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TECHNOLOGY REPORT

An Improved Flp Deleter Mouse in C57Bl/6 Based on Flpo Recombinase

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Summary: Recently, a codon improved version of the Flpe site specific recombinase, termed Flpo, was reported as having greatly improved performance in mammalian cell applications. However, the degree of improvement could not be estimated because essentially no Flpe activity was observed. Here, we compare Flpe and Flpo accurately in a mammalian cell assay to estimate that Flpo is about five times more active than Flpe and similar to Cre and Dre. Consequently, we generated a Flpo deleter mouse line from the JM8 C57Bl/6 ES cells used in the EUCOMM and KOMP systematic knock-out programs. In breeding experiments, we show that the Flpo deleter delivers complete recombination using alleles that are incompletely recombined by a commonly used Flpe deleter. This indicates that the Flpo deleter is more efficient. genesis 48:512–520, 2010.

Key words: site specific recombination; conditional mutagenesis; genome engineering; allele design

Site specific recombinases (SSRs) are versatile and powerful tools for genome engineering in cells and model organisms from *E. coli* to plants and mammals (Branda and Dymecki, 2004; Glaser *et al.,* 2005). These remarkable enzymes locate two recombination target sites (RTs), which are usually DNA sequences 32bp long or longer, from amongst the rest of the genome and mediate precise recombination between them. The most useful SSRs for genome engineering bind their RTs as homodimers and recombination is mediated through a tetrameric synapse formed after contact between two homodimer-bound RTs (Grindley *et al*., 2006).

Tyrosine recombinases are the major SSRs for genome engineering and Cre, from the P1 coliphage, is pre-eminent. Flp recombinase from the *S. cerevisiae* 2 micron circle was the second tyrosine recombinase shown to work in mammalian cells (O'Gorman *et al.,* 1991). However, we found that Flp had a temperature optimum of 30°C, concordant with its yeast origin but suboptimal for application in mammalian cells (Buchholz *et al*., 1996). We also found the same limitation for two other yeast SSRs and failed to find another SSR as good as Cre from amongst several prokaryotic candidates (Ringrose *et al*., 1997; unpublished). Consequently, we applied molecular evolution to identify an altered Flp recombinase, termed Flpe, which had a temperature optimum at 37°C (Buchholz *et al*., 1998). In addition to the four amino acid changes that conveyed a higher temperature optimum, the addition of the SV40 Large T nuclear localization sequence (nls) and expression from the CAGGs promoter further improved performance in mammalian cells (Schaft *et al*., 2001). Nevertheless, by several empirical criteria, we concluded that Cre was clearly the more effective recombinase in mammalian cells and the search to find another remarkable SSR continued. This search has recently uncovered Dre recombinase (Anastassiadis *et al*., 2009; Sauer and McDermott, 2004).

Recently, it was reported that a codon optimized version of Flpe, termed Flpo, delivered greatly improved performance in mammalian cells (Raymond and Soriano, 2007). Flpo has the same amino acid sequence as Flpe

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and only the codon usage was altered to improve expression in mammalian cells. In the mammalian cell assay used by Raymond and Soriano, (2007), Flpo appeared to be at least an order of magnitude better than Flpe. This assay utilized a host cell line containing a stably integrated FRT recombination reporter, which was transfected with Flpe or Flpo expression plasmids carrying a selectable marker. After selection for the selectable marker of the expression plasmid to create a "double-stable" cell line, recombination was scored showing about 80% Flpo recombination. However, almost no Flpe recombination was observed, which is unexpected because a double-stable experiment allows recombination events to accumulate through cell divisions and near complete recombination is expected using any reasonably active Flp, including wt Flp, or the even weaker Flp F70L (Buchholz *et al*., 1996). Because we were concerned that the Flpe part of this experiment did not properly reflect Flpe activity, we initiated the experiments reported here.

Comparison of Flpe and Flpo in ES Cells

To compare Flpe and Flpo, we cloned Flpo into our best Flpe expression vector (pCAGGs-Flpe-IRES-puro; (Schaft *et al*., 2001). These two plasmids, pCAGGs-Flpe and pCAGGs-Flpo, are identical except for the codon changes in the Flpo coding region. The plasmids were compared by transient expression in two different ES cell lines. Both lines carried randomly integrated copies of the PGK-paZ-22 FRT recombination reporter (Buchholz *et al*., 1996) shown to be single copy by Southern blotting (clones 1 and 10; Fig. 1a; data not shown). Forty-eight hours after lipofection of increasing amounts of pCAGGs-Flpe or Flpo in triplicate, extracts were harvested for a β-galactosidase assay (Fig. 1b). By this test, overall Flpo delivered about five times more recombination than Flpe.

Next, we compared pCAGGs-Flpe and pCAGGs-Flpo in a colony scoring assay using electroporation rather than lipofection. We also included the PGK-Flpo expression vector described by Raymond and Soriano (2007). In this assay, we plated at lower dilution so that most colonies would arise from single cells. After 10 days, colonies were stained for β-galactosidase expression (Fig. 1c) and scored for complete staining reflecting early and complete recombination, mosaic staining reflecting incomplete recombination, or no staining reflecting no recombination (Fig. 1d). By this test, Flpo again delivered about a fivefold improvement over Flpe when expressed from either pCAGGs-Flpo or PGK-Flpo.

