Supplementary Data

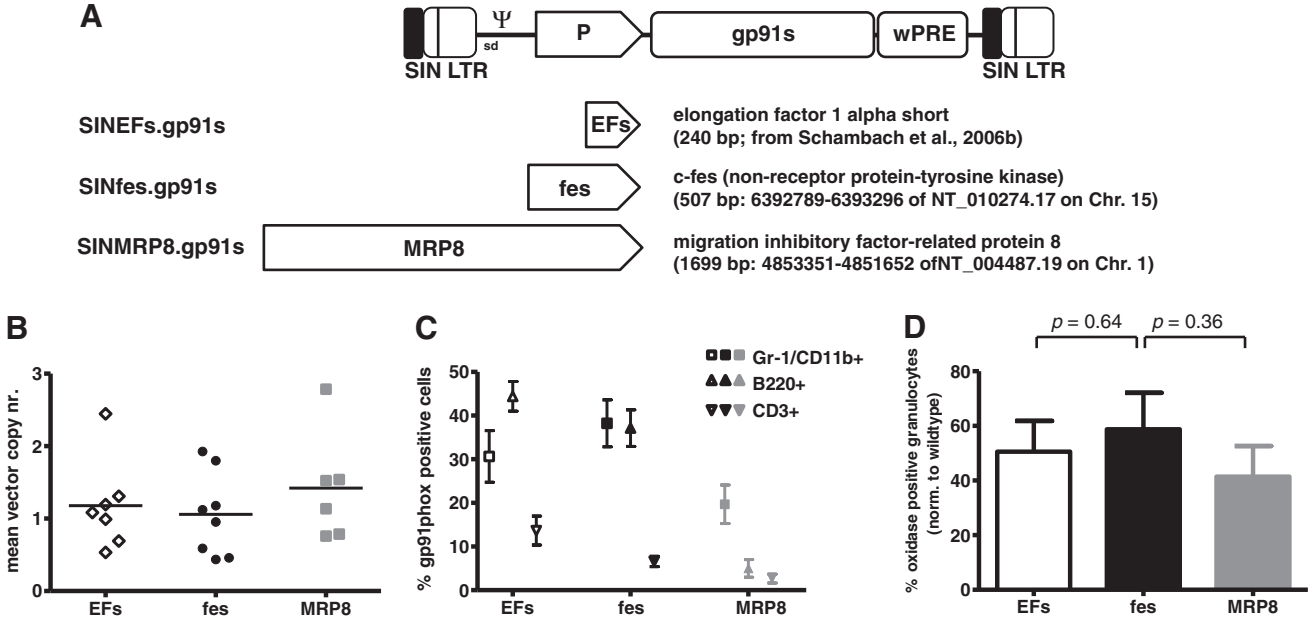
Supplementary Materials and Methods

Animals

The gp91^{phox}-deficient mice with X-linked form of chronic granulomatous disease (X-CGD) (B6.129S6-Cybb$^{tm1Din/J}$) (Pollock *et al.,* 1995) and Bl/6-CD45.1 mice (B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ; Jackson Laboratory, Bar Harbor, ME), as well as NSG.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice (Jackson Laboratory), were housed in microisolator cages provided with autoclaved food and drinking tap water *ad libitum*. All animal experimental procedures were approved by the regional council (Regierungspräsidium, Darmstadt, Germany) and performed in compliance with the local animal experimentation guidelines. Animals were 7–13 weeks old at the time of transplantation.

Retroviral vectors and vector production

The self-inactivating (SIN) gammaretroviral vector SINfes.gp91s was constructed from the SERS11.SF.GFP.W vector backbone (Schambach *et al.,* 2006) by inserting an optimized cDNA sequence for human gp91^{phox} (Moreno-Carranza *et al.,* 2009) into the *Xba*I and *Sal*I unique restriction sites. The internal spleen focus-forming virus (SFFV) promoter/enhancer was replaced by a 507 bp fragment from the human *c-FES* promoter (Heydemann *et al.,* 1996, 2000) (GenBank Accession No. NM_002005). SINEFs.gp91s and SINMRP8.gp91s were cloned accordingly using a 240 bp fragment from the EF1a locus (Schambach *et al.,* 2006) or a 1699 bp fragment from the human *MRP8* gene (GenBank Accession No. NM_002964) (Lagasse and Clerc, 1988), respectively. The SIN vectors also contain the safety-improved woodchuck hepatitis virus post-transcriptional regulatory element (wPRE) (Schambach *et al.,* 2006) downstream of the gp91s cDNA sequence. The SF91eGFP control vector has been described previously (Modlich *et al.,* 2008). Retroviral cell-free vector supernatants were generated by transient co-transfection of 293T packaging cells with each transfer vector, together with packaging constructs coding for the gag-pol proteins (pcDNA3.MLVgp) and the gibbon ape leukemia virus (GALV) or vesicular stomatitis virus glycoprotein (VSV-G) envelope, as previously described (Schambach *et al.,* 2006). VSV-G vector supernatants were concentrated 60-fold by ultracentrifugation at 50,000 *g* for 2 hr at 4°C. Viral titers were determined by flow cytometry on the myelomonocytic cell line PLB 985 X-CGD (Zhen *et al.,* 1993) and ranged



