Title
Floxin, a resource for genetically engineering mouse ESCs

Journal
Nature Methods, 7(1)

ISSN
1548-7091

Authors
Singla, Veena
Hunkapiller, Julie
Santos, Nicole
et al.

Publication Date
2010

DOI
10.1038/nmeth.1406

Peer reviewed

HHS Public Access

Author manuscript

Nat Methods. Author manuscript; available in PMC 2010 July 01.

Published in final edited form as:

Nat Methods. 2010 January ; 7(1): 50–52. doi:10.1038/nmeth.1406.

Floxin, a resource for genetically engineering mouse embryonic stem cells

Veena Singla[1], **Julie Hunkapiller**[1], **Nicole Santos**[1], **Allen Seol**[1], **Andrew R. Norman**[1,2], **Paul Wakenite**[3], **William C. Skarnes**[4,5], and **Jeremy F. Reiter**[1,6]

[1]Department of Biochemistry and Biophysics, Cardiovascular Research Institute, University of California, San Francisco, San Francisco, California 94158-2324

[3]Department of Human Genetics, University of Chicago, Chicago, Illinois

[4]Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1HH, United Kingdom

Abstract

We describe a method for the highly efficient and precise targeted modification of gene trap loci in mouse embryonic stem cells (ESCs). Through the Floxin method, gene trap mutations are reverted and new DNA sequences inserted using Cre recombinase and a shuttle vector, pFloxin. Floxin technology is applicable to the existing collection of 24,149 compatible gene trap cell lines, which should enable the high-throughput modification of many genes in mouse ESCs.

Genetic modification of mice and of embryonic stem cells (ESCs) is an important source of insight into the functions of vertebrate genes. Current methods commonly used for genetically modifying ESCs are transgene insertion and homologous recombination. Transgene insertion involves integration at a random location in the genome. Homologous recombination can create targeted mutations, but only about 1.5% of ESC clones, on average, correctly integrate the construct and different targeting constructs must be created for each gene.

A complementary approach to generating loss-of-function mutations in ESCs is gene trapping1–3. Below, we describe the gene trap vectors pGTLxf and pGTLxr, which allow post-insertional modification of the trapped locus using an accompanying technology called

[5]Correspondence should be addressed to W.C.S. (skarnes@sanger.ac.uk). [6]Correspondence should be addressed to J.F.R. (jeremy.reiter@ucsf.edu).

[2]Current address: Department of Developmental Biology, Stanford University School of Medicine, Stanford, California 94305–5329

Authors' Contributions

V.S., J.H., W.S., and J.R. conceived and designed the experiments. P.W. conceived and designed the first Floxin vector. V.S., J.H., N.S, A.S., W.S., A.N. and J.R. performed the experiments. J.R. and V.S. wrote the paper.

Editorial Summaries

AOP: An efficient system for the reversion and modification of mouse gene trap alleles is presented. It is applicable to available collections of gene trap embryonic stem cell lines.

Issue: An efficient system for the reversion and modification of mouse gene trap alleles is presented. It is applicable to available collections of gene trap embryonic stem cell lines.

Floxin (Flanked lox site insertion), based on recombination mediated cassette exchange (RMCE).

We show a schematic of mutation, reversion, and modification of a generic, autosomal *Your Favorite Gene* (*YFG*) (Fig. 1a–d). A pGTLxf or pGTLxr gene trap in an intron of *YFG* results in expression of a YFG-βgeo fusion protein in the gene trap line $YFG^{Gt/+}$ (Fig. 1a). To revert this loss-of-function mutation, transiently expressed Cre excises the floxed splice acceptor, leaving a single Lox71 site (Fig. 1b)4. In the absence of the splice acceptor, $YFG^{Rev/+}$ cells lose β*geo* expression and reactivate *YFG* expression.

To permit modification of a gene trap locus, pFloxin vectors contain a Lox66 site (Fig. 1c) so that Cre-mediated recombination between the pFloxin Lox66 and the genomic Lox71 site of revertant cells results in directional insertion of the pFloxin sequence (Fig. 1d). Recombination between Lox66 and Lox71 sites produces one inactive Lox site and one LoxP site (Fig. 1d), making this integration irreversible5.