To compare the Flpo to the other two recombinases, Dre and Cre, we used the already existing Rosa26-rox ES cell line (Anastassiadis *et al*., 2009) and generated Rosa26-FRT and Rosa26-loxP reporter lines by gene targeting in R1 ES cells. The gene targeting efficiency was ~ 30% and established recombination reporters for Flp, Cre, and Dre that are identical except for the respective 34 bp RTs (data not shown). Two clones for each reporter line (Rosa26-rox, Rosa26-loxP, and Rosa26-FRT) were used for transient transfection with increasing amounts of the corresponding recombinase expression vector (CAGGs-Dre-IRES-puro, CAGGs-Cre-IRES-puro, CAGGs-Flpe-IRES-puro and CAGGs-Flpo-IRES-puro) in triplicate and cells were harvested for β-galactosidase assays (Fig. 2a,b). In this test, Flpo was again superior to Flpe and similar to Cre and Dre.

These data indicate that Flpo delivers more recombination in mammalian cells than Flpe and could therefore be the most effective version of Flp for mammalian genome engineering. Because Flpo and Flpe are identical proteins, we presume that the improvement is due to about a fivefold improved expression level of Flpo protein. However, the absence of a useful Flp antibody, despite many attempts, prevented us from examining this detail.

Generation of CAGGs-FIpo ES Cells and Mice

We established a new Flp deleter mouse line by randomly integrating pCAGGs-Flpo into C57Bl/6N JM8.F6 ES cells, which is the cell line selected for the international programs, EUCOMM and KOMP, to create conditional knock-out alleles in every mouse gene for public availability (Pettitt *et al*., 2009; Skarnes *et al*., 2010 [Nature in press]; http://www.knockoutmouse.org/). pCAGGs-Flpo-IRES-puro was linearized with *Spe*I and cells were selected with 1 μg/ml puromycin after electroporation. Colonies were picked and screened by transient transfection using the Flp-reporter PGK-paZ-22. All clones showed β-galactosidase activity and two were selected for injection into 8-cell, C57Bl/6 albino, embryos (Pettitt *et al*., 2009; Poueymirou *et al*., 2007). Chimaeras were crossed to C57Bl/6 albino and germline transmission for one of these clones was obtained. After breeding, homozygous pCAGGS-Flpo mice were obtained at Mendelian frequencies thereby establishing that the transgene was not obviously mutagenic (data not shown).

The functionality of the Flpo deleter mouse line was tested by crossing to the RC::Fela reporter line (Fig. 3a) (Jensen *et al*., 2008) and analyzing E10.5 embryos. We crossed both Flpo males to Fela females and Fela males to Flpo females (Fig. 3b). Embryos were analyzed at E10.5 for GFP expression and genotype by PCR. We observed homogenous GFP fluorescence and complete recombination without any mosaicism in all embryos that contained both the Fela recombination reporter and the pCAGGS-Flpo transgene (Fig. 3b,c; data not shown).

Previously in collaboration with the Dymecki lab, we generated the ACTB::FLPe deleter mouse line (Rodriguez *et al*., 2000). In our hands, this line has achieved complete deletion of various FRT flanked cassettes from many, but not all, alleles. For example, we found that alleles of *TFIIA* and *Mll1* showed only mosaic deletion by *ACTB::FLPe* of their FRT-flanked cassettes. To evaluate whether the *CAGGs-Flpo* deleter was improved over *ACTB::FLPe*, we crossed male deleters to female *TFIIA* heterozygotes and evaluated recombination by PCR in E11.5 embryos. In all cases where the embryo carried both the *TFIIA* allele (Fig.