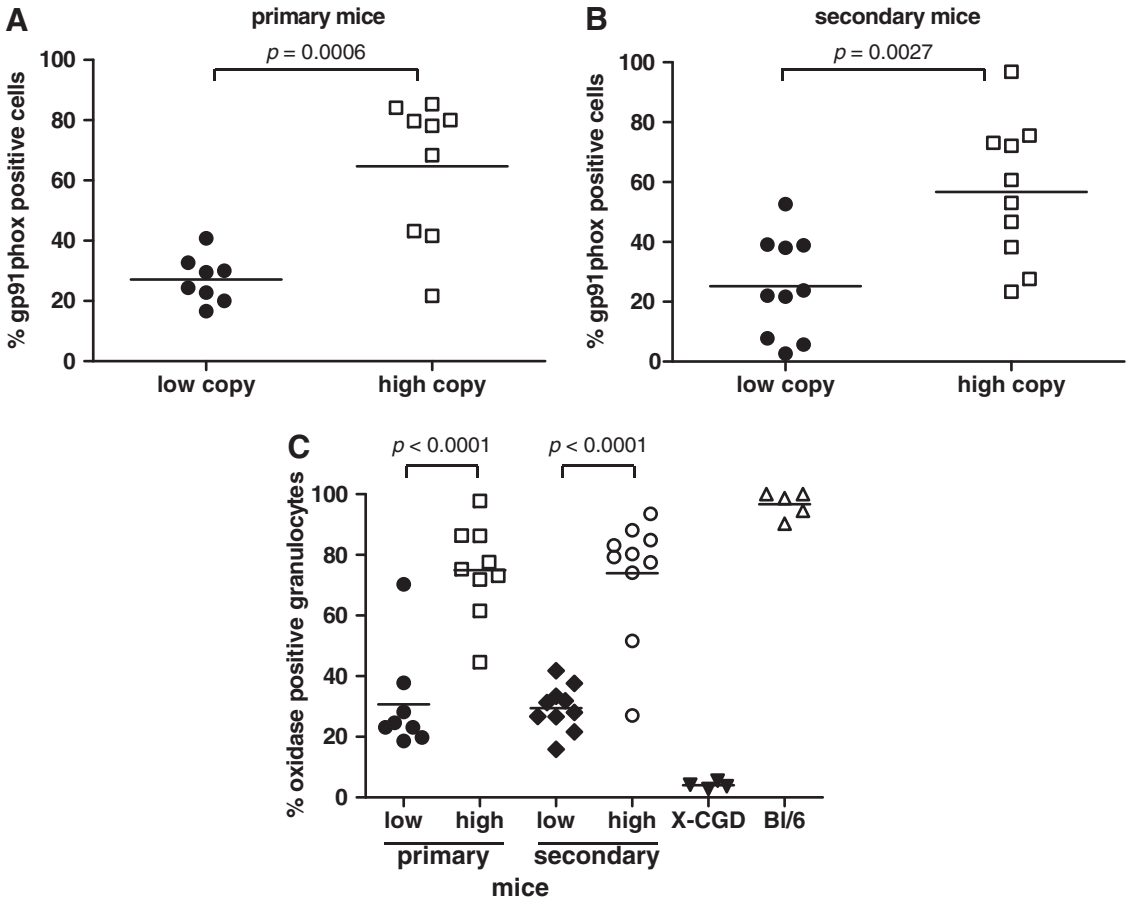
SUPPLEMENTARY FIG. S1. Schematic design of SIN gammaretroviral vectors tested in this study. **(A)** The structure of the vectors used in this study is shown as provirus with the SIN LTRs, splice donor (sd) site, and packaging signal (Ψ) in the leader region, internal promoter (P), gp91^{phox} transgene (gp91s), and wPRE. The relative lengths and annotations of the three internal promoters (EFs, fes, MRP8) are also indicated. **(B–D)** Comparison of *in vivo* performance of the SINEFs.gp91s, SINfes.gp91s, or SINMRP8.gp91s vectors 6–8 weeks after transplantation of transduced X-CGD lineage-negative cells in gp91$^{phox-/-}$ mice. **(B)** Proviral vector copy number in peripheral blood cells from reconstituted mice as determined by qPCR. **(C)** Gp91^{phox} expression in granulocytes (Gr-1^{+}/CD11b^{+}), B (B220^{+}), and T (CD3^{+}) cells of transplanted animals as measured by flow cytometry (mean ± SEM, n = 6–8 per group). **(D)** Reconstitution of superoxide production in granulocytes of transplanted animals. The data were normalized to the respiratory burst activity in wild-type animals (mean ± SEM, n = 6–8 per group). p-Values according to two-tailed unpaired t-test. LTRs, long terminal repeats; qPCR, quantitative polymerase chain reaction; SEM, standard error of the mean; SIN, self-inactivating; wPRE, woodchuck hepatitis virus post-transcriptional regulatory element; X-CGD, X-linked form of chronic granulomatous disease.