Floxin vectors also permit expression of defined sequences at the modified locus (Fig. 1c–d). Although Floxin can facilitate many kinds of gene modifications, we illustrate here the production of carboxy- and amino-terminally tagged alleles of YFG. The pFloxin-YFG-Flag inserted cDNA is spliced so that the inserted sequence is expressed as a fusion with upstream exons from the endogenous promoter. The other Floxin vector, pFloxin-IRES-HA-YFG, contains an IRES element to initiate translation of an amino-terminally tagged version of YFG. The line $YFG^{IRES\text{-}HA\text{-}YFG/+}$ expresses full length *HA-YFG* under the control of the endogenous promoter and the IRES element. The Floxin vectors also include β*Actin* promoters that reactivate β*geo* expression and thus permit pharmacological selection of correct insertions (Fig. 1c–d).

RMCE has been shown previously to function robustly in ESCs with varied vector designs6–11. To date, the BayGenomics and Sanger Institute gene trap efforts have generated 24,149 gene trap cell lines with the pGTLxf and pGTLxr vectors, representing 4,528 individual genes12. A list of the gene trap alleles is reported here (Supplementary Table 1), and the cell lines are available to the community through the International Gene Trap Consortium (IGTC) database (www.genetrap.org).

Here, we demonstrate modification of eight genomic loci (*Sall4*, *Suz12*, *Ofd1*, *Gli2*, *Tardbp*, *Sntb2*, *Pex14* and *Tet1*) using gene trap cell lines and the Floxin system. We note that $Ofd1^{Gt}$ ($Ofd1^{Gt(RRF427)Byg}$; MGI names for all alleles are provided in Supplementary Table 2) cells are hemizygous as the *Ofd1* gene is X-linked and the E14 gene trap ESCs are male. Consequently, $Ofd1^{Gt}$ cells do not produce any Ofd1 protein (Fig. 1e).

To remove the exogenous splice acceptor, we electroporated gene trap cells with an expression construct for nuclear Cre recombinase. On average, 45% of colonies screened showed proper excision of the splice acceptor (Table 1). Revertant cells no longer displayed β-galactosidase activity or neomycin resistance (Fig. 2a, Supplementary Fig. 1d), and reversion caused loss of the β*geo* transcript (Supplementary Fig. 1e). Genomic PCR and Southern blot confirmed correct excision of the splice acceptor in revertant cells (Supplementary Fig. 1f–g).

Using the Floxin strategy, we generated cell lines expressing wild type Ofd1, Suz12, Sall4, Gli2, or Tardbp with carboxy-terminal tags, and lines expressing *eGFP* at the *Sntb2*, *Pex14*, or *Tet1* genomic loci. Revertant lines were co-electroporated with the appropriate pFloxin or pFloxin-IRES construct and a nuclear Cre expression construct and selected with neomycin. On average, 86% of resultant ESC colonies contained the correctly integrated pFloxin construct (Table 2), and β-galactosidase activity was re-activated (Fig. 2a). Genomic PCR and Southern blot confirmed integration in Floxin cell lines (Supplementary Fig. 1g, Supplementary Fig. 2a–c). These data indicate that Floxin-mediated targeted insertion occurs efficiently and accurately in many different genomic contexts.

Quantitative RT-PCR and immunoblot indicated that revertant alleles are expressed at wild type levels in $Sall4^{Rev/+}$ and $Suz12^{Rev/+}$(Fig. 2b and Supplementary Fig. 2d). In contrast, $Ofd1^{Rev}$ cells expressed lower levels of Ofd1 (at the expected size of 110 kD) than wild type cells (Fig. 2c). Ofd1 is essential for the formation of the primary cilium13, and wild type ESCs possess primary cilia whereas $Ofd1^{Gt}$ cells do not. However, $Ofd1^{Rev}$ cells possessed primary cilia, indicating that reversion of the gene trap mutation restored gene function (Fig. 2d). RT-PCR and sequencing indicated that the reduced *Ofd1* expression is attributable to the use of cryptic splice acceptor sites present in the β*geo* cassette (data not shown). Moreover, the β*geo* transcript is detectable by Northern blot in a subset of revertant lines (Supplementary Fig. 1e). The use of these cryptic splice acceptor sites in certain revertant cell lines suggests that locus-dependent factors affect the restoration of normal expression.