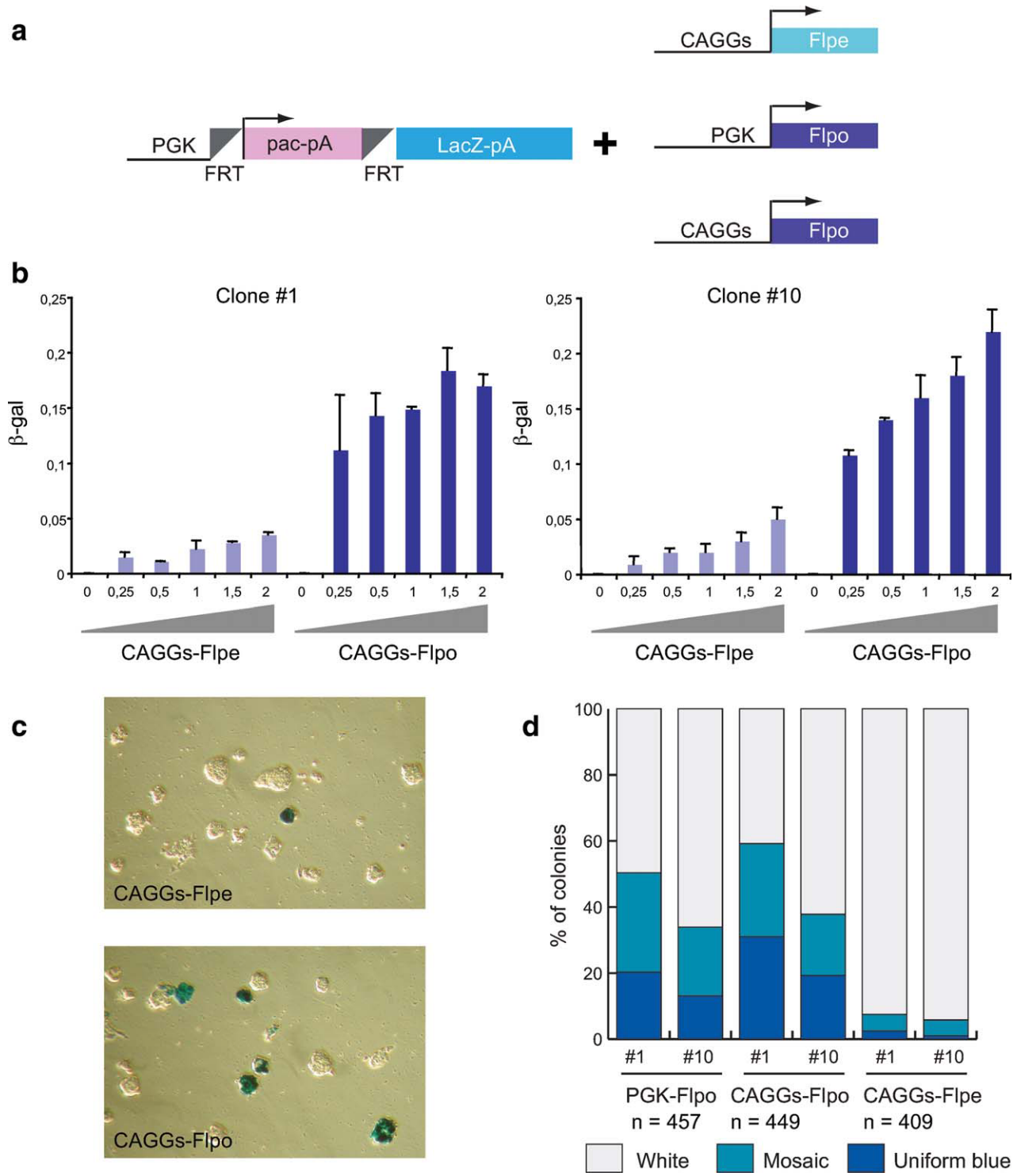

FIG. 1. Comparison of Flpo and Flpe. (**a**) Scheme of the Flp-recombinase reporter vector PGK-paZ-22 and the recombinase expression vectors for Flpo and Flpe. PGK, phosphoglycerate kinase promoter; CAGGs, the composite promoter cytomegalovirus (CMV) enhancer/β-actin promoter (**Kiwaki** *et al.*, 1996); pac, puromycin acetyltransferase gene; pA, polyadenylation signal. (**b**) Two PGK-paZ-22 reporter ES cell lines (#1 and #10) were lipofected in triplicate with different amounts of expression vectors (0 up to 2 μg DNA) as indicated. Cell extracts were tested for β-galactosidase activity. (**c**) Representative images of in situ β-galactosidase staining of a reporter ES cell line electroporated with Flpo or Flpe expression plasmids and plated in limited dilution. (**d**) Percentages of colonies from the experiment of (c) grouped as unrecombined (white), partially recombined (i.e., mosaic; light blue), and fully recombined (dark blue). *n* = the number of colonies counted.