between $0.5–1\times10^6$ TU/ml and 2.8×10^8 TU/ml for concentrated VSV-G pseudotyped. Supernatants were used for transduction on the same day or stored at −80°C until use. For the study on human cells, the SINfes.gp91s vector supernatant was supplied as a pre-GMP lot by EUFETS GmbH, Idar-Oberstein. The GALV pseudotyped vector supernatant was obtained from a stable producer cell line (PG368-23–522 K#4) (Loew *et al.*, 2010) and concentrated by cross-flow filtration with a hollow fiber module. The titer of this vector preparation was 1.3×10^6 transducing units per ml.

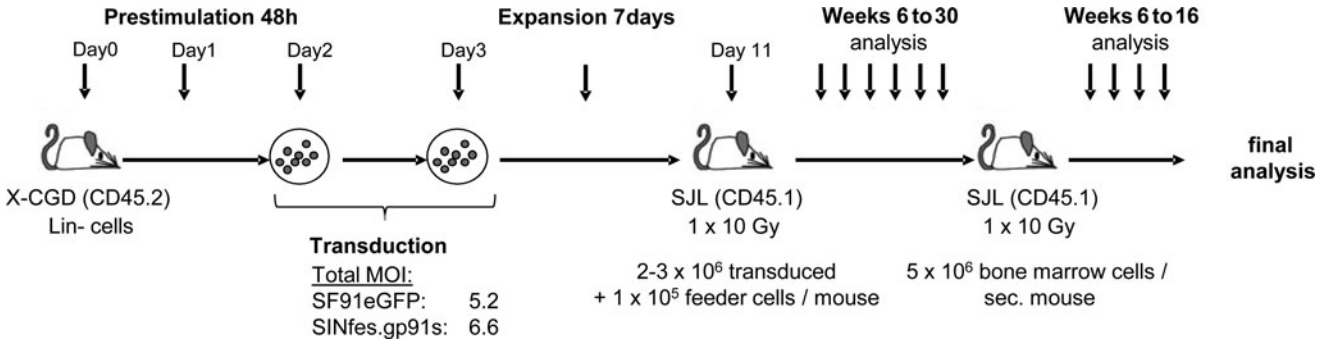
Isolation and culture of hematopoietic progenitor cells and retroviral transduction

Lineage-negative (lin−) cells were isolated from complete bone marrow (BM) flushed from femora and tibiae of untreated X-CGD mice by magnetic sorting using lineage-specific antibodies (Lineage Cell Depletion Kit; Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). Lin− cells ($0.8–1.0\times10^6$ cells/ml) were prestimulated for 2 days in StemSpan serum-free medium (StemCell Technologies, Grenoble, France) supplemented with 1% penicillin/streptomycin and 10 ng/ml murine stem cell factor (SCF), 20 ng/ml murine thrombopoietin (TPO), 10 ng/ml human fibroblast growth factor-1 (FGF-1) (all from Peprotech, Hamburg, Germany), and 20 ng/ml murine insulin-like growth factor-2 (IGF-2) (R&D Systems, Wiesbaden, Germany). Human $CD34^+$ cells from cryopreserved granulocyte-colony stimulating factor (G-CSF)–mobilized peripheral blood of healthy or X-CGD donors were thawed and purified using the human CD34 Microbead Kit (Miltenyi Biotec GmbH) according to manufacturer's instructions and cultured in StemSpan serum-free medium (StemCell Technologies) containing 300 ng/ml human SCF, 300 ng/ml humanFlt3 ligand, 100 ng/ml human TPO, and 60 ng/ml human IL-3 (all from Peprotech). Granulocytic differentiation was induced 3 days later by changing the medium to RPMI containing 20% fetal calf serum, 10 ng/ml human G-CSF, and 4 m*M* glutamine and penicillin/streptomycin (100 U/m each) for 7–10 days. The use of these cells was approved by the Ethics Review Board of the University of Frankfurt Medical School. Informed consent was obtained in accordance with the Declaration of Helsinki.