Floxin-inserted alleles were expressed at levels equivalent to the revertant alleles. The tagged alleles for both $Sall4^{Sall4TAP/+}$ and $Suz12^{Suz12TAP/+}$ were expressed at wild type levels (Fig. 2b and Supplementary Fig. 2d). Although Ofd1-Myc protein levels were lower than that of wild type (Fig. 2c), production of Ofd1-Myc was also sufficient to support ciliogenesis (Fig. 2e) and Ofd1-Myc, like endogenous Ofd1, localized to the centrosome (Fig. 2f). Suz12-TAP, like endogenous Suz12, localized to the nucleus (Fig. 2g), and ESC differentiation led to downregulation of endogenous Suz12 and Suz12-TAP to similar extents (Fig. 2h)14. Together, these data indicate that Floxin cells produce modified proteins that function and are regulated similar to wild type proteins.

In addition to tagged versions of endogenous genes, the Floxin technology can insert exogenous DNA into loci. $Sntb2^{IRESeGFP/+}$, $Pex14^{IRESeGFP/+}$ and $Tet1^{IRESeGFP/+}$ lines expressed eGFP under the control of endogenous regulatory elements (Supplementary Fig. 2e).

The presence of vector sequences may affect normal gene expression15. Therefore, we included Frt sites in the gene trap and Floxin vectors to allow for Flp-mediated removal of the β*Actin* promoter and β*geo* cassette (Supplementary Fig. 3a). We electroporated $Ofd1^{Ofd1myc}$ or $Gli2^{Gli2TAP/+}$ cells with a FLPo expression construct16 and identified cells in which the β*Actin*-β*geo* cassette was successfully excised by loss of β-galactosidase activity (Fig. 2a). Genomic PCR verified correct excision (Supplementary Fig. 3c). Removal of vector sequences increased the expression of Ofd1-Myc (Supplementary Fig. 3d).

To assess whether the genetic manipulations associated with gene trapping, reversion, and the Floxin process affected ESCs, we performed karyotyping and evaluated pluripotency by three methods. The five Floxin lines evaluated showed normal euploid karyotypes (data not shown). All Floxin lines had normal ES colony and cell morphology (Supplementary Fig. 4a). Additionally, Floxin lines had expression levels similar to wild type ESCs for three regulators of pluripotency, *Oct4*, *Sox2* and *Nanog* (Supplementary Fig. 4b), and could differentiate into cell types that express *Fgf5*, *Afp* and *T/Brachyury*, markers of embryonic ectoderm, endoderm and mesoderm, respectively (Supplementary Fig. 4c). While these assays suggest that the Floxin process does not adversely affect ESC pluripotency, germline competency of Floxin cells has not been systematically evaluated. Other studies have shown that three genetic manipulations do not necessarily limit germline transmission7, 17.

A detailed understanding of gene function requires the generation of a range of alleles. The Floxin strategy described here allows for high throughput modification of ESC loci harboring insertions of the pGTLxf or pGTLxr gene trap vectors. The Floxin system allows for Cre-mediated reversion of the gene trap mutation and subsequent insertion of new DNA of interest into the genomic locus. Genes of interest are cloned with standard molecular biology techniques into pFloxin shuttle vectors. With each manipulation, the presence or absence of β*geo* expression assists in the selection or identification of the desired cells.

We have demonstrated the generation of tagged or reporter alleles at eight loci, and shown how the technology can be used to model a human genetic disease in ESCs, study protein localization, and report on the dynamics of protein expression (Supplementary Table 3). The gene trap allele can also be converted into a wide variety of other tailored alleles, such as missense, deletion, and domain swap alleles, and Floxin can be used to insert non-homologous DNA sequences into endogenous loci. This approach could be utilized for generating alleles expressing other exogenous proteins such as Cre, rtTA, ΦC31 integrase, Alkaline phosphatase, or Diptheria toxin under the control of tissue- or cell type-specific promoters.

The Floxin strategy has several advantages over previously described approaches for recombinase-mediated insertion of exogenous DNA elements6–8, 10. First, reversion allows for confirmation that observed cellular phenotypes are due to the gene trap mutation, as demonstrated for the role of *Ofd1* in ciliogenesis. Second, inserted DNA sequences can be expressed either as a direct fusion to upstream exons, or as a separate cistron. Third, Frt sites allow for removal of the β*Actin*-β*geo* cassette, abrogating interference from vector prokaryotic sequences. Lastly, the Floxin technology is compatible with the extensive collection of 24,149 characterized and validated gene trap lines available to the community.

