Supporting Information

SI Materials and Methods

Recombinant DNA construction. All PCR for DNA construction was done with Phusion DNA polymerase (Finnzymes, Finland). All DNA fragments that were amplified by PCR were completely sequenced after cloning. For primer sequences used in construction see Table S4.

pBT296 (*pBS-U-attP-FRT5-pSV40-Neo-pA-FRT5)*: Into a modified pBluescript, we subcloned:
1) a unique sequence "*U*" from the promoter of yeast *his3* gene:
(GGTGATAGGTGGCAAGTGGTATTCCGTAAGGATATC).
2) the single "full-length" *attP* site from *pTA-attP* (gift of Michelle Calos) (Ref. 1).
3) *FRT5* (GAAGTTCCTATTCCGAAGTTCCTATTCTTCAAAAGGTATAGGAACTTC) (Ref. 2, 3)-flanked neomycin resistance gene driven by an *SV40* promoter.

pBT298 *(pBS-U-attPx3-FRT5-pSV40-Neo-pA-FRT5):* Same as *pBT296*, but the "full-length" *attP* site was replaced by three sequential *attP* sites (70 bp each, sequence of a single site: CGGGAGTAGTGCCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGGCGTAGGG TCG), synthesized by Celtek Genes (Nashville, TN).

pBT305 *(pBS-U-attPx3-FRT5-pSV40-Neo-pA-PL-FRT5):* PL represents a polylinker: SbfI-HpaI-AatII. The plasmid was generated by inserting annealed oligos PR402 and PR403 into the XbaI site of *pBT298*, thereby destroying the XbaI sites on both ends of the PL.

pBT307 *(pBS-U-attPx3-FRT5-pSV40-Neo-pA-ϕC31o-pA-FRT5):* ϕC31o was amplified by PCR from *pPGKPhiC31obpA* (Addgene plasmid 13795) (Ref. 4) and subcloned into *pBT305*.

pBT308b *(pTOPO-pVasa*): A previously described fragment of the murine VASA promoter (5) was amplified by PCR from genomic DNA of the FVB strain and TOPO-cloned into *pTOPO* (Invitrogen).

pBT309a *(pBS-U-attPx3-FRT5-pSV40-Neo-pA-pVasa-ϕC31o-pA-FRT5)*: *pVasa* was subcloned from *pBT308b* into *pBT307*.

pBT310 *(pBS-U-attP-FRT5-pSV40-Neo-pA-pVasa-ϕC31o-pA-FRT5):* The NheI/AscI fragment from *pBT309a* was subcloned into the NheI/AscI-digested *pBT296*.

pBT311 *(pH11-U-attPx3-FRT5-pSV40-Neo-pA-pVasa-ϕC31o-pA-FRT5*): The SwaI/AscI fragment from *pBT309a* was subcloned into the PmeI/AscI-digested *pHipp11* (Ref. 6).

pBT312 *(pH11-U-attP-FRT5-pSV40-Neo-pA-pVasa-ϕC31o-pA-FRT5*): The SwaI/AscI fragment from *pBT310* was subcloned into the PmeI/AscI-digested *pHipp11* (Ref. 6).

pBT313 *(pR26-U-attPx3-FRT5-pSV40-Neo-pA-pVasa-ϕC31o-pA-FRT5*): The SwaI/AscI fragment from *pBT309a* was subcloned into the SwaI/AscI-digested *pROSA26* (Ref. 7).

pBT314 *(pR26-U-attP-FRT5-pSV40-Neo-pA-pVasa-ϕC31o-pA-FRT5*): The SwaI/AscI fragment from *pBT310* was subcloned into the SwaI/AscI-digested *pROSA26* (Ref. 7).

pBT316 *(pattB-pCA-GFP-pA)*: The SalI fragment from *pTA-attB* (1) containing the "full-length" *attB* site was subcloned into the SalI site of *pBT255* (*pCA-GFP4m-pA*).

pBT317 (*pET-ϕC31o-pA* – **for preparation of ϕC31o mRNA**): ϕC31o gene was amplified by PCR from *pPGKϕC31obpA* (Addgene plasmid 13795) (Ref. 4) using primers PR437 and PR438. The PCR was digested with BamHI and MseI and cloned into BamHI/NdeI-digested *pET11ϕC31pA* (8).

pBT340 *(pattB-pCA-GFP-pA-FRT5-pPGK-Flpo-pA)*. A fragment containing *FRT5-pPGK-Flpo-pA* was PCR amplified from *pPGKFLPobpA* (Addgene plasmid 13793) (Ref. 4) and cloned between NotI and AscI sites of *pBT316*.

pBT344 *(pattB-pCA-GFP-pA-FRT5* – **for cloning any DNA fragment to be integrated as a full plasmid using ϕC31; the bacterial backbone can be subsequently removed by crossing to *GFP-Flpo* mice***)*: The *pPGK-Flpo-pA* portion was removed from *pBT340*.

pBT346 *(pλ-attB-pCA-GFP-pA* – **for producing attB-containing minicircle *in vitro*)***:* I-SceI restriction site and the λ *attL1* were amplified from *pENTR-TopoD* using PR493 and PR494. The PCR product was digested with Acc65I and XhoI, and inserted into the Acc65I/XhoI-digested *pBT316* to generate a construction intermediate. Subsequently, the λ *attR1* site was amplified from *pENTR-TopoD* using PR495 and PR496 and cloned into the SacI/NotI-digested construction intermediate to generate *pBT346*.

pBT366 *(pattB-Hb9-GFP-pA-FRT5)*. The filled-in *Hb9-GFP* XhoI fragment from *pHB9-EGFP* (Addgene plasmid 16275) (Ref. 9) was subcloned into the PacI/AscI digested and filled-in *pBT344*.

pBT374 *(pattB-pCA-GFP-pA-attB^{Swa})*: The filled-in SalI fragment containing the *attB* site from *pBT316* was subcloned into the SwaI site of *pBT316*. This plasmid was used for initial cassette exchange tests, but for future use we recommend *pBT378* below, as it contains more convenient restriction sites.

pBT378 *(pattB-pCA-GFP-pA-attB* – **for ϕC31-mediated cassette exchange**): It contains more convenient restriction sites than *pBT374* that enable replacement of the *pCA-GFP-pA* insert with an insert of choice (ClaI, HindIII, PacI, PmeI, PstI between the first *attB* and *pCA*, and SwaI, AscI, SpeI and NotI between the *pA* and the second *attB*). It was created by subcloning the filled-in SalI fragment containing the *attB* site from *pBT316* into the BstXI-linearized and filled-in *pBT316*.

Preparation of plasmid DNA by a modified Qiagen mini-prep procedure. We started from 4 ml of DH5α bacterial culture grown in LB broth for not more than 10 h at 37ºC. We collected the bacteria in 2 ml tubes by spinning 2 ml of culture twice in the same tube. We doubled the recommended volumes of P1, P2 and N3 (Qiagen). After loading the samples onto the Qiagen

mini-prep columns, we washed the columns twice with buffer PB and then twice with buffer PE (Qiagen). The PB washes diminish but do not abolish RNase contamination. We eluted the DNA in 3-fold diluted EB (Qiagen). The plasmid DNA yield from a single prep was usually in the range of 7.5 to 25 μg. To obtain more DNA, several preps can be performed at the same time and pooled. We determined the concentration using the Nanodrop spectrophotometer (Thermo Scientific) and used at least 5-20 μg in 200 μl of solution for subsequent extractions to remove residual RNase (see below).