On days 2 and 3, transduction of lin− or $CD34^+$ cells was performed on RetroNectin-coated ($10\,\mu g/cm^2$; TaKaRa, Otsu, Japan) culture dishes by centrifugation with VSV-G (multiplicity of infection [MOI] of 6.6) or GALV pseudotyped



SUPPLEMENTARY FIG. S2. Reconstitution of gp91 expression and NADPH oxidase activity in the peripheral blood of SINfes.gp91s-transplanted X-CGD mice. **(A** and **B)** Percentage of $gp91^{phox}$-positive cells in peripheral blood of primary **(A)** and secondary **(B)** mice for low- and high-vector copy animals 16–19 weeks after transplantation. **(C)** Percentage of granulocytes with NADPH oxidase activity in the peripheral blood of primary and secondary transplanted animals 16–19 weeks after transplantation. Nontransplanted animals were included for background signals (X-CGD) or as positive controls (Bl/6), respectively. *p*-Values according to two-tailed unpaired *t*-test. NADPH, nicotinamide dinucleotide phosphate.



SUPPLEMENTARY FIG. S3. Outline of the *in vivo* genotoxicity study.

viral particles (MOI of 4.3) at 800 *g* for 60 min at 32°C. Non-concentrated ecotropic supernatants were preloaded three times on the retronectin-coated plates (final MOI of 15). In the genotoxicity study, transductions were performed in one well for each recipient mouse separately. Transduced cells were incubated under the same culture conditions as above plus 10 μg/ml heparin (Sigma, Taufkirchen, Germany) for 2–7 additional days before proceeding to further experiments.

In vivo *assessment of transduced cells*

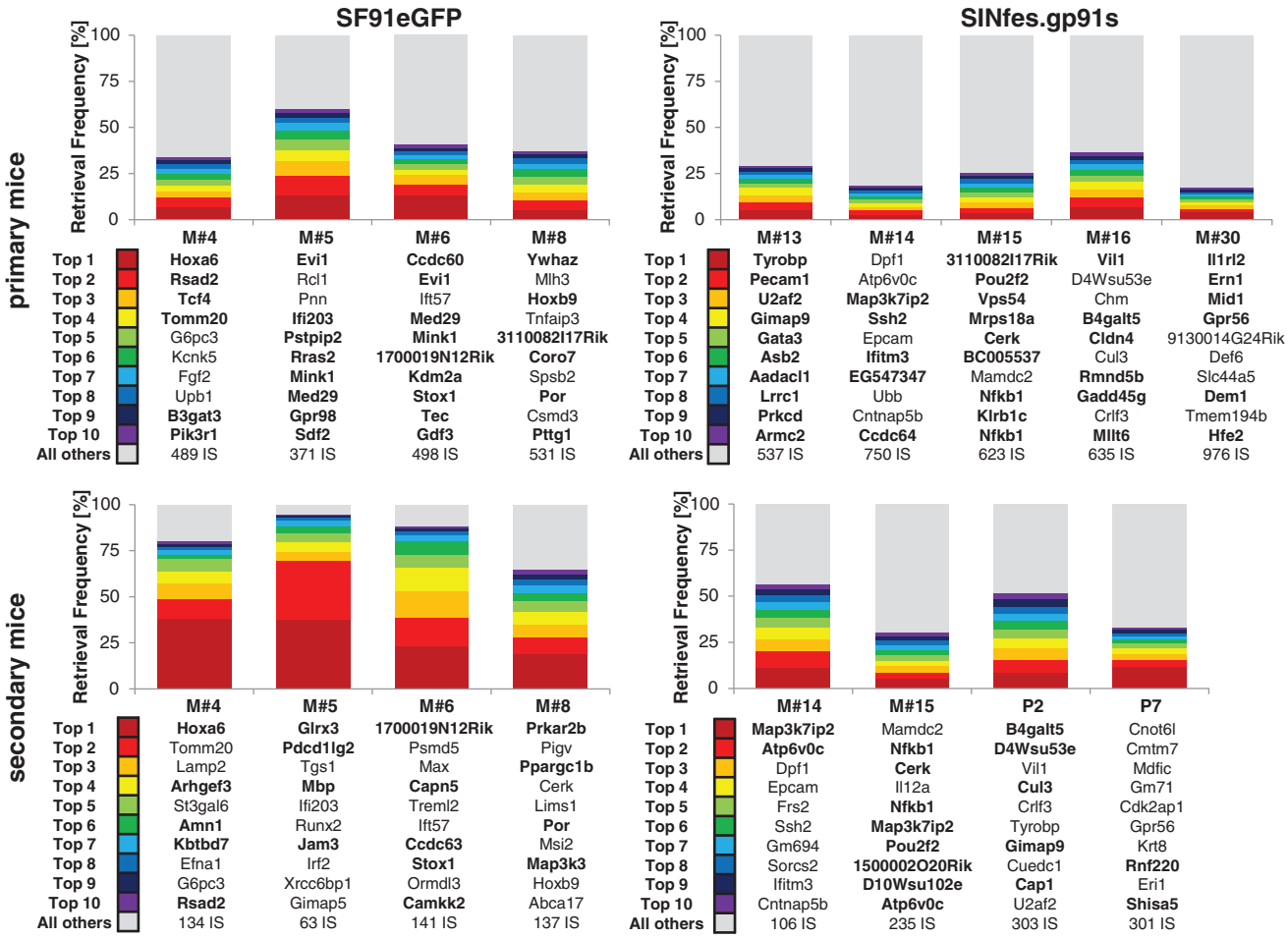
After expansion, transduced murine lin− cells were harvested, and 1×10^6 (efficacy study) or $2–3\times10^6$ cells (genotox study) were transplanted into lethally irradiated X-CGD or Bl/6-CD45.1 recipient mice (single dose of 10 Gy) via tail vein injection. For the genotox study, the test cells were transplanted concomitantly with 1×10^5 BM mononuclear helper cells from the Bl/6-CD45.1 recipient strain. In case of the $CD34^+$ cells, $3–10\times10^6$ cells where transplanted into irradiated (2.5 Gy) NOD/*scid*/IL-2Rγ null recipient mice. All transplanted mice were kept in individually ventilated cages, and drinking water was supplemented with 1.6 g/liter neomycin for the following 3 weeks.