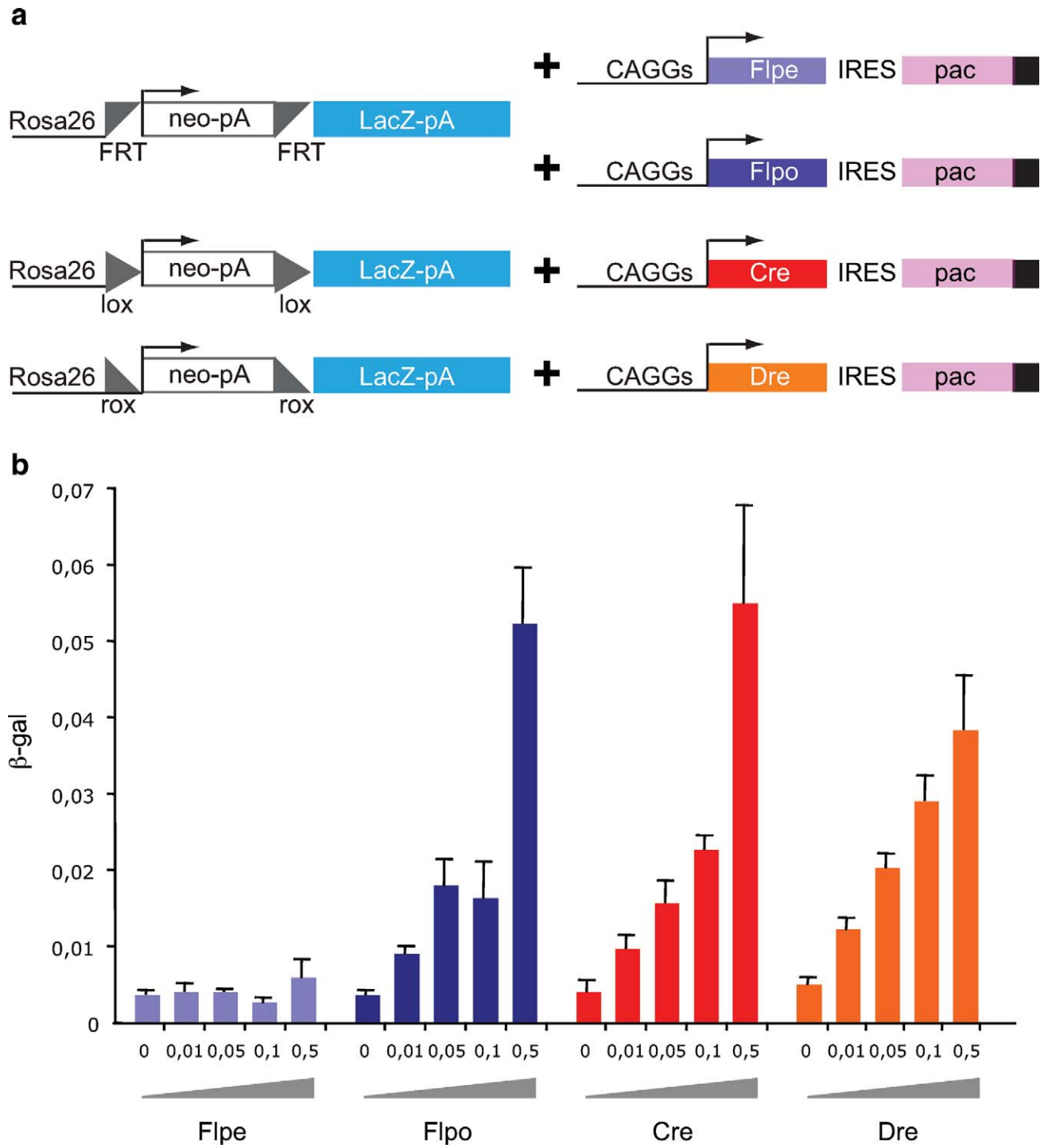

FIG. 2. Comparison of Flpe, Flpo, Dre, and Cre. (**a**) Schematic presentation of the three recombinase reporter constructs and the corresponding recombinase expression vectors. (**b**) β-galactosidase activity of transiently transfected reporter lines with different amounts of expression vectors (0, 10, 50, 100, and 500 ng DNA per 6-well). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

4a) and the Flpo transgene, complete recombination was observed. In contrast, only mosaic recombination was observed in *TFIIA*/FLPe embryos (Fig. 4b,c). We quantified the amount of the FRT-flanked cassette that remained after recombination using Q-PCR on genomic DNA isolated from the tail of E11.5 embryos. In all 10 embryos that were *ACTB::FLPe/+: TFIIA/+* we detected the unrecombined cassette at levels that varied between 20 and 70% of