The Floxin strategy of reversion and new DNA insertion are both highly efficient and reproducible at a variety of loci. By avoiding the most laborious aspects of traditional gene replacement strategies, the Floxin system allows new alleles to be engineered with minimal effort.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Ofd1^{Gt} (RRF427), *Sall4*$^{Gt/+}$(XE027), *Suz12*$^{Gt/+}$(XG122), *Gli2*$^{Gt/+}$ (XG045), and *Tardbp*$^{Gt/+}$ (RRB030) E14 ESC lines were obtained from BayGenomics. *Sntb2*$^{Gt/+}$ (XC195), *Pex14*$^{Gt/+}$ (XC197), *Tet1*$^{Gt/+}$ (XD006), *Flna*^{Gt} (XC373), *Btf3*$^{Gt/+}$ (XD028), *Nsd1*$^{Gt/+}$ (XC030), and *XD052*^{Gt} (XD052) were provided by W.C.S. Cells were cultured on 0.1% gelatin in GMEM supplemented with 10% FBS, glutamine, pyruvate, NEAA, βME, and LIF.

Plasmids and Vector Construction

pPGK-NLS-Cre expresses a fusion of the bacteriophage P1 recombinase Cre with a SV40 large T antigen nuclear localization signal18. pFloxin vectors were built from the pBluescript backbone by PCR amplification and insertion of an *Engrailed2* splice acceptor sequence, SV40 polyA signal and, in the case of pFloxin-IRES, an IRES. The tandem stop codons and multiple cloning site polylinker were synthesized as oligonucleotides and ligated into the vectors. All pFloxin constructs were produced in *rec*A1 Stbl2 cells (Invitrogen) according to manufacturer's protocol, except cultures were grown at 37° in LB medium. GenBank accession numbers for pFloxin, pFloxin-IRES, pFloxin-TAP, and pFloxin-IRES-eGFP are EU916834, EU916835, EU916836, and GQ357182 respectively. The vector pFloxin-MCS2-IRES-MCS (GenBank number GU180239) with two multiple cloning sites and an IRES allows insertion of homologous cDNA as well as a reporter regulated by the same promoter.

cDNA constructs and cloning

Suz12 and *Sall4* cDNAs were gifts from Miguel Ramalho-Santos. To make *Sall4*$^{Sall4TAP/+}$ and *Suz12*$^{Suz12TAP/+}$ cell lines, primers were used to amplify and add appropriate restriction sites to exons 2–4 of *Sall4*, and exons 8–16 of *Suz12* (Supplementary Table 4). The cDNAs were then cloned into pFloxin-TAP, in frame with the TAP tag. *Ofd1* cDNA was cloned from RNA of E11.5 mouse embryos using 1 Step RT-PCR kit (Invitrogen). Further amplification and addition of the Myc tag was performed with the Expand High Fidelity kit (Roche). Quik Change II XL site directed mutagenesis kit (Stratagene) was used to repair missense mutations introduced during PCR. Final products were confirmed by sequencing.

Electroporation and Selection

For reversion: 60 μg of pGK-NLS-Cre DNA was ethanol precipitated, and the pellet washed 3 times with 70% ethanol. The pellet was dried in tissue culture hood for 30' at room temperature and resuspended in 100 μl calcium-magnesium free PBS overnight at room temperature. On the morning of electroporation, new media was added to the gene trap cells grown to 70–90% confluency in a T75 flask. After 3–4 hours, cells were trypsinized, counted, and washed with PBS. 10^7 cells were added to a chilled electroporation cuvette with DNA and electroporated at 240 V, 500 μF, exponential (Bio-Rad GenePulser Xcell). Electroporated cells were incubated 20' at room temperature, and 10^5 cells were split evenly between 10 10-cm plates. Colonies were picked and transferred to a 48 well plate after 3–5 days.

For Floxin: pGK-NLS-Cre and pFloxin DNA were prepared as above. We electroporated revertant cell lines with both DNAs as above, except 10^7 cells were plated evenly between 6 10-cm plates. We began selection with 150–350 μg/ml G418 (Invitrogen) one day after electroporation, and picked colonies after 6–7 days.