Analysis of immune reconstitution and oxidase activity

Animals were regularly monitored for their health status (e.g., body weight, peripheral blood parameters). Cellular composition, chimerism, transgene expression, and oxidase activity were determined by flow cytometry at the indicated time points post-transplantation as previously described (Brendel *et al.,* 2012). For final analysis (30 weeks), primary hosts were sacrificed and thoroughly necropsied. Peripheral blood, spleen, and BM samples were analyzed as stated above. For secondary transplants, 5×10^6 BM cells after erythrocyte lysis were injected into secondary Bl/6-CD45.1 recipients, which were assayed for an additional 16 weeks. Analysis of human cells in immunodeficient mice was based on an APC-labeled antihuman CD45 antibody (BD, Heidelberg, Germany) and a CD11b-PECy7 conjugate (eBioscience, Frankfurt, Germany). BM cells were isolated from transplanted immunodeficient mice and kept in RPMI media containing 20% fetal calf serum, 50 ng/ml human G-CSF, 4 m*M* glutamine, and penicillin/streptomycin 100 units/ml each for 1–2 weeks for granulocytic differentiation. Engraftment was determined by flow cytometry using the isotype-specific CD45.2 antibody.

Ear skin inflammation and Aspergillus fumigatus *challenge*

BM harvested from X-CGD mice (CD45.2) treated 3 days previously with 5-fluorouracil were prestimulated for 2 days with IL-6 and SCF (Goebel *et al.,* 2005), transduced with ecotropic envelope-packaged SINfes.gp91s and injected intravenously into F1 chimeric (CD45.1/CD45.2) CGD animals at 0.5×10^6 to 2×10^6 cells per recipient animal. Recipient mice were preconditioned with 60 mg/kg busulfan, given as a split dose of 30 mg/kg on day −2 and day −1 before transplant. Transduction efficiency was estimated by the nitroblue tetrazolium assay in BM polymorphonuclear leukocytes or by staining for $gp91^{phox}$ expression using the monoclonal antibody 7D5 in Gr1+ cells after *in vitro* differentiation of transduced cells and found to be 65% and 39%, respectively. Transplanted animals were examined for engraftment and superoxide production for up to 7 months. Donor leukocyte chimerism was ≥80% for all transplanted cell doses. Some animals were challenged with heat-killed *A. fumigatus* hyphae as previously described (Petersen *et al.,* 2002). Essentially, mice were anesthetized with intraperitoneal Avertin (2.5% vol/vol in 0.9% NaCl, 0.015–0.025 ml/g body weight), and the dorsal aspect of one ear was injected with 50 μl of a solution containing 2.5 mg of *A. fumigatus*, with the contra lateral ear injected with phosphate buffered saline (PBS) vehicle. At 3 and 30 days postinjection, all mice were examined for clinical evidence of inflammation at the injection sites. Three days after injection, ear inflammation was assessed by measuring ear thickness using spring-loaded calipers (Mitutoyo, Aurora, IL). At 30 days postinjection, the mice were sacrificed by Avertin overdose and CO_2 asphyxiation. A 5 mm punch biopsy was then taken of each ear, and each biopsy was weighed, measured for thickness, and processed for histological evaluation. Biopsy specimens were evaluated for the presence or absence of inflammatory cells and were graded on a 0–3 scale, with a score of 0 indicating no inflammation (e.g., as seen in ears injected with PBS), and a score of 3+ denoting maximum inflammations as observed in X-CGD controls. Other animals were challenged with 200 *A. fumigatus* conidia by direct intratracheal instillation, a dose previously shown to establish chronic *A. fumigatus* pneumonia in 100% of X-CGD mice (Dinauer *et al.,* 2001). The number of conidia in the inoculum was confirmed by plate culture. As prophylaxis against secondary bacterial infection, mice were given intramuscular injection of ceftriaxone (Rocephin; Hoffman-La Roch, Nutley,