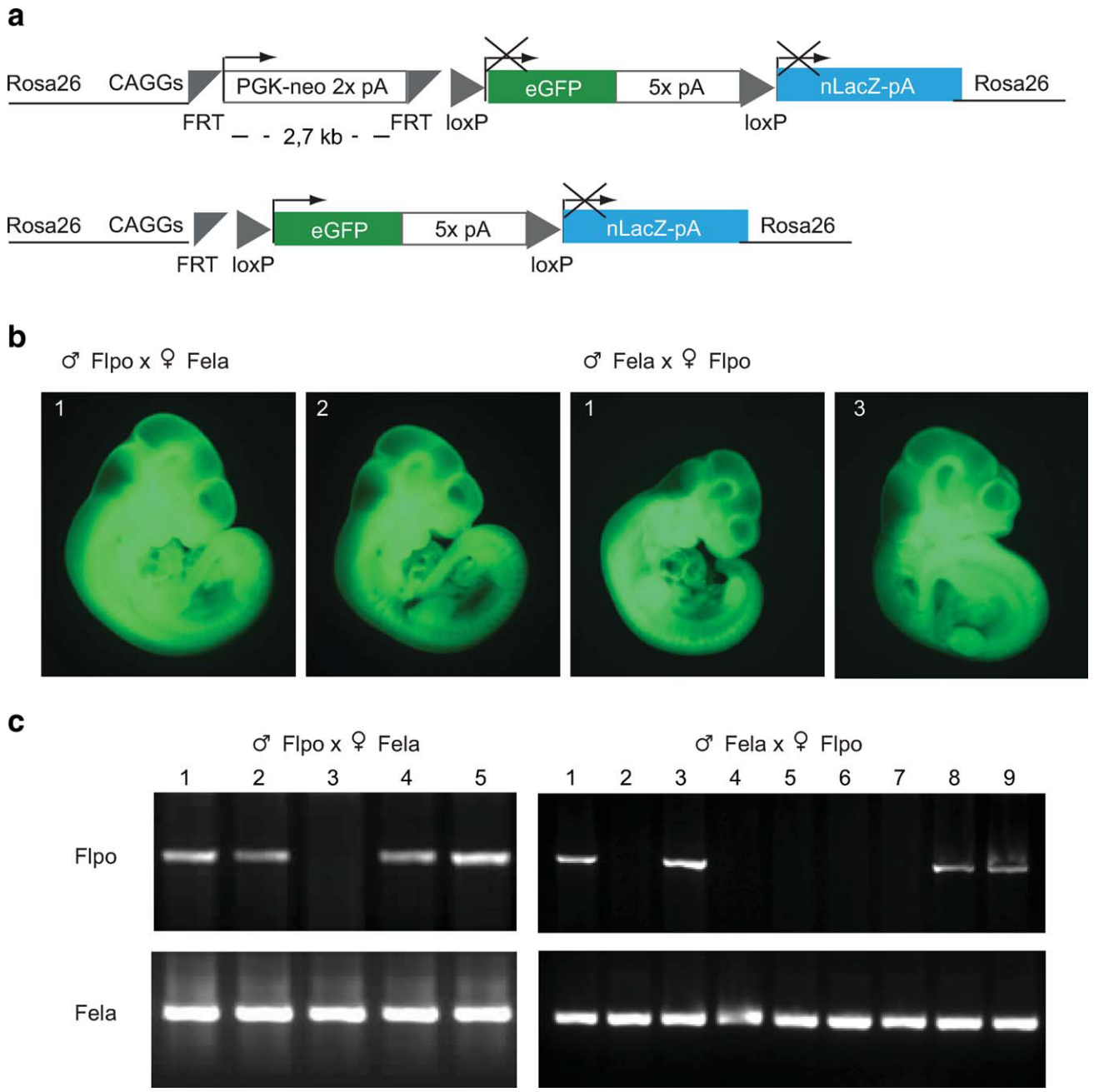

FIG. 3. Functional testing of the Flpo deleter mouse. (**a**) Scheme of the RC::Fela reporter allele in the Rosa26 locus before and after Flp recombination, which turns on eGFP (enhanced green fluorescent protein) expression. (**b**) eGFP epifluorescence in selected E10.5 embryos from Flpo × Fela (embryos 1 and 2; left panels) and Fela × Flpo (embryos 1 and 3; right panels) crosses as indicated. (**c**) Genotyping of yolk sacs from the Fela and Flpo crosses confirms that embryos 1 and 2 (left panel) and 1 and 3 (right panel) contained both the Flpo and Fela alleles.

the control level set at 100% for *TFIIA* heterozygotes without Flpe. In all 9 embryos that were *CAGGs-Flpo/+: TFIIA/+* we could not detect any Ct values, indicating that recombination was complete (Fig. 4c).

Similarly, the *CAGGs-Flpo* or *ACTB::FLPe* deleters were crossed to *Mll1* heterozygotes (Fig. 5a). Embryos (E10.5) were stained for β-galactosidase expression and genotyped by PCR. Although *ACTB::FLPe* did provoke recombination, a significant amount of β-galactosidase expression remained and was mosaic (Fig. 5c), whereas Flpo deleter action appeared to completely remove β-galactosidase expression from the *Mll1* heterozygous embryos (Fig. 5b).

Taken together, the above results indicate that Flpo is more efficient than Flpe for applications in mammalian cells and we report a new Flp deleter mouse line that appears to be more efficient than a currently popular Flpe deleter. Recently, a Flpo deleter mouse was generated by pronuclear injection of a PGK-Flpo construct in