For FLP removal of vector sequence: 120 μg pPGK-FLPo (Addgene plasmid 13793) was prepared as above16. We electroporated and plated Floxin cell lines as described in the reversion section above.

β-galactosidase Activity Assays

Galacto-Light Plus System (Applied Biosystems) was used to assay cell lysates from a 24 or 48-well plate according to manufacturer's protocol.

Quantitative PCR

RNA was extracted from ESCs or embryoid bodies using RNeasy Plus (Qiagen). First strand cDNA synthesis was performed using extracted RNA with iScript (Biorad) or First Strand cDNA Synthesis (Fermentas). Transcript levels were measured in triplicate using a 7300 Real-time PCR machine (Applied Biosystems) and then normalized to β*Actin* levels.

Antibodies

Antibodies to Ofd1 were generated by Covance by immunizing rabbits with the peptide [H]-CDTYDQKLKTELLKYQLELKDDYI–[NH2] corresponding to amino acids 340–362 of murine Ofd1. Antibody was used at 1:5000 for Western blotting and 9.2 μg for IP. Other antibodies used were: acetylated Tubulin (Sigma T6793) used at 1:1000, γ–Tubulin (Santa Cruz sc-7396) 1:200, GFP (Aves Labs GFP-1020) 1:500, Centrin1 (Abcam ab11257) 1:200, Suz12 (Santa Cruz sc-46264) 1:200, Flag (Sigma F7425) 1:500 for IF. Flag resin (Sigma) was used for IP. Myc antibody (Novus Biologicals NB600-335) was used at 1:1000 for immunoblotting, 1:200 for IF, and 3 μg for IP. TAP antibody (GenScript A00683) was used at 1:500 for immunoblotting. Secondary antibodies were conjugated with Alexa Fluor 488 or 555 (Invitrogen) and used at 1:400.

Immunofluorescence and Microscopy

For ESC ciliation studies: ESCs were plated on coverslips coated with poly-D-lysine and 1% Matrigel (BD) and treated with 0.5 mM mimosine (Sigma) overnight to arrest cells. Cells were fixed 5' in 4% PFA, washed in PBS, and fixed 2–3' in −20° methanol. The cells were then washed in PBS with 0.1% Triton-X100 (PBST), blocked in 2% BSA in PBST, and incubated with primary antibodies in block for 1 hr at RT. The cells were washed in PBST, incubated with secondary antibodies in block for 30' at RT, and mounted with Vectashield hardset with DAPI (Vector labs). Slides were viewed on Deltavision microscope (Applied Precision) and images were processed with Deltavision and Metamorph (Molecular Devices) software.

For GFP staining: Cells were plated on coverslips as above and cultured overnight. Cells were fixed 5' in 4% PFA and stained as above. Slides were viewed on an Axio Observer D1

(Zeiss) microscope and images were processed with Axiovision (Zeiss), ImageJ (NIH), and Metamorph (Molecular Devices) software.

For TAP staining: Cells were plated on coverslips as above and cultured overnight. The cells were fixed in 100% methanol for 5'and stained as above. Slides were viewed on Nikon C1 confocal and images were processed with Nikon EZ-C1 software.

For cellular morphology assessment: Cells were plated in 6 well plates and viewed on an Axio Observer D1 (Zeiss) microscope.

ESC differentiation

ESCs were plated in suspension culture in ultra-low adherence six well plates (Corning) at 5×10^5 per well (differentiation assay to asses Suz12 expression) or 10^6 per well in duplicate (pluripotency assessment) to induce embryoid body formation. Suspension media consisted of 10% FBS, nonessential amino acids, pyruvate, glutamine, and βME in GMEM and was changed every other day. For the differentiation assay to assess Suz12 expression, protein lysate was collected on 0, 4, 8, and 12 days following initial plating. For pluripotency assessment, RNA was collected 7 days after plating.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The authors thank K. Thorn and the University of California, San Francisco Nikon Imaging Center for assistance with confocal microscopy, and the members of the Reiter lab for critical reading. Grants from the National Science Foundation (V.S., N.S.), NIH (RO1AR054396), CIRM (RN2-00919), the Burroughs Wellcome Fund, the Packard Foundation, and the Sandler Family Supporting Foundation (J.F.R.) funded this work.