Phenol/chloroform extraction of plasmid DNA. At least 5 μg of DNA in 200 μl of solution were extracted twice with a phenol:chloroform (50:50) mix and twice with chloroform only. The DNA was mixed with 1/10 volume of 3M sodium-acetate pH 5.2, precipitated with 2.7 volumes of ethanol, and subsequently dissolved in sterile and RNase-free microinjection TE buffer (miTE; 0.1 mM EDTA, 10 mM Tris pH 7.5). The DNA was filtered through a sterile 0.2 μm filter (Millipore, Cat. No. SLGV004SL) and the concentration was determined using the Nanodrop spectrophotometer (Thermo Scientific).

Preparation of minicircle DNA. We started from 4 μg of *pBT346* plasmid DNA purified by the modified Qiagen mini-prep procedure above. The 200 μl-recombination reaction consisted of 40 μl of LR clonase II (Invitrogen, Cat. No. 11791-020) and 160 μl of the DNA diluted in miTE buffer. The reaction was incubated for 3 h at 25ºC in the PCR machine. The reaction was purified using the QIAquick PCR Purification kit (Qiagen) and the DNA was eluted in 35 μl of 3-fold diluted EB (Qiagen). The DNA was digested in a 50 μl reaction with 20U each of SacI and KpnI. The DNA was analyzed on 1% agarose gel (Figure S3) and the minicircle DNA was purified using the MinElute Gel Extraction kit (Qiagen). The DNA was eluted in 12 μl of 3-fold diluted EB (Qiagen), filtered through a sterile 0.2 μm filter (Millipore, Cat. No. SLGV004SL) and the concentration was determined using the Nanodrop spectrophotometer (Thermo Scientific). The overall yield of the DNA with this procedure is about 3% of the starting DNA. The DNA was diluted to 6 ng/μl in miTE buffer and stored at -80ºC before injection. We have noticed that this DNA is more difficult to microinject than plasmid DNA.

Preparation of mouse genomic DNA No. 1 – for genotyping by long-range PCR. Tissue samples from mouse pups (~5 mm of each tail tip) were collected in 1.5-ml tubes. Each tail was digested in 0.5 ml of lysis buffer (TrisHCl pH 8-8.5, 100 mM; EDTA pH 8, 5 mM; SDS, 0.2%; NaCl, 200 mM; proteinase K, 0.2 mg/ml) at 55ºC overnight. The digestion was centrifuged on the next day for 5 min. at ≥10,000 g, and 450 μl of the supernatant were transferred to a new tube. After adding 450 μl of 5M NaCl, the tubes were rocked for 5 min. at room temperature. The samples were centrifuged at ≥10,000 g for 10 min. 750 μl of the supernatant were transferred to a new tube and precipitated with 750 μl of isopropanol. The samples were centrifuged for 15 min at ≥10,000 g at room temperature. The pellet was washed with 500 μl of 70% ethanol and the tubes were air dried for 5-10 min. The pellet was dissolved in 200 μl of TE (1 mM EDTA, 10 mM Tris pH 7.5), and extracted twice with a phenol:chloroform (50:50) mix and twice with chloroform only. The DNA was precipitated with 1/10 volume of 3M sodium-acetate pH 5.2 and 2.7 volumes of ethanol, and dissolved in 200 μl of TE. 1 μl of this solution was used as template in long-range PCR (see below).

Preparation of mouse genomic DNA No. 2 – for genotyping by short-range PCR. Tissue samples from embryos or pups (~2 mm of each tail tip) were collected in 96-well plates, so that many subsequent steps could be done with a multi-channel pipet. The plate was sealed with the plastic cover (ThermalSeal, E&K Scientific, Cat. No. 100-THER-PLT) and briefly centrifuged before the next step to make sure that the tissue samples were on the bottom of the wells. Each tissue sample was lysed with 120 μl of 50 mM NaOH. The plate was sealed with a new plastic cover, incubated in PCR machine at 95ºC for 38 min., briefly centrifuged to collect possible condensation, and the cover was peeled away. At this moment, some gas may be released from the samples and cause droplets of lysate to come close to the rim of the wells. We collected any solution that was close to the rim of the wells by blotting it away carefully with a kimwipe. The lysates were neutralized with 30 μl of 1 M Tris (pH 7.5), tightly sealed with a new plastic seal, vortexed (using a flat head vortex), and briefly centrifuged. 1 μl of this prep was used for PCR (Materials and Methods).

Long-range genomic PCR. We used LA Taq (Takara Bio; Cat Nos. RR02AG and RR002M) and the following primers for *H11* 5' arm: PR374 and PR432; *H11* 3' arm: PR351 and PR422; *Rosa26* 5' arm: Rosa3 and PR432, and *Rosa26* 3' arm: PR351 and PR395. The complete PCR reactions had a volume of 20 μl, and contained 1 μl of genomic DNA that was prepared by the DNA preparation protocol No. 1 above. For *H11* 5'arm, we used the LA PCR buffer II and the following program: 94ºC, 3 min., 40 cycles of: [94ºC, 20 sec.; 60ºC, 30 sec.; 68ºC, 5 min. 30 sec.], 72ºC, 15 min. For *H11* 3' arm, we used the GC buffer I and the following program: 94ºC, 3 min., 40 cycles of: [94ºC, 30 sec.; 56ºC, 30 sec.; 72ºC, 3 min. 30 sec.], 72ºC, 15 min. For *Rosa26* 3' arm, we used the LA PCR buffer II and the following program: 94ºC, 3 min., 40 cycles of: [94ºC, 20 sec.; 58ºC, 30 sec.; 68ºC, 5 min.], 72ºC, 15 min. For *Rosa26* 5' arm, we used the GC buffer I and the following program: 94ºC, 3 min., 40 cycles of: [94ºC, 30 sec.; 60ºC, 30 sec.; 72ºC, 2 min.], 72ºC, 5 min.

Short-range genomic PCR. All short-range PCRs were performed in 20 μl reactions containing 1 μl of prepared DNA (see Preparation of mouse genomic DNA No. 2 above), using Taq polymerase (Qiagen), and the following program: 94ºC, 3 min.; 32 cycles of [94ºC, 20 sec., 60ºC, 25 sec., 72ºC, 45 sec.]; 72ºC, 5 min. Taq polymerase from Qiagen has proven more reliable than polymerases from other manufacturers with this particular DNA preparation. The products were analyzed on a 2% agarose gel.

Primer combinations and expected product sizes for the PCRs used in this study are:

PCR1 in Fig. 1 and Fig. S1; 5'-junction: PR425 and PR436. Expected sizes are: 147 bp, 217 bp, 287 bp, and 244 bp, for insertion into the first *attP*, second *attP*, third *attP*, or full-length *attP*, respectively.

PCR2 in Fig. 1 and Fig. S1; 3'-junction: PR522 and PR387. Expected sizes are: 371 bp, 301 bp, 231 bp, and 313 bp, for insertion into the first *attP*, second *attP*, third *attP*, or full-length *attP*, respectively.

PCR3 in Fig. 1; 5'-junction*:* PR425 and PR551. Expected sizes are 395 bp, 465 bp, 535 bp, and 492 bp, for insertion into the first *attP*, second *attP*, third *attP*, or full-length *attP*, respectively.