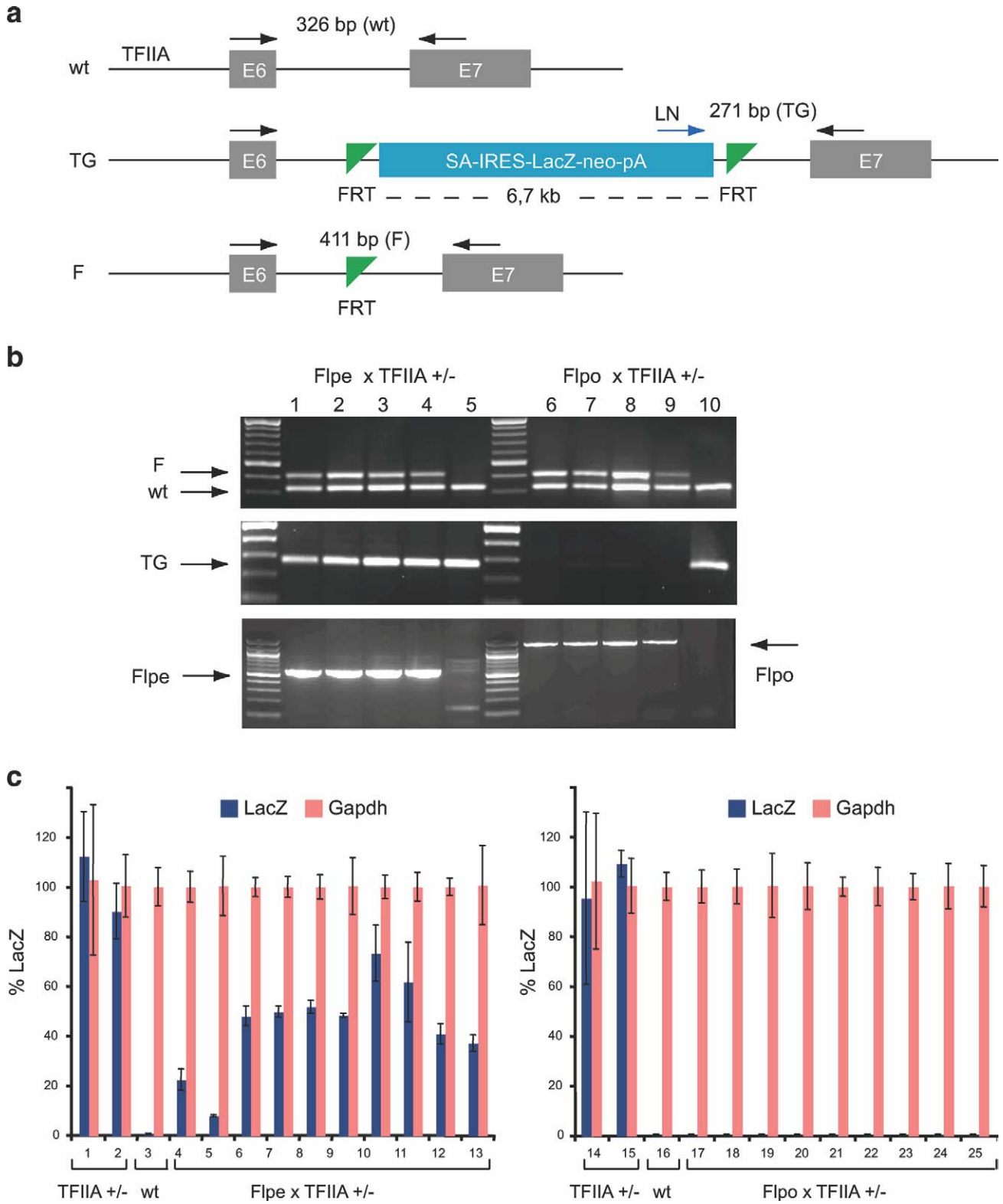

FIG. 4. Complete recombination by Flpo but not Flpe of a targeted TFIIA allele. (**a**) Scheme of the TFIIA allele before and after Flp recombination. The LacZ cassette flanked by FRT sites is 6.7kb (**Testa** *et al.*, 2004) and was inserted in intron 6 of the *TFIIA* locus by targeting (unpublished). The arrows represent the PCR primers used for detecting recombination (E6 + E7 = wt or F for Flp recombined; LN + E7 = TG). (**b**) The three panels show PCR for detecting recombination in E11.5 yolk sac DNA from a litter each of Flpe or Flpo crosses as indicated. All samples (1–10) were heterozygous for the targeted *TFIIA* allele and Flpe or Flpo except for lanes 5 (no Flpe) or Lane 10 (no Flpo). The top panel shows PCR products from primer pairs E6 and E7 showing the recombined allele (F) at 411 bp and the wt allele at 326 bp. The middle panel shows the product of PCR primers LN and E7 to detect the FRT-flanked cassette. All embryos that contain Flpe (1–4) also showed amplification of the cassette indicating that recombination was incomplete. There was no amplification in all embryos that contain Flpo (6–9) indicating that recombination was complete. The lower panel shows PCR detection of the Flp transgenes. (**c**) Q-PCR for quantifying the relative amount of FRT-flanked cassette using DNA prepared from E11.5 tail. Blue bars show the amount of the FRT-flanked cassette (LacZ-cassette) and red bars show the control (house keeping gene, *Gapdh*). Samples Nr. 1, 2, 14, and 15 are *TFIIA* heterozygous that did not contain Flpe or Flpo and serve as a positive control. Samples Nr. 3 and 16 are wt embryos and serve as a negative control. Samples Nr. 4–13 are Flpe + ; TFIIA +/− and Nr. 17–25 are Flpo + ; TFIIA +/−. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