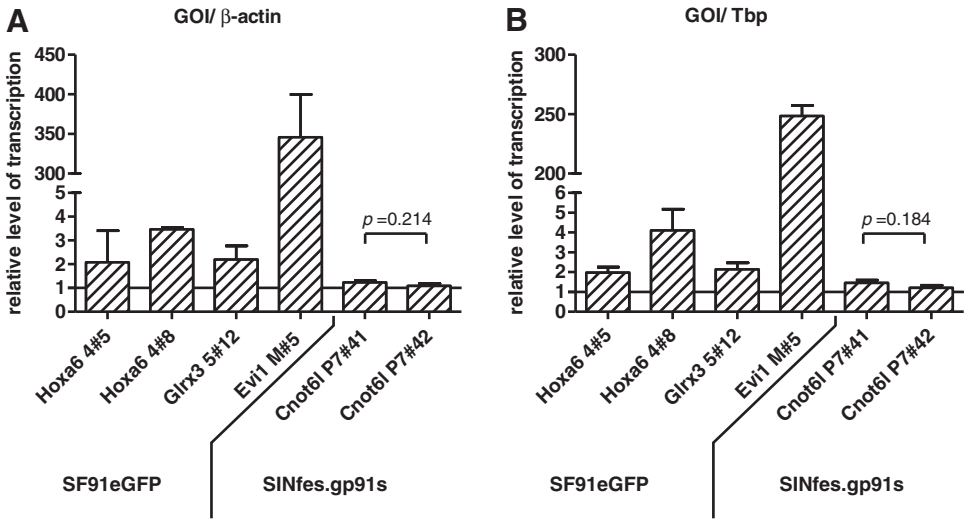


SUPPLEMENTARY FIG. S4. Contribution of clones with individual integration sites to gene-marked hematopoiesis in transplanted primary (upper row) and secondary animals (lower row). Depicted is the retrieval frequency of individual integration sites in samples from SF91eGFP-transplanted (left panels) and SINfes.gp91s-transplanted (right panels) animals. The clonal contribution of individually gene-marked cells was assessed by counting identical integration site sequences after 454 pyrosequencing of LAM-/LM-PCR amplicons. The relative contribution of individual amplicons is given as the percentage of all insertion site sequence reads encountered in the particular sample. The 10 most frequent retroviral integration site (RIS) are ranked from 1 to 10, according to the retrieval frequency. Genes highlighted in bold face/characters were associated with the 10 most abundant RIS in more than one sample. M#xx indicates the identity of the primary recipient; in P2 animals, bone marrow samples from M#13 and M#16 were pooled and used for transplantation; for P7 animals, bone marrow samples from M#29 and M#30 were pooled and used for transplantation. LAM-/LM-PCR, linear amplification-mediated/linker-mediated PCR.

NJ) 1.25 mg per animal immediately before infection and again 24 hr later, followed by oral tetracycline (Polyotic; American Cyanamid, Wayne, NJ) 5 mg/ml in the drinking water for the remainder of the experiment. Mice were examined daily and sacrificed 14 days after fungal challenge. Lungs were fixed for histologic examination of paraffin-embedded sections stained with hematoxylin and eosin for assessment of pathologic changes. Lung histology was scored in a blinded manner, following a scoring system correlating with histopathologic severity. Three cross sections were scored between 0 (no inflammation) to 4 in three categories: focal granulomas and/or microabcesses, multifocal granulomas and/or microabscesses, or diffuse purulent granulomatous inflammation covering the lung lobe. A composite disease score reflected the total numerical score from these three categories.