PCR4 in Fig. 1; 3'-junction: PR488 and PR387. In the case of cassette exchange with *pBT374*, expected sizes are 502 bp, 432 bp, and 362 bp, for insertion into the first *attP*, second *attP*, and third *attP*, respectively. For insertions of the minicircle derived from *pBT346*, expected

sized are: 463 bp, 393 bp, 323 bp, and 405 bp for insertion into the first *attP*, second *attP*, third *attP*, and full-length *attP*, respectively.

PCR4' in Fig. S1; 3'-junction**:* This PCR can be used instead of ***PCR4. It detects the same junction, but instead of PR488, it uses PR487. The expected sizes for minicircle insertions are: 544 bp, 474 bp, 404 bp, and 405 bp for insertion into the first *attP*, second *attP*, third *attP*, and full-length *attP*, respectively.

PCR5 in Fig. S1; 3'-junction: PR21 and PR387. Expected sizes are 498 bp, 428 bp, 358 bp, and 440 bp, for insertion into the first *attP*; second *attP*, third *attP*, or full-length *attP*, respectively. The products will be obtained only if the full plasmid is integrated.

PCR6 in Fig. S1; internal: FACS G5' and GFP2-Hermie. Expected size: 420 bp. This PCR amplifies a portion of the GFP cDNA.

PCR7+8 in Fig. S1: SH176, SH178 and PR432. The expected sizes are: 147 bp for any knockin or site-specifically integrated allele into *H11* that has the unique sequence "U" at 5' end (see plasmids above), 321 bp for wt, 726 bp for *H11P*, and 687 bp for *H11P3*.

PCR9 in Fig. S1; 3' junction: PR522 and PR428. Expected sizes are: 178 bp, 248 bp, 318 bp, and 260 bp for insertion into the first *attP*; second *attP*, third *attP*, or full-length *attP*, respectively.

To confirm that a particular insertion into *H11P(3)* detected by ***PCR1*** and ***PCR2*** originated from the plasmid, we performed an additional PCR for the 3' junction, ***PCR5*** (**Fig. S1**). The products were indeed obtained only when the full plasmid was integrated. This PCR was also used to test the cassette exchange founders that were positive for ***PCR1*** and ***PCR2*** and negative for ***PCR3*** and ***PCR4***. Indeed, all those founders were positive for ***PCR5***, thereby confirming that they contain only integration of the plasmid bacterial backbone. For detection of integration into the *H11PNVϕ* or *H11P3NVϕ* alleles we used ***PCR1*** and ***PCR6***, and instead of ***PCR2*** we used ***PCR9***. For detection of any knock-in or site-specifically integrated allele into *H11* we used ***PCR7+8***. For detection of the *H11P* or *H11P3* Flp-out alleles we used ***PCR8***.

For the majority of integrants that were analyzed in detail by sequencing of the recombinant junctions (22 out of 28 founders in **Table S2**), ϕC31 catalyzed precise recombination between *attP* and *attB*. In 6 cases, integration appeared to occur at two different *attP* sites, or it caused the deletion of one or more *attP* sites (for example, see **Fig. S1B**, top panel). These imprecise events occurred only when transgenesis was performed on embryos with three tandem *attP* sites.

For detection of site-specific integration into *R26P3NVϕ* we used ***PCR1***. For detection of any knock-in or site-specifically integrated allele into *Rosa26* we used primers: Rosa10, Rosa11, and PR432. Expected sizes are: 168 bp for any knockin or site-specifically integrated allele into *Rosa26* that has the unique sequence "U" at 5' end (see plasmids above), 330 bp for wt, and 696 bp for *R26P3*. For detection of the *R26P* or *R26P3* Flp-out alleles we used primers Rosa10 and Rosa11.

Quantitative PCR. Quantitative PCR was performed as previously described (46, 47). Each sample was tested in triplicate both for GFP and for the internal control. The primers used for GFP were: LL84 and LL85, and they generate a product of 187 bp. The internal control primers were: IMR0015 and IMR0016, and they generate a product of 200 bp.

Gene targeting in mouse ES cells. After electroporation of targeting constructs *pBT311*, *pBT312*, *pBT313*, and *pBT314* into mouse ES cells of 129 strain origin (10), individual G418-

resistant clones were evaluated for homologous recombination by long-range PCR (see above). The clones containing correctly recombined targeting vectors were used to generate mouse chimeras by injection into C57BL/6 blastocysts. The chimeras were crossed to B6D2 F1 females (F1 females from a cross between C57BL/6J and DBA2/J mice; Stock No. 100006, Jackson Lab). Agouti F1 progeny were genotyped for the presence of approproate knockin allele using the same long-range PCRs that were used for screening ES cells. Subsequent genotyping was performed with short-range PCR described above.

Microinjection for generation of site-specific integrants, additional notes. We have tested Qiagen maxi-prep DNA that was filtered through the 0.2 μm filter for injections. We noticed that although this DNA was RNase-free, it was more difficult to inject. Phenol/chloroform extractions followed by filtration as described above greatly facilitated the injection of this DNA.

We would not recommend the use of homozygous *attP*-containing F0 animals that do not contain site-specific integrations and were obtained from injections in subsequent injections, as they may contain random insertions or conversions of *attPx3* into *attPx2* or a single *attP*, which, although infrequent, have been observed. The use of these animals for subsequent injections is recommended only after proper control PCRs exclude the animals with undesirable events mentioned above.

To test for integrity of RNA after each injection, we analyzed the remaining DNA/RNA injection mix on 1% agarose gel (after incubation with the Ambion loading buffer as described for the analysis of *in vitro* transcribed RNA).

Microinjection of Flpo mRNA for generation of Flp-out alleles. To remove the *NV*ϕ cassette, which is flanked by *FRT5* sites (2, 3), we injected the capped *in vitro* transcribed Flpo mRNA obtained from *pFlpo* (Addgene plasmid 13792) (Ref. 4) at 48 ng/μl in miTE into the cytoplasm of *attP*-homozygous embryos. The average efficiency of Flp-out was 25.6% (32 out of 125) for *H11P(3)NV*ϕ and 7.4% (6 out of 81) for *R26P(3)NV*ϕ. None of the animals obtained were homozygous Flp-outs (n=206) based on the PCR that can detect both the Flp-out and non-Flp-out alleles. We mated the animals containing the same Flp-out allele to each other to create homozygous Flp-out mouse lines.

Generation of *GFP-Flpo* transgenic mice. *GFP-Flpo* mice were generated as random integrants from an experiment in which *pBT340* was co-injected with ϕC31o mRNA into *H11P3NV*ϕ homozygous embryos of mixed background in an attempt to achieve site-specific integration of this plasmid and subsequent removal of the bacterial backbone by Flpo-mediated self-excision. The site-specific integration was not successful (0/106 F0 founders screened). However, several random insertions were retained for further characterization, in order to select an efficient Flpo line that can be detected by ubiquitous GFP expression (the progeny can be screened for GFP fluorescence with a UV lamp). The activity of one of the *GFP-Flpo* lines was initially evaluated by analyzing the Flp-out frequency for the *H11P3NV*ϕ allele (removal of the *NV*ϕ cassette) to create the *H11P3* allele. As all F1 progeny from this founder, which was generated by a random insertion of *pBT340* into *H11P3NV*ϕ homozygous embryos, were heterozygous for the *H11* knock-in, we were able to establish the efficiency of Flp-out after a single cross to wt mice. We analyzed all F1 progeny that was negative for *GFP-Flpo*, and by the nature of the cross, heterozygous for the *H11* knock-in allele to detect *H11P3* and *H11P3NV*ϕ

alleles. Based on this experiment, the efficiency of Flp-out was 100%, as only *H11P3* and no *H11P3NVϕ* alleles could be detected among the F1 progeny.