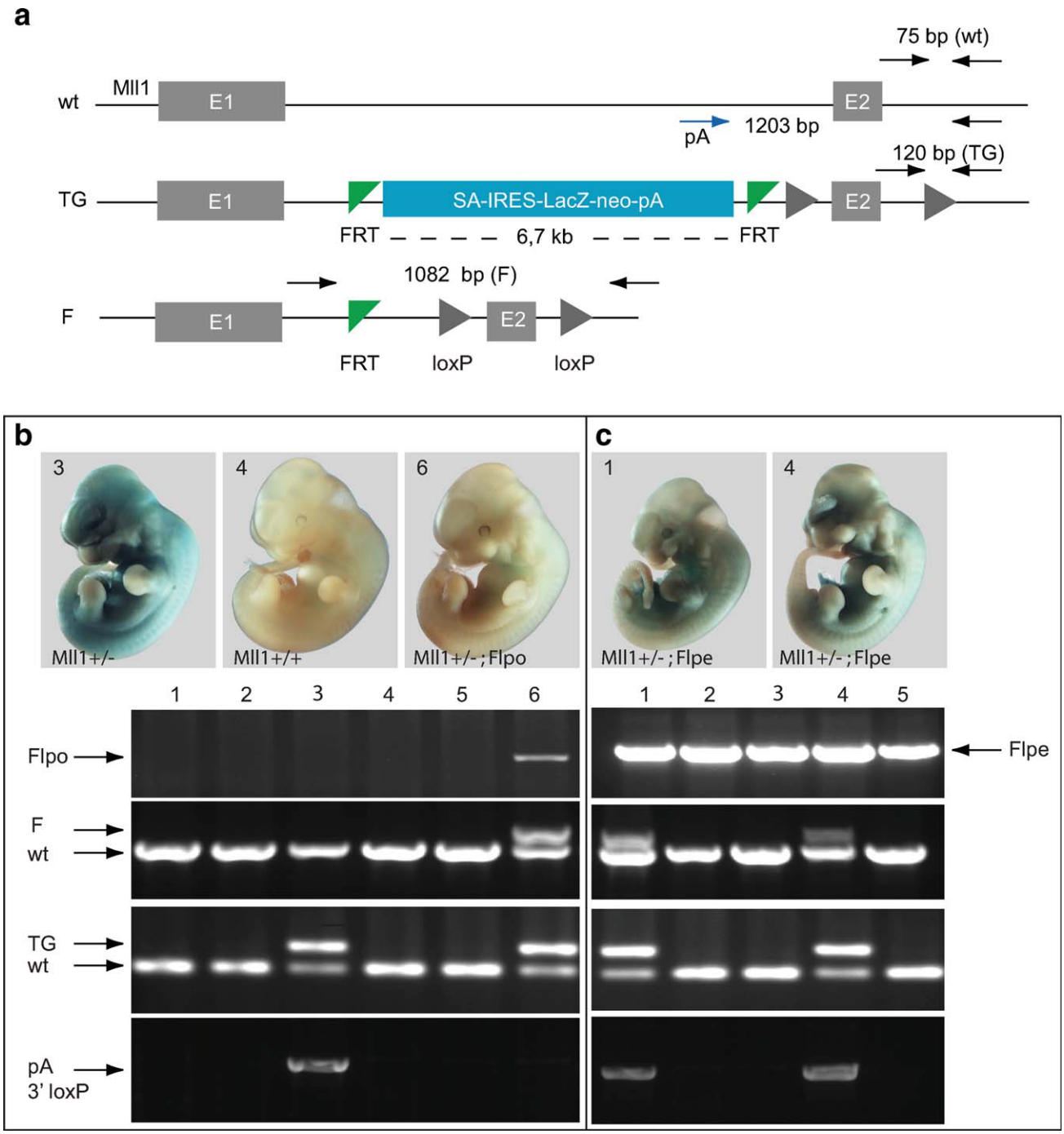

FIG. 5. Complete recombination by Flpo but not Flpe of a targeted *Mll1* allele. (**a**) Scheme of the *Mll1* allele before and after Flp recombination. The LacZ cassette flanked by FRT sites is 6.7kb (**Testa** *et al.,* 2004) and was inserted in intron 1 of the *Mll1* locus by targeting (unpublished). The arrows represent the PCR primers used for detecting recombination. Wild type (wt) and targeted (TG) alleles were distinguished using the PCR primers that flank the loxP site in the second intron. The presence of the LacZ cassette was detected using primers pA and the one 3′ of the second intron loxP site. Flp recombination (F) was detected using the primer upstream of the 5′ FRT site and the one 3′ of the second intron loxP site. (**b**) Embryos (E10.5) were taken from a single litter of *Flpo*/+ *x Mll1*/+ and genotyped as shown in the panels below. Embryo 3 carried the targeted Mll1 allele but not Flpo and therefore showed strong β-galactosidase expression. Embryo 4 did not contain either the *Mll1* or *Flpo* alleles, and therefore represents zero β-galactosidase expression. Embryo 6 contained both the *Mll1* and *Flpo* alleles. It shows no β-galactosidase expression indicating complete Flp recombination. (**c**) As for (b) except embryos were taken from a single litter of *ACTB::FLPe*/*hom x Mll1*/+. Embryos 1 and 4 contained both *Flpe* and *Mll1* alleles, however, showed significant β-galactosidase expression indicating incomplete Flp recombination.

C57Bl/6J fertilized oocytes (Wu *et al*., 2009). In concordance with our results, one of those PGK-Flpo lines showed complete recombination of FRT-flanked reporters and it was superior to the Flpe deleter line. The mice described here will be available soon through Mutant Mouse Regional Resource Center (MMRRC) and European Mutant Mouse Archive (EMMA).

METHODS

Plasmids

Plasmids were constructed using standard or recombineering (Zhang *et al*., 1998) methods available on request. The CAGGs-Flpo-IRES-puro expression vector was constructed by replacing Flpe in CAGGs-Flpe-IRES-puro (Schaft *et al*., 2001). The Rosa reporter constructs (Rosa-loxP-neo-loxP-LacZ, Rosa-FRT-neo-FRT-LacZ) were constructed by inserting the respecting cassettes into a Rosa26 targeting vector.