REFERENCES

1. Mitchell KJ, et al. Functional analysis of secreted and transmembrane proteins critical to mouse development. Nat Genet. 2001; 28:241–249. [PubMed: 11431694]
2. Stanford WL, Epp T, Reid T, Rossant J. Gene trapping in embryonic stem cells. Methods Enzymol. 2006; 420:136–162. [PubMed: 17161697]
3. Hansen J, et al. A large-scale, gene-driven mutagenesis approach for the functional analysis of the mouse genome. Proc Natl Acad Sci U S A. 2003; 100:9918–9922. [PubMed: 12904583]
4. Sauer B. Manipulation of transgenes by site-specific recombination: use of Cre recombinase. Methods Enzymol. 1993; 225:890–900. [PubMed: 8231893]
5. Albert H, Dale EC, Lee E, Ow DW. Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome. Plant J. 1995; 7:649–659. [PubMed: 7742860]
6. Araki K, et al. Exchangeable gene trap using the Cre/mutated lox system. Cell Mol Biol (Noisy-le-grand). 1999; 45:737–750. [PubMed: 10512203]
7. Hardouin N, Nagy A. Gene-trap-based target site for cre-mediated transgenic insertion. Genesis. 2000; 26:245–252. [PubMed: 10748462]
8. Osipovich AB, Singh A, Ruley HE. Post-entrapment genome engineering: first exon size does not affect the expression of fusion transcripts generated by gene entrapment. Genome Res. 2005; 15:428–435. [PubMed: 15741512]
9. Schnutgen F, et al. Genomewide production of multipurpose alleles for the functional analysis of the mouse genome. Proc Natl Acad Sci U S A. 2005; 102:7221–7226. [PubMed: 15870191]

10. Taniwaki T, et al. Characterization of an exchangeable gene trap using pU-17 carrying a stop codon-beta geo cassette. Dev Growth Differ. 2005; 47:163–172. [PubMed: 15840001]
11. Xin HB, et al. Gene trap and gene inversion methods for conditional gene inactivation in the mouse. Nucleic Acids Res. 2005; 33:e14. [PubMed: 15659575]
12. Skarnes WC, et al. A public gene trap resource for mouse functional genomics. Nat Genet. 2004; 36:543–544. [PubMed: 15167922]
13. Ferrante MI, et al. Oral-facial-digital type I protein is required for primary cilia formation and left-right axis specification. Nat Genet. 2006; 38:112–117. [PubMed: 16311594]
14. Pasini D, Bracken AP, Hansen JB, Capillo M, Helin K. The polycomb group protein Suz12 is required for embryonic stem cell differentiation. Mol Cell Biol. 2007; 27:3769–3779. [PubMed: 17339329]
15. Wilson TJ, Kola I. The LoxP/CRE system and genome modification. Methods Mol Biol. 2001; 158:83–94. [PubMed: 11236673]
16. Raymond CS, Soriano P. High-efficiency FLP and PhiC31 site-specific recombination in mammalian cells. PLoS ONE. 2007; 2:e162. [PubMed: 17225864]
17. Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci U S A. 1993; 90:8424–8428. [PubMed: 8378314]
18. Gu H, Zou YR, Rajewsky K. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. Cell. 1993; 73:1155–1164. [PubMed: 8513499]