Removal of bacterial backbone from site-specific transgenes by crossing to *GFP-Flpo* mice. The bacterial backbone can be removed from site-specific transgenes if the plasmid that was used to generate the transgene contains an *FRT5* site between the 3' end of the transgene and the plasmid bacterial backbone (e.g., *pBT344* or *pBT366*). This procedure requires two crosses: first one to create double heterozygous animals containing a site-specifically integrated allele and *GFP-Flpo*, and the second one to remove the *GFP-Flpo* transgene. For both *H11P3-pCA-GFP-BB* and *H11P3-pHb9-GFP-BB*, all progeny from the second cross that did not contain *GFP-Flpo*, but contained the site-specific integration allele, had the bacterial backbone removed (i.e., detected by genotyping as *H11P3-pCA-GFP/wt* and *H11P3-pHb9-GFP/wt*, respectively). Therefore, with this crossing scheme and our *GFP-Flpo* mice, the corresponding alleles without the bacterial backbone were generated at 100% efficiency.

We also examined the F1 progeny from the cross of *H11P3-pHb9-GFP-BB/wt* (male) to *GFP-Flpo* (female) for possible bacterial backbone excision by maternal contribution of the Flp recombinase. We did not detect any Flp-out in F1 animals from this cross that were positive only for the site-specifically inserted *Hb9* allele.

We also compared the efficiency of our *GFP-Flpo* line with *Rosa-Flpe* (Jackson Labs, Stock No. 003946) (Ref. 11). *Rosa-Flpe* generated Flp-out only in a small minority of F2 progeny following the two-cross scheme described above.

Tissue preparation and immunohistochemistry. Tissues were obtained from postnatal day 21 (± 2 days) mice that were transcardially perfused with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). The tissues were post-fixed overnight in 4% PFA, washed once with PBS and cryoprotected in 30% sucrose overnight. The tissues were embedded in OCT (Tissue-Tek) and stored at -80ºC before cryosectioning. Tissue sectioning was performed using a Leica cryostat. The livers and hearts were sectioned coronally to obtain 10 μm-thick sections, washed in PBS, stained with DAPI and mounted in Fluorogel (Electron Microscopy Sciences, Cat. No. 17985-11). The livers and hearts were imaged with a fluorescence microscope (Nikon). The brains were sectioned sagittally to obtain 30 μm-thick sections, the sections were washed 3 times in PBS and incubated overnight at 4ºC with chicken anti-GFP antibody (Aves Labs) at 1:500 dilution and monoclonal mouse anti-calbindin antibody (Sigma) at 1:3000 dilution. Following incubation with fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch) and DAPI, the slides were washed 3 times in PBS, mounted in Fluorogel, and imaged with a Zeiss confocal microscope.

E11 embryos were dissected in ice-cold PBS, fixed for 2 h at 4ºC with shaking, washed 3 times in ice-cold PBS, and cryoprotected in 30% sucrose overnight. Embryos were embedded into OCT coronally and sectioned at 12 μm thickness with a Leica cryostat. The sections were washed 3 times in PBS and incubated overnight at 4ºC with chicken anti-GFP antibody (Aves Labs) at 1:500 dilution and polyclonal rabbit anti-N-terminal Hb9 antibody (generous gift of S. Arber) (Refs. 9, 12) at 1:1000 dilution. Following incubation with fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch) and DAPI, the slides were washed 3 times in PBS, mounted in Fluorogel, and imaged with a Zeiss confocal microscope.

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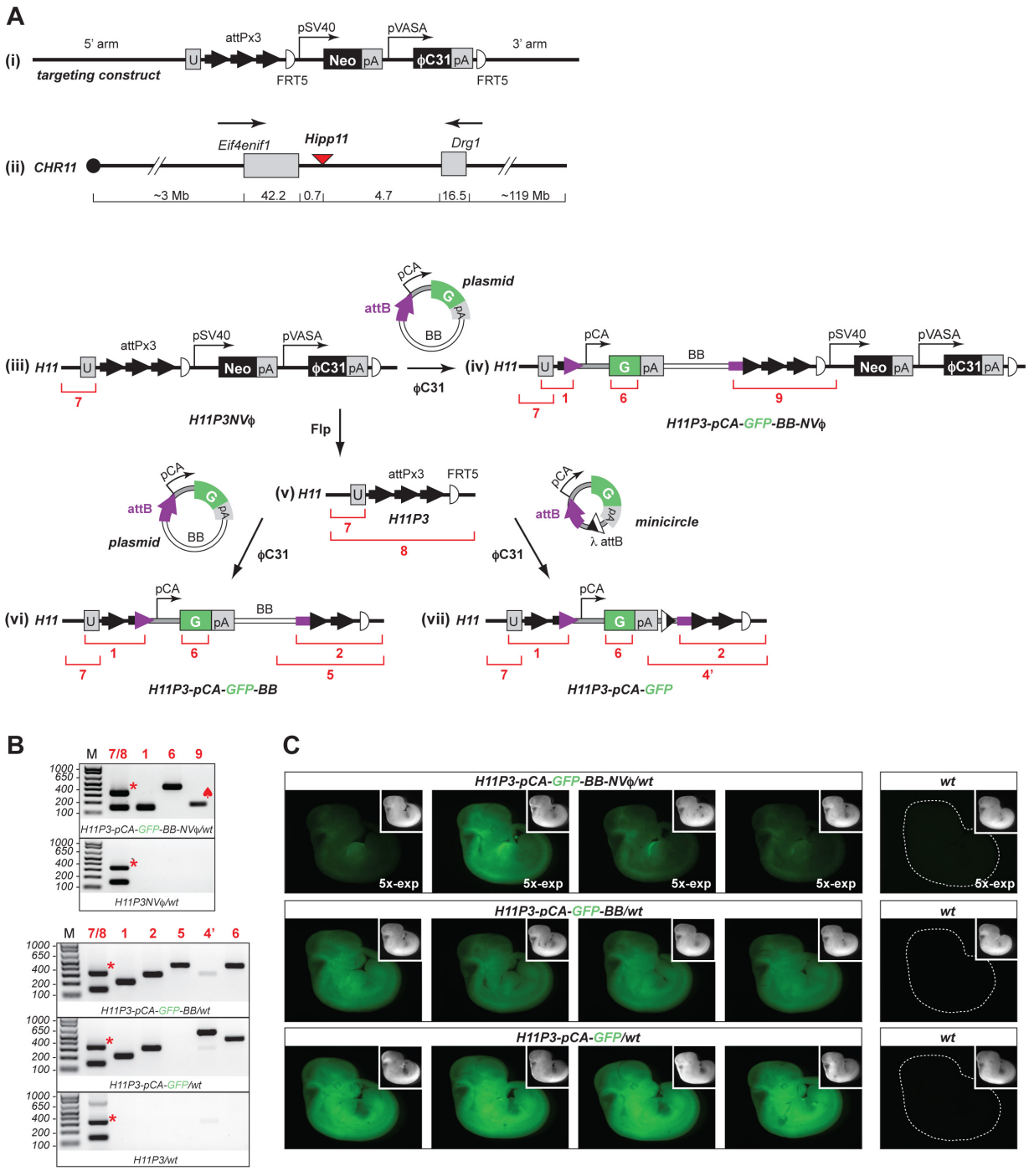