In vitro *immortalization assay*

The *in vitro* immortalization assay was performed as described previously (Modlich *et al.*, 2006, 2009). Briefly, 100,000 murine lin− BM cells were transduced with an MOI of 2×10 in a serum-free medium (StemCell Technologies Stem Span medium containing 50 ng/ml mSCF, 100 ng/ml hFlt-3 ligand, 100 ng/ml hIL-11, 10 ng/ml mIL-3, 1% penicillin/streptomycin, and 2 m*M* glutamine) and expanded for 2 weeks in a differentiation-inducing medium (10% FCS, 2 m*M* glutamine, 1% penicillin/streptomycin, 50 ng/ml mSCF, 100 ng/ml hFlt-3 ligand, 100 ng/ml IL-11, 10 ng/ml mIL-3), followed by a replating step (100 cells/well on 96-well plates). Two weeks after replating, wells with proliferating cells were counted and the replating frequency was calculated based on Poisson distribution using L-Calc (StemCell



SUPPLEMENTARY FIG. S5. No transcriptional dysregulation of gene expression by the integrated SINfes.gp91s vector. Real-time reverse transcriptase PCR was used to assess transcription levels of genes adjacent to selected predominant integration sites in SF91eGFP- and SINfes.gp91s-transplanted animals. The RNA levels of the GOI were normalized according to the $\Delta\Delta C_t$ method using internal *β-actin* **(A)** or TATA-box binding protein (*Tbp*) **(B)** transcripts as controls. Mean values of at least three independent assays ± SEM are shown. *p*-Values (two-tailed unpaired *t*-test) are given for *Cnot6l* transcripts in P7#41 vs. P7#42 mice. GOI, gene of interest.

Technologies, Vancouver, Canada). Four days after transduction, aliquots of cells were taken to isolate DNA and vector copy numbers were determined by quantitative polymerase chain reaction (PCR) and used for normalization of the replating frequencies. Cells of replated wells were expanded, DNA was collected, and insertion sites were determined.

Quantitative PCR

Mean vector copy number was assayed by quantitative PCR for the wPRE on genomic DNA prepared from peripheral blood, spleen, and BM samples of transplanted animals. The PCR was performed in a Roche LightCycler480 machine in a duplex reaction using the following primers and probes: WPRE.for, 5′-ATTGCCACCACCTGTCAACT-3′; WPRE.rev, 5′-GCAACCTAGCCCCTGTCC-3′; WPRE.probe, universal probe #95 (Roche Diagnostics, Mannheim, Germany; catalog no. 04692128001); murine samples: m-b-actin.for, 5′-AGAGGGAAATCGTGCGTGAC-3′; m-b-actin.rev, 5′-CAATAGTGATGACCTGGCCGT-3′; b-actin probe, 5′-TexasRed-CACTGCCGCATCCTCTTCCTCCC-3′-BHQ2; human samples (Epo receptor gene): hEPOR_7/5_se, 5′-ATGCCAGACTAGACCCAGAC-3′; hEPOR_7/5_rev, 5′-GGAAAGGAACTAACAAAGGGAC-3′; hEPO_7/5.probe, 5′-TexasRed-TCTTGGGGACTTTCACCTGATTTTCCTTCTAC-3′-BHQ2. As references for human- or murine-derived samples, genomic DNA isolated from a PLB985 clone or a Baf3 clone was used respectively, each containing a single vector integration. To determine chimerism after transplantation of male cells in female hosts, amplicons for the *SRY* gene were quantified using SRY_82.for, 5′- AGCCTCATCGGAGGGCTA-3′; SRY_82.rev, 5′-AGGCAACTGCAGGCTGTA-3′ primers; and the universal probe #82 (Roche Diagnostics; catalog no. 04689054001).

