp band was amplified by primers B048/B045, whereas the original gene trap insertion led to a 631-bp band.

end of genes. From the GGTC library, the existence of larger numbers of β -geo gene traps, selected using vectors with Kozak consensus translational start sequences in different reading frames inserted in identical introns of several genes, support our view: for this analysis, only clones with a clear genomic tag were chosen. Of a total of 5220 introns trapped, 3472 were trapped in only one, 1083 in two and 665 in all three reading frames. Of the clones, which were trapped in only one frame, 63% were either in the first or second intron (referred to as 5' insertion), while 84 and 87% of hits with multiple frames were in the 5'-end of genes. As likely reasons for this, we suspect initiation of translation starting at the Kozak site of the hygromycin resistance cassette, especially in cases where the first exon is untranslated (e.g. Tardbp clone; Figure 2) as well as alternative splicing. In addition, we do not rule out the presence of alternative promoters, which were recently predicted for 40–50% of all human and mouse genes (33). Therefore, fusions with endogenous peptides are possible, but cannot be predicted. In case fusions are not desired, the use of T2A cassettes will ensure the production of two independent peptides (34).

A seemingly similar RMCE system with even slightly higher efficiencies (75%) of their exchange vector pEXCH1 was recently introduced by Cobellis *et al.* (35). The exchange vector pEXCH2, however, which was designed to carry the transgenic cargo, uses a non-removable TK promoter-driven antibiotic resistance cassette, which selected higher numbers of random insertions and therefore had exchange efficiencies of a lower rate (7%) when compared to pEx-FLP. In addition,

pEXCH vectors are Gateway-incompatible and do not allow for conditional gene expression.

The first-generation of conditional gene trap vectors (8) bears a 50% risk of losing a 5' Frt site, which most likely occurs during reverse transcriptase activity in packaging cells. This risk was reduced to 10% after exchange of the spacer between 5' Frt and 5' F3 site (unpublished results). The loss of the 5' Frt site may lead to continuous F3/F3 recombination and therefore rotation of the original trap or—after RMCE—the replacement vector, until transient FLP recombinase expression is lost. RMCE events on inverted β -geo FIE vectors with deletions of the 5' Frt site result in, depending on the presence of FLP recombinase, rotating hygromycin cassettes, of which only correctly orientations are selected by the antibiotic. After sequencing of 5' FIE arrays, we identified one clone (Tardbp; Figure 2), which lacked the 5' Frt site. However, also in this case, overall exchange efficiencies were 40–50%.

In summary, the RMCE system presented here extends the possibilities for further conditional or non-conditional gene expression, restricted only by the availability of FIE gene trap clones. First, it represents an alternative to conventional transgenic technology with entire control over copy number and endogenous expression already *in vitro*. This should circumvent, e.g., epigenetic inactivation as a result of high-copy number followed by cosuppression of transgenes (36). Second, FIE-RMCE facilitates genetic pathway dissection by allowing e.g. replacement of presumed upstream transcriptional regulators by their potential targets. Third, this system provides a simple

method in order to replace given mouse genes with the human counterpart on the transcriptional level. As we demonstrate, it is especially suited for the expression of human disease-causing genes containing point mutations in the corresponding cells and tissues of the mouse. The combined GGTC and EUComm FLEx libraries currently represent more than 791 independent confirmed or candidate genes for human genetic diseases. Fourth, this system will be particularly of interest to further extend the catalogue of Cre-expressing mouse lines.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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