Figure S1. Site-specific transgenesis – proof of principle.
(***A***) Generation of *H11* knockin alleles containing ϕC31 *attP* sites. (**i**) A schematic of the recombinant DNA construct for introduction of three *attP* sites and the *NVϕ* cassette into *H11* via homologous recombination in mouse ES cells. Two versions, containing either a single "full-length" *attP* site or three tandem short *attP* sites, were generated. (**ii**) mouse chromosome 11 with the *Hipp11 (H11)* locus designated as a red triangle. The scale below is in kilobases, except where megabases (Mb) are indicated. (**iii**) The *H11P3NVϕ* allele resulting from homologous

recombination between (**i**) and (**ii**). (**iv**) *H11P3-pCA-GFP-BB-NVϕ* allele obtained by ϕC31-catalyzed site-specific insertion of the *pattB-pCA-GFP* plasmid *(pBT316)* into the first *attP* site. All three *attP* sites are suitable recipients for the transgene and the site used in any particular case can be determined by PCR. (**v**) *H11P3* locus that was generated from (**iii**) by Flpo mRNA injection into the cytoplasm of mouse embryos carrying (**iii**) (see SI Materials and Methods). The *H11* locus with a single *attP* site and the *Rosa26* locus with either a single or three *attP* sites were generated in the same manner. (**vi**) and (**vii**), two products obtained by ϕC31-catalyzed site-specific insertion of the *pattB-pCA-GFP* plasmid (*pBT316*, left) or the *attB-pCA-GFP* minicircle (generated from *pBT346*, right). The corresponding alleles are: *H11P3-pCA-GFP-BB* (left) and *H11P3-pCA-GFP* (right), respectively.

(***B***) PCR results on N1 animals confirming site-specific integrations. The DNA template for each PCR panel was obtained from a mouse of the genotype designated below each gel. The red numbers correspond to the PCR products designated on the schemes by red brackets and numbers in (*A*). The primer set #9 amplified a band smaller than expected due to the deletion of two *attP* sites during integration (spade). The wt *H11* locus is also amplified by primer set #8 to generate a 321 bp band (asterisk, see schematic (**v**)).

(***C***) GFP expression in N2 mouse embryos at embryonic day 11. Each row shows representative embryos from a single pregnancy with genotypes designated above. Images were obtained under identical conditions, except that "5x-exp" designates five-fold longer exposure time than for the rest of the images. Insets represent the corresponding bright field images of each embryo.

Abbreviations: pSV40, *SV40* promoter; pVASA, *VASA* promoter; U, unique sequence; FRT5, a mutant version of *FRT* that is compatible with itself but not with wt *FRT*; pCA, β-actin promoter and CMV enhancer; G, GFP; pA, *polyA* signal; BB, plasmid bacterial backbone; attB and attP, ϕC31 *attB* and *attP* sites.

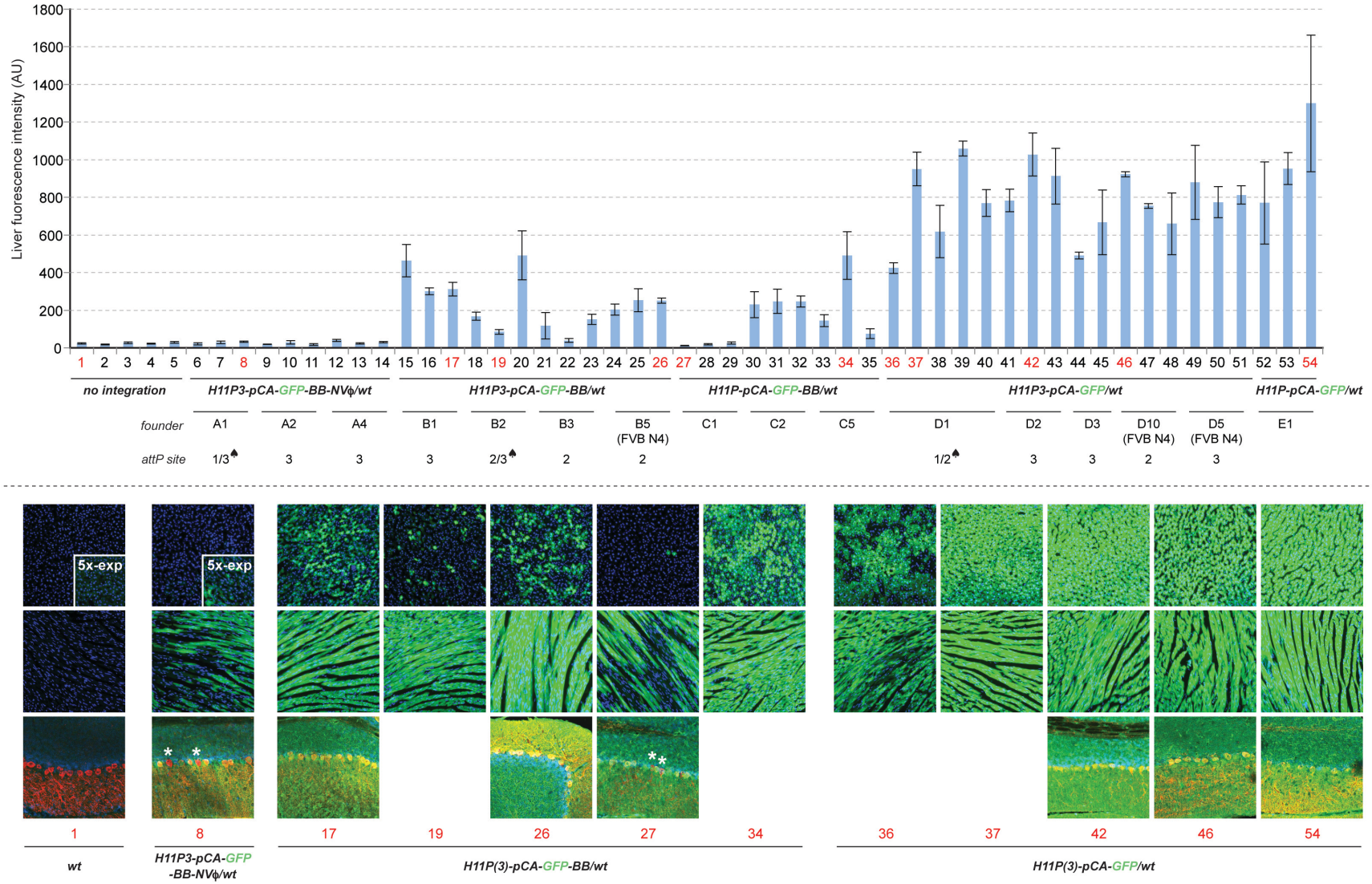

Figure S2. GFP expression in individual adult animals carrying *pCA-GFP* site-specific transgenes introduced by ϕC31 integrase-mediated transgenesis. Each column on the graph represents average fluorescence in arbitrary units (AU) in the GFP channel for liver sections from individual animals represented by numbers and genotypes below ("no integration" represents *wt*, *H11P3NVϕ/wt*,

H11P3/wt and *H11P/wt* genotypes). The animals are grouped according to the genotype and founder (designated further below). For each founder, one of the three sites from *H11P3* into which the site-specific integration occurred is indicated below. In cases labeled by spades, recombination appeared to occur at two different *attP* sites, or it resulted in deletion of some of the *attP* sites. For a subset of animals, representative images of liver and heart sections are shown below. The images show native GFP fluorescence in green and nuclei stained by DAPI in blue. Further below, corresponding confocal images of cerebellar sections are shown for a subset of animals. The sections are stained by anti-GFP antibody (green), anti-calbindin (red) for Purkinje cells, and DAPI (blue). The numbers below the images correspond to the numbers of individual animals below the chart. The Purkinje cells in animals #8 and #27 that appear calbindin(+) but GFP(-) are indicated by asterisks. GFP expression in the liver appears most sensitive to the presence of the bacterial backbone and the *NVϕ* cassette. 17 out of 19 transgenic animals (#36-54), which do not contain the *NVϕ* cassette and the bacterial backbone, show uniform GFP expression. The exceptions are animals #36 (the strongest variability observed in this set) and #38. The tissue section images for animals #1, 8, 17, and 42 also appear in Fig. 2.