Reverse transcriptase PCR

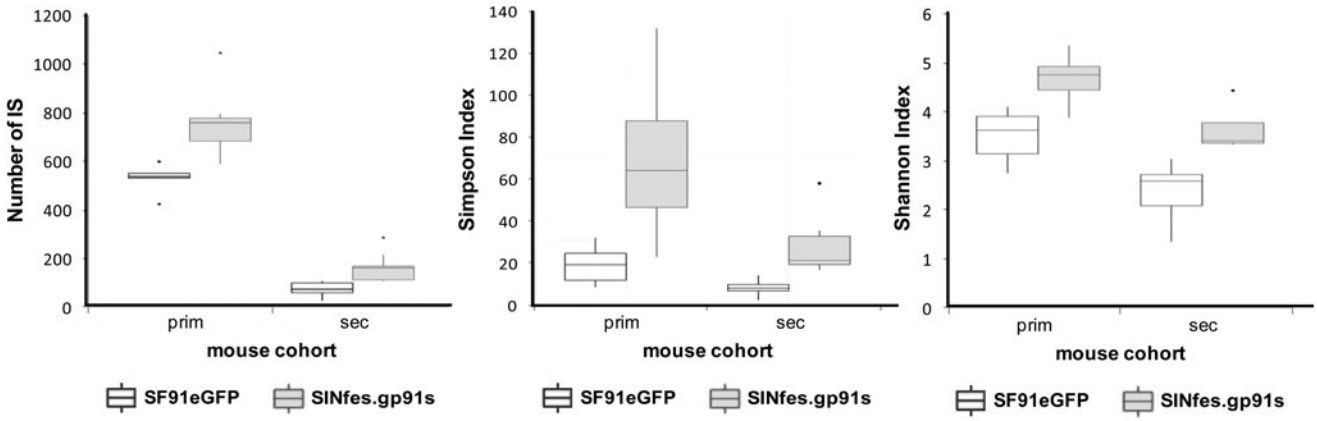
Total RNA was extracted from indicated samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and subsequent DNaseI treatment. For samples with less than 5×10^5 cells, the RNAqueous-Micro Kit (Ambion, Darmstadt, Germany) was used. Reverse transcription was performed with random primers using the RETROscript Kit (Ambion) according to the manufacturer's instructions. About 5 μl of the cDNA was applied as template in duplex reverse transcriptase PCRs normalizing the total amount of cDNA by *β*-actin or TATA box binding protein (Tbp) as reference genes. For amplification, the following primer–probe ready-to-use mixes were used (Applied Biosystems, Darmstadt, Germany): *Cnot6l* (Mm00523339_m1), *Evi1* (Mm00514814_m1), *Glrx3* (Mm0071228X_m1), *Hoxa6* (Mm00550244_m1), and *Tbp* (Mm00_m1). For *beta-actin*, we used m-b-actin.for, 5′-AGAGGGAAATCGTGCGTGAC-3′; m-b-actin.rev, 5′-CAATAGTGATGACCTGGCCGT-3′; b-actin probe, 5′-TexasRed-CACTGCCGCATCCTCTTCCTCCC-3′-BHQ2.

Bisulfite conversion and sequencing

Between 100 and 500 ng of genomic DNA or extracts from a single BM colony was used for bisulfite conversion with the EpiTect Bisulfit Kit (Qiagen) according to the manufacturer's protocol. The product was used as template for a nested or seminested PCR amplification of promoter sequences. Primer sequences for the SFFV promoter enhancer were described by Stein *et al.* (2010). For the fes promoter, we used FES-BS-for3, 5′- ATATTTTGGGTTTTTTTGGGTTTAG-3′, and FES-BS-rev1, 5′- TTCAAACCCAACCACACC-3′ for the first, and FES-BS-for1, 5′- TGGGTTTAGTATTTGTTTGGGG-3′, and FES-BS-rev1-2, 5′- CCCAACCACACCAAAATC -3′ for the second PCR. Reaction products were cloned into the TOPO-TA plasmid (Invitrogen, Darmstadt, Germany) and plasmids from individual clones were sequenced.

High-throughput integration site analysis

Linear amplification-mediated (LAM) PCR and 454 pyrosequencing were performed as described before (Schmidt



SUPPLEMENTARY FIG. S6. Clonal diversity. **(A)** Total number of integration sites per mouse. Integration sites were obtained from serially transplanted SF91eGFP and SINfes.gp91s mice by LAM-/LM-PCR and subsequent 454 sequencing. **(B)** Simpson index of primary and secondary animals. The Simpson index is used to measure the degree of clonal dominance in transplanted animals, with a low value indicating high dominance. **(C)** Shannon index of primary and secondary animals. The degree of heterogeneous clonal repopulation with vector-transduced cells in SINfes.gp91s and SF91eGFP mice was determined by calculating the Shannon index. prim, primary mouse samples; sec, secondary mouse samples.

et al., 2007; Paruzynski *et al.,* 2010). In brief, linear amplification of vector–genome junctions using vector-specific primers was followed by magnetic capture and double-strand synthesis. A restriction digestion was performed using non-CG motif restriction enzymes for optimal accessibility to viral integration sites (Gabriel *et al.,* 2009). After ligation of an enzyme-specific linker cassette, the resulting fragments were amplified by two nested exponential PCRs using linker- and vector-specific primers. Purified LAM-PCR and linker-mediated PCR products were prepared for 454 sequencing adding GS FLX-specific adaptors and unique barcodes to each sample (Paruzynski *et al.,* 2010). Sequences retrieved from 454 sequencing were bioinformatically processed (Arens *et al.,* 2012) for subsequent analyses. (Data of unique LAM-PCR amplicons are available as open access database at https://consert.gatc-biotech.com/lampcr/. For username and password, please contact the corresponding author.)

Statistical analysis

Experimental data are plotted as means ± standard error of the mean. Student's two-tailed *t*-test, one-way analysis of variance, or χ^2-test was used for comparison of differences between groups as indicated. A p-value < 0.05 was considered significant.

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