Cell Culture and Transfections

R1 and JM8.F6 embryonic stem cells (mES), were cultured on Mitomycin-C inactivated mouse embryonic fibroblasts (MEFs) using the following ES-medium (DMEM, 15% fetal calf serum [FCS], 2 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin/streptomycin, 100 μM nonessential amino acids (all Invitrogen), 100 μM β-mercaptoethanol) containing leukemia inhibitory factor (LIF). ES cells (10^7) were electroporated using a BIORAD electroporator (250 V, 500 μF) with 40 μg linearized constructs (CAGGs-Flpo-IRES-puro, PGK-paZ-22) and selected with 1 μg/ml puromycin (Sigma). The Rosa reporter-targeting constructs were digested with *Sfi*I and *Psh*AI and after electroporation cells were selected with 200 μg/ml G418 (Invitrogen). ES cells were transiently transfected using Lipofectamine 2000 (Invitrogen) following the manufacturers instructions.

Southern Blots

For the identification of ES cell clones with single copy integrations of the Flp-reporter PGK-paZ-22, genomic DNA was extracted from cells by proteinase K digestion and isopropanol precipitation. DNA was restricted overnight with EcoRV, separated on 0.8% agarose gels and blotted to nylon membranes (PALL). A probe specific for *LacZ* was labeled with ^{32}P by random priming (Roche Diagnostics). For correct targeting events of the Rosa reporter constructs, DNA was digested with *Eco*RI and the membranes were hybridized with a Rosa26 5′probe.

β-Galactosidase Assays

For β-galactosidase assays, whole cell extracts were prepared by freezing and thawing in 0.1 M Tris pH 7.8 containing 1 mM PMSF, 1 mM DTT, and Protease inhibitors (Sigma). Protein concentrations were determined by O.D. at 230 and 260 nm and calculated using the following formula: Concentration (μg/ml) = $100 \times (183 \times A_{230} - 75.8 \times A_{260})$. For each sample, 5 μg total protein was added in 800 μl of Solution I (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, and 50 mM β-mercaptoethanol [fresh]). Then 200 μl Solution II (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 2 mg/ml ONPG) was added and incubated at 37°C. The reaction was stopped with 500 μl 1M Na_2CO_3 and the O.D. was measured at 420 nm. For β-galactosidase staining, cells were rinsed twice with PBS and fixed with 2% formaldehyde; 0,1% glutaraldehyde for 2 min, washed three times with PBS and stained at 37°C in the dark with PBS containing 2 mM $MgCl_2$, 2.1 mg/ml K-ferrocyanide, 1.6 mg/ml K-ferricyanide and 1 mg/ml X-Gal. Embryos were fixed for 1.5 hours in 0.2% glutaraldehyde and washed three times for 5 min in wash buffer (2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet-P40 in PBS). They were then incubated in staining solution (1 mg/ml X-gal, 2.1 mg/ml K-ferrocyanide, and 1.6 mg/ml K-ferricyanide in wash buffer) O/N at 37°C, protected from light. Embryos were washed three times with PBS and postfix was done O/N at 4°C in 0.1% glutaraldehyde, 2% PFA/PBS.

Generation of Mice and Genotyping

CAGGs-Flpo-IRES-puro mice were generated by injection of JM8.F6 ES cells into 8-cell stage embryos. All manipulations were done in the Transgenic Core Facility (TCF) of the MPI-CBG, Dresden. Chimaeras were crossed to albino C57Bl/6 mice and were screened by PCR for germline transmission using the following primers:

Flpo1: 5′-GCTATCGAATTCCACCATGGCTCCTAAGAAGAA-3′ and
Flpo2: 5′-CAATGCGATGAATTCTCAGATCCGCCTGTTGATGTA-3′.

Genotyping of the Flpe embryos was done using the primers:

Flpe1: 5′-CCTAAGGTCCTGGTTCGTCA-3′ and
Flpe2: 5′-TTGTTGCTTTTTGCGTCTTG-3′.

Flp-mediated recombination in the TFIIA locus was detected using the following primers:

E6: 5′-AAGCTCCTGTTATCCAGCAGGTAA-3′,
E7: 5′-GTGAAATCCCTCCAGGAAGTGGGG-3′, and
LN: 5′-GGGGGTACCGCGTCGAGTTTAAA-3′.

Flp-mediated recombination in the Mll1 locus was detected using the following primers:

Loxd-se: 5′-CCTGCTGCCTGTGCTTGTAAA-3′,
Loxd-as: 5′-GTAGAAACCTACTTCCCATGCC-3′,
Mll-Flp: 5′-GAGGTAAGGAGAGTTTTTGCT-3′, and
pA: 5′-GCATTCTAGTTGTGGTTTGTC-3′.

Quantification

qPCR analysis was done in triplicates using LacZ and Gapdh specific primers. Ct values for LacZ were normalized against the values of the house keeping gene (Gapdh). Fold differences in expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method using as reference the positive control samples (Livak and Schmittgen, 2001).

GAPDH(se): 5′-TCACCACCATGGAGAAGGC-3′,
GAPDH(as): 5′-GCTAAGCAGTTGGTGGTGCA-3′,
Q-LacZ-1: 5′-AAATATGATGAAAACGGCAACC-3′, and
Q-LacZ-2: 5′-AACAGGTATTCGCTGGTCACTT-3′.

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