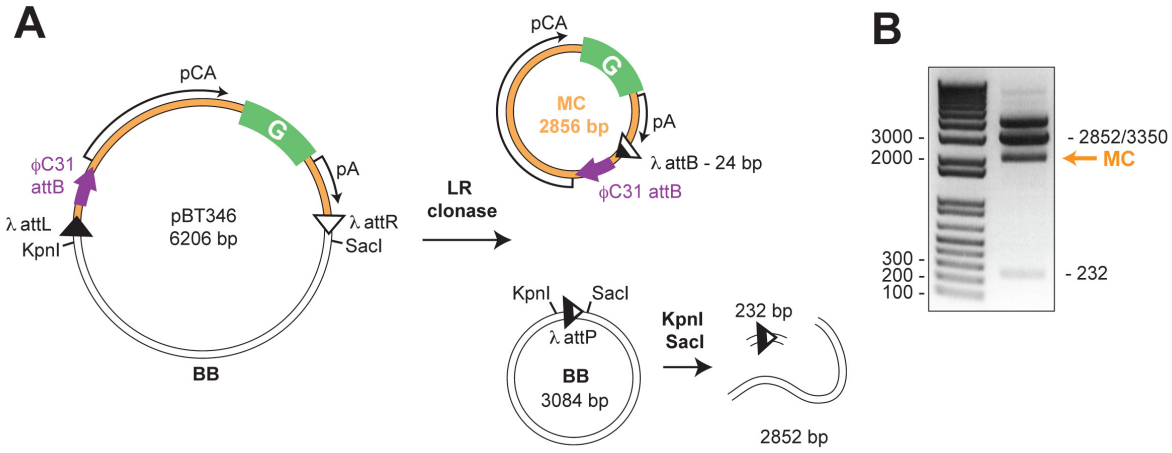

Figure S3. Generation of minicircle DNA with λ integrase and excisionase (LR clonase, Invitrogen) *in vitro*. (***A***) From left to right: The starting plasmid (*pBT346*) contains λ *attL* and *attR* sites, which recombine in the LR clonase-catalyzed reaction to generate two minicircles: one (*MC*) contains the ϕC31 *attB* site and *pCA-GFP*, and the other contains the plasmid bacterial backbone (*BB*). After recombination, the DNA is treated with appropriate restriction endonucleases to selectively digest the *BB* minicircle and the starting plasmid. (***B***) The recombined and digested DNA is run on 1% agarose gel. *MC* DNA (orange arrow) migrates faster than the linear *BB* or plasmid DNA and is purified from the gel for microinjection.

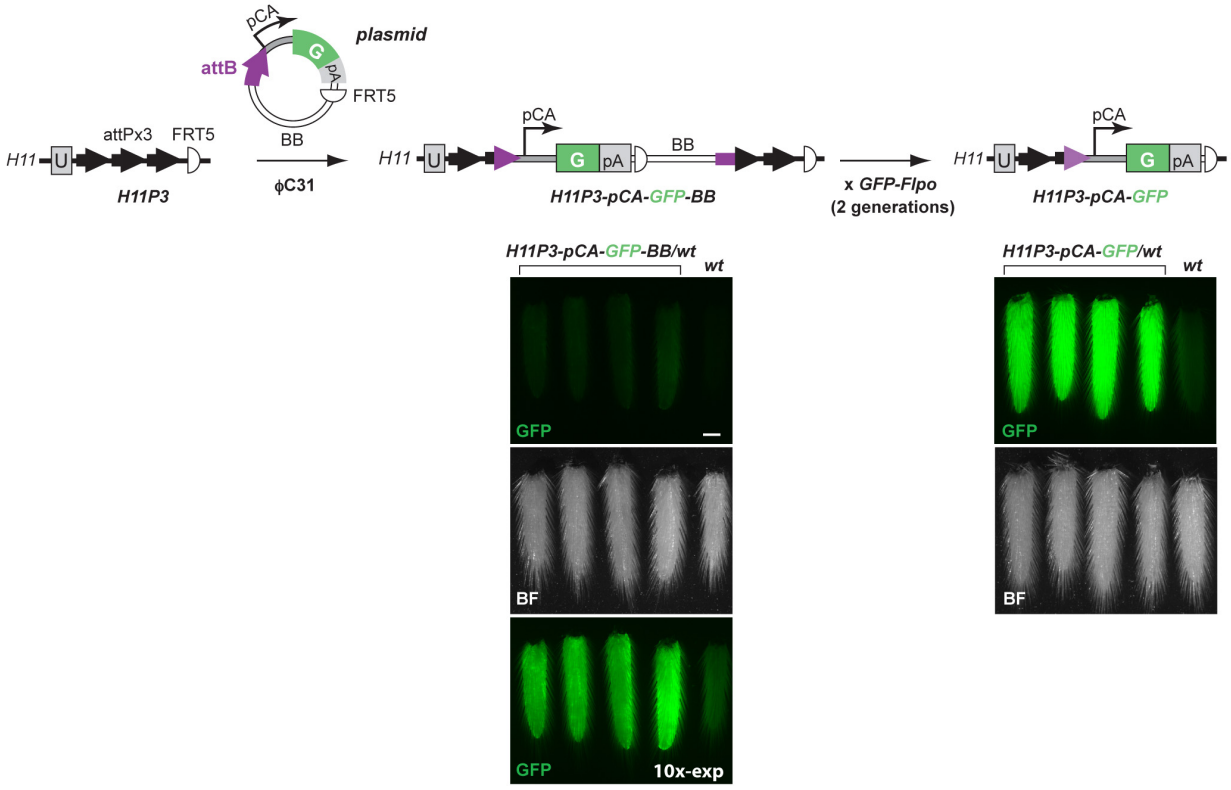

Figure S4. GFP expression from a site-specifically integrated *H11P3-pCA-GFP-BB* transgene increases upon the removal of the bacterial backbone. Top, schematic of the generation of the *H11P3-pCA-GFP-BB* transgene (from *pBT344*) and subsequent derivation the *H11P3-pCA-GFP* transgene from it, by crossing to the *GFP-Flpo* transgenic mouse to remove the bacterial backbone (SI Materials and Methods). Below, four tail tips for each genotype designated above and a tail tip of a wt littermate were imaged for GFP fluorescence using identical imaging conditions. Bright field (BF) images of the same tails are shown further below. 10x-exp, the same tails above were imaged for GFP fluorescence with 10-times longer exposure. Scale bar, 1 mm.

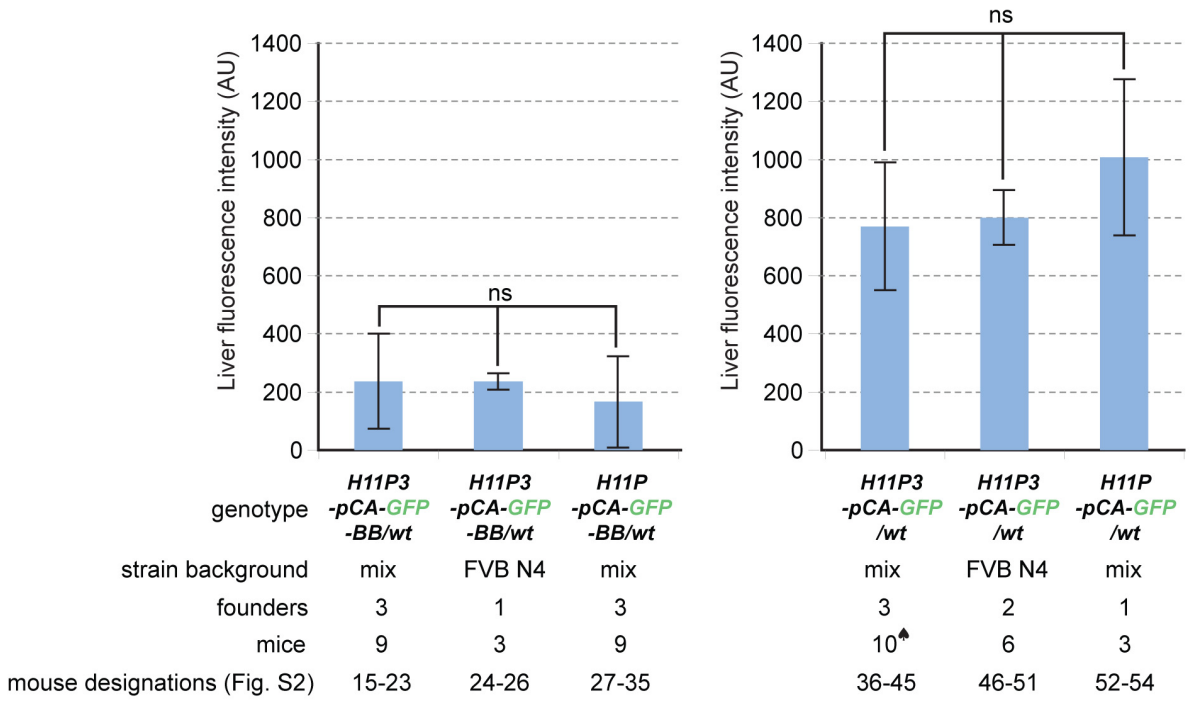

Figure S5. Average GFP fluorescence in livers does not differ between mice containing an insertion of the same transgene into one of the three *attP* sites from *H11P3* or insertion into a single *attP* site from *H11P* (compare 1st and 3rd columns in both graphs). The GFP fluorescence is also not affected by the genetic background of the injected embryos (compare 1st and 2nd columns in both graphs). Each dataset is represented by mean ± standard deviation. The numbers of individual animals and founders analyzed for each genotype are listed below the genotypes. When samples from multiple founders were combined to obtain an average, each founder was represented by the same number of animals except in the case labeled by a spade. Mouse designations are numbers used to represent each mouse in Fig. S2. Statistical comparisons were performed with one-way ANOVA.

Row	DNA[a]	DNA type	DNA size (kb)	Strain	Back-ground	Embryos injected (n)	Embryos implanted (n)	Implanted/ Injected (%)	F0 (n)	F0/ Implan-ted (%)	SS (n)	SS% (of injected)	SS% (of implan-ted)	**SS% (of F0)**	R (n)[h]	R% (of implan-ted)	R% (of F0)	Experi-ments (n)
1	***attB*-*pCA-GFP***	minicircle	~3	*H11P*	mix	141	115	82	21	18	1	0.7	0.9	**4.8**	1	0.9	4.8	4
2	***attB*-*pCA-GFP***	minicircle	~3	*H11P3*	mix	168	136	81	39	29	4	2.4	2.9	**10.3**	1	0.7	2.6	4
3	***attB*-*pCA-GFP***	minicircle	~3	*H11P3*	FVB N4	122	115	94	15	13	6	4.9	5.2	**40.0**	3	2.6	20.0	3
4	***attB*-*pCA-GFP*-*attB***	plasmid	~6	*H11P3*	FVB N4	128	119	93	38[c]	32	6[e]	15.8	5.0	**15.8**	1	0.9	2.6	1
5	***attB*-*pCA-GFP*, no RNA**	plasmid	~6	*H11P3NVϕ*	mix	160	78	49	32[c]	41	0	0.0	0.0	**0.0**	5	6.4	15.6	1
6	***attB*-*pCA-GFP***	plasmid	~6	*H11P3NVϕ*	mix	292	223	76	64[d]	29	10	3.4	4.5	**15.6**	4	1.8	6.3	3
7	***attB*-*pCA-GFP***	plasmid	~6	*H11PNVϕ*	mix	140	89	64	30[c]	34	2	1.4	2.2	**6.7**	0	0.0	0.0	2
8	***attB*-*pCA-GFP-FRT5***	plasmid	~6	*H11P*	mix	264	232	88	51	22	5[f]	1.9	2.2	**9.8**	3[g]	0.9	5.9	5
9	***attB*-*pCA-GFP-(FRT5)*[b]**	plasmid	~6	*H11P3*	mix	142	129	91	61	47	4[f]	2.8	3.1	**6.6**	9[g]	9.9	14.8	4
10	***attB*-*pCA-GFP-FRT5***	plasmid	~6	*H11P3*	FVB N4	50	43	86	8	19	3[f]	6.0	7.0	**37.5**	1[g]	0.0	10.3	1
11	***attB*-*pHB9-GFP-FRT5***	plasmid	~14	*H11P3*	FVB N4	305	267	88	66[d]	25	2[f]	0.7	0.7	**3.0**	2[g]	0.7	3.0	2
12	***attB*-*pCA-GFP***	plasmid	~6	*R26P3NVϕ*	mix	83	63	76	22[c]	35	2	2.4	3.2	**9.1**	2	3.2	9.1	1

Abbreviations: F0, embryos or animals obtained from injections; SS, site-specific integration; R, random integration; mix, mixed background of 129, C57BL/6 and DBA2; FVB N4, mice of the mixed background were outcrossed for 4 generations to the FVB strain and then intercrossed.

[a] All DNA was injected at the final concentration of 3 ng/μl. All DNA was coinjected with ϕC31o mRNA at the final concentration of 48 ng/μl, except for the experiment in row 5. [b] Both FRT and non-FRT versions of *pattB-pCA-GFP* (*pBT316 or pBT344*) were used. [c] F0s were analyzed only as E10 or E11 embryos. [d] F0s were analyzed either as E10 or E11 embryos or as live pups. [e] The 6 founders with cassette exchange listed here contain *pCA-GFP* without the bacterial backbone; 5 more founders with cassette exchange contained only the bacterial backbone. Therefore the total number of founders with cassette exchange is 11 (29%). [f] One F0 with site-specific insertion, also contains a random insertion. [g] One random insertion was found in a founder that also contains a site-specific insertion. [h] The number of random insertions may be somewhat underreported when F0 embryos were included in the analysis, as random insertions are not detectable by GFP-specific PCR if they occur in embryos that also contain site-specific insertions.

Table S1. Stepwise efficiency of site-specific integration

Transgene	Founder	Back-ground	Sex	Insertion into attP 1, 2 or 3	GT of SS (Y/N)[a]	N1 (n)	N1 SS (n)	N1 SS (%)[c]	N2/3 (n)	N2/3 SS (n)	N2/3[d] SS (%)	R (Y)	N1 R (n)	N1 R (%)
H11P3-pCA-GFP-BB-NVϕ	A1	mix	M	1/3[b]	Y	55	36	65.5	25	15	60.0		0	0
	A2	mix	M	3	Y	62	12	19.4***	16	10	62.5		0	0
	A3	mix	M	1/3[b]	Y	36	10	27.8*					0	0
	A4	mix	M	3	Y	63	29	46.0	14	4	28.6		0	0
H11P3-pCA-GFP-BB	B1	mix	M	3	Y	143	19	13.3***	48	20	41.7	Y	11	7.7
	B2	mix	M	2/3[b]	Y	29	11	37.9	85	36	42.4		0	0
	B3	mix	Fem	2	Y	44	9	20.5***	76	34	44.7		0	0
	B4	mix	Fem	2	Y	20	4	20.0*					0	0
	B5	FVB N4	M	2	Y	24	9	37.5					0	0
	B6	FVB N4	Fem	2	N									
	B7	FVB N4	Fem	2/3[b]	Y	8	3	37.5				Y	3	37.5
H11P-pCA-GFP-BB	C1	mix	Fem	n/a	Y	15	11	73.3					0	0
	C2	mix	M	n/a	Y	44	9	20.5***					0	0
	C3	mix	Fem	n/a	Y	9	4	44.4				Y	2	22.2
	C4	mix	Fem	n/a	N	1	0	0					0	0
	C5	mix	M	n/a	Y	67	14	20.9***	62	29	46.8		0	0
H11P3-pCA-GFP	D1	mix	Fem	1/2[b]	Y	20	8	40.0	25	15	60.0		0	0
	D2	mix	M	3	Y	33	10	30.3	12	4	33.3		0	0
	D3	mix	Fem	3	Y	23	5	21.7*					0	0
	D4	mix	M	3	N	59	0	0***					0	0
	D5	FVB N4	Fem	3	Y	32	6	18.8**					0	0
	D6	FVB N4	Fem	3	Y	41	10	24.4**					0	0
	D7	FVB N4	M	3	N									
	D8	FVB N4	Fem	3	Y	14	2	14.3*					0	0
	D9	FVB N4	M	1/3[b]	N									
	D10	FVB N4	Fem	2	Y	27	8	29.6	20	10	50.0		0	0
H11P-pCA-GFP	E1	mix	M	n/a	Y	39	11	28.2*					0	0
H11P-pHB9-GFP	F1	FVB N4	Fem	2	Y	45	9	20.0***					0	0

Abbreviations: SS, site-specific integration; R, random integration; GT, germline transmission; mix, mixed background of 129, C57BL/6 and DBA2; FVB N4, the mice of the mixed background were outcrossed for 4 generations to the FVB strain and then intercrossed; N1, progeny from the first generation where a founder was crossed to CD1 wt animal; N2 or N3, progeny from the second or third generation where N1 or N2 animals were crossed to CD1 wt animals, respectively.

[a] The founders that did not transmit were ether sterile (D7 and D9), died upon delivery (C4), or cannibalized the pups (B6). [b] Based on site-specific PCR, integration appeared to occur at two different *attP* sites, or it caused the deletion of one or more *attP* sites.

[c] Frequency of germline transmission for site-specific integrations from some founders was sub-Mendelian, suggesting mosaicism in the founders (Fisher's exact test, *, p<0.05; **, p<0.01; ***, p<0.001). [d] Frequency of transmission for site-specific integrations for subsequent generations (N2 and/or N3) is statistically indistinguishable from Mendelian transmission (Fisher's exact test).

Table S2. Complete list of transgenic founders (n=28) and their germline transmission efficiency.

Row	Pooled rows from Table 1[a]	Strain	Background	F0 (n)[a]	SS F0 (n)[a]	Significant?[b]	**SS% (from F0)**
1	1, 7, 8	*H11P(NVϕ)*	mix	102	8	} ns	**7.8**
2	2, 6, 9	*H11P3(NVϕ)*	mix	164	18		**11.0**
3	3, 10	*H11P3*	FVB N4	23	9	} p=0.0015	**39.1**

Abbreviations: SS, site-specific integration; mix, mixed background of 129, C57BL/6 and DBA2; FVB N4, mice of the mixed background were outcrossed for 4 generations to the FVB strain and then intercrossed.
[a] Pooled results from Table 1 for the same strain and background (regardless of the presence of the *NVϕ* cassette) used for injections of 3-6 kb DNA. The pooling did not include Table 1 entry 4 (as the mechanism of integration may be different); entry 11 (as the DNA used is dramatically larger); entry 5 (as no RNA was coinjected); and entry 12 (as it used the *Rosa26* locus). [b] Statistical significance was evaluated using Fisher's exact test. ns, not-significant; α=0.05.

Table S3. Comparisons of integration efficiencies: single vs. triple *attP* alleles, and mixed vs. FVB background.

Name	Sequence
21	ctgcaaggcgattaagttgg
351	aataaGCTAGCctcgagGATATCctgtgccttctagttgccag
374	atgtgaggcaggagatgagagaggaatgactggtcac
387	gtgggactgctttttccaga
395	gttgagggcaatctgggaaggt
402	ctagCCTGCAGGaattaaGTTAACaattaaGACGTC
403	ctagGACGTCttaattGTTAACttaattCCTGCAGG
422	ccatttttagtacccctctacactcctcc
425	ggtgataggtggcaagtggtattc
428	ccgaaaagtgccacctgaataat
432	GATATCCTTACGGAATACCACTTGCCACCTATCACC
436	atcaactaccgccacctcgac
437	AACCAACCttaaCCGCCACCATGGATACCTAC
438	AATAggatccTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTctcgagTCACACTTTCCGCTTTTTCTTAGG
487	tccccctgaacctgaaacat
488	gcaatagcatcacaaatttcacaa
493	aaagaGGTACCagttacgctagggataacagggtaatatagCAAATAATGATTTTATTTTGACTGATAG
494	aaataCTCGAGagcctGCTTTTTTGTACAAAGTTG
495	aagaaGCGGCCGCacaagtttgtacaaaaaagcTGAACG
496	AAGAAgagctcCATAGTGACTGGATATGTTGTGTTTTA
522	CGATGTAGGTCACGGTCTCG
551	GGCTATGAACTAATGACCCCGTA
FACS G5'	CTTCAAGTCCGCCATGCCCGA
GFP2-Hermie	TCCAGCAGGACCATGTGATCGC
IMR0015	CAAATGTTGCTTGTCTGGTG
IMR0016	GTCAGTCGAGTGCACAGTTT
LL84	AAGTCGTGCTGCTTCATGTG
LL85	ACGTAAACGGCCACAAGTT
Rosa10	CTCTGCTGCCTCCTGGCTTCT
Rosa11	cgaggcggatcacaagcaata
Rosa3	ccactgaccgcacggggattc
SH176	tggaggaggacaaactggtcac
SH178	ttccctttctgcttcatcttgc

Table S4. List of primers used in this study.