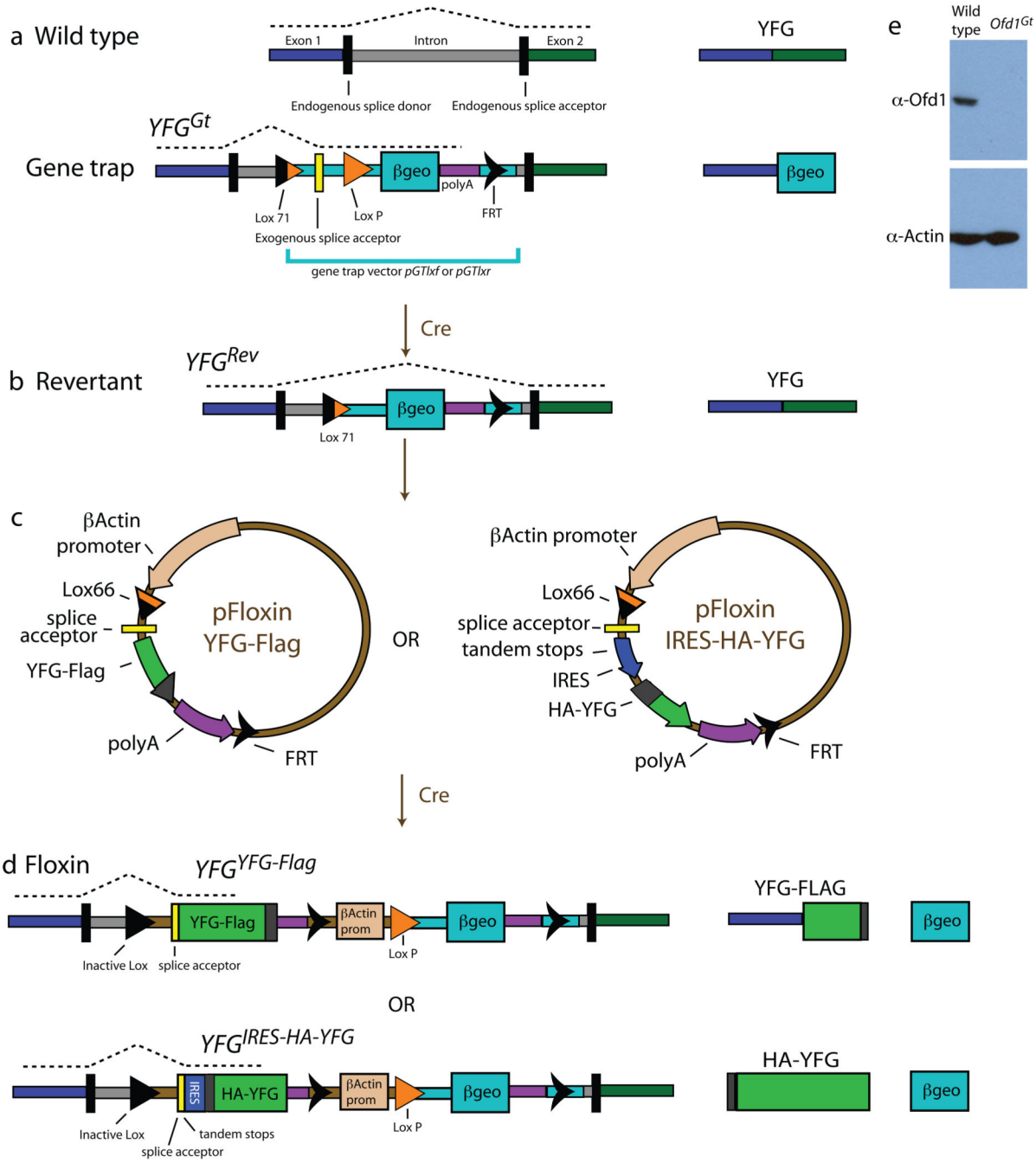

Figure 1. The Floxin strategy for reversion and modification of gene trap loci

(**a**) In the wild type allele of *Your Favorite Gene* (*YFG*), exons 1 and 2 are spliced together and translated to produce full length YFG protein. In the gene trap line of genotype $YFG^{Gt/+}$, a splice acceptor (yellow box) flanked by Lox71 and LoxP sites co-opts splicing to create a fusion between β*geo* and the 5' endogenous exon. β*geo* encodes a fusion of β-galactosidase and neomycin resistance. (**b**) Reversion of the gene trap mutation to yield revertant line YFG^{Rev} (genotype $YFP^{Rev/+}$). Cre recombines the Lox71 and LoxP sites, restoring endogenous splicing and wild type expression of *YFG*. (**c**) Vectors for Floxin-mediated

cassette insertion. Cre recombines the genomic Lox71 and Floxin Lox66 sites to integrate the pFloxin construct into the genomic locus. In these examples, Floxin mediates insertion of exon 2 of YFG with a C-terminal Flag tag (left) or the full-length cDNA for YFG with an N-terminal HA tag (right). (**d**) Schematic showing Floxin alleles that express tagged YFG under the control of endogenous regulatory elements and re-express β*geo*. (**e**) Immunoblots with the indicated antibodies ESCs of the $Ofd1^{Gt}$ genotype. 10 μg protein per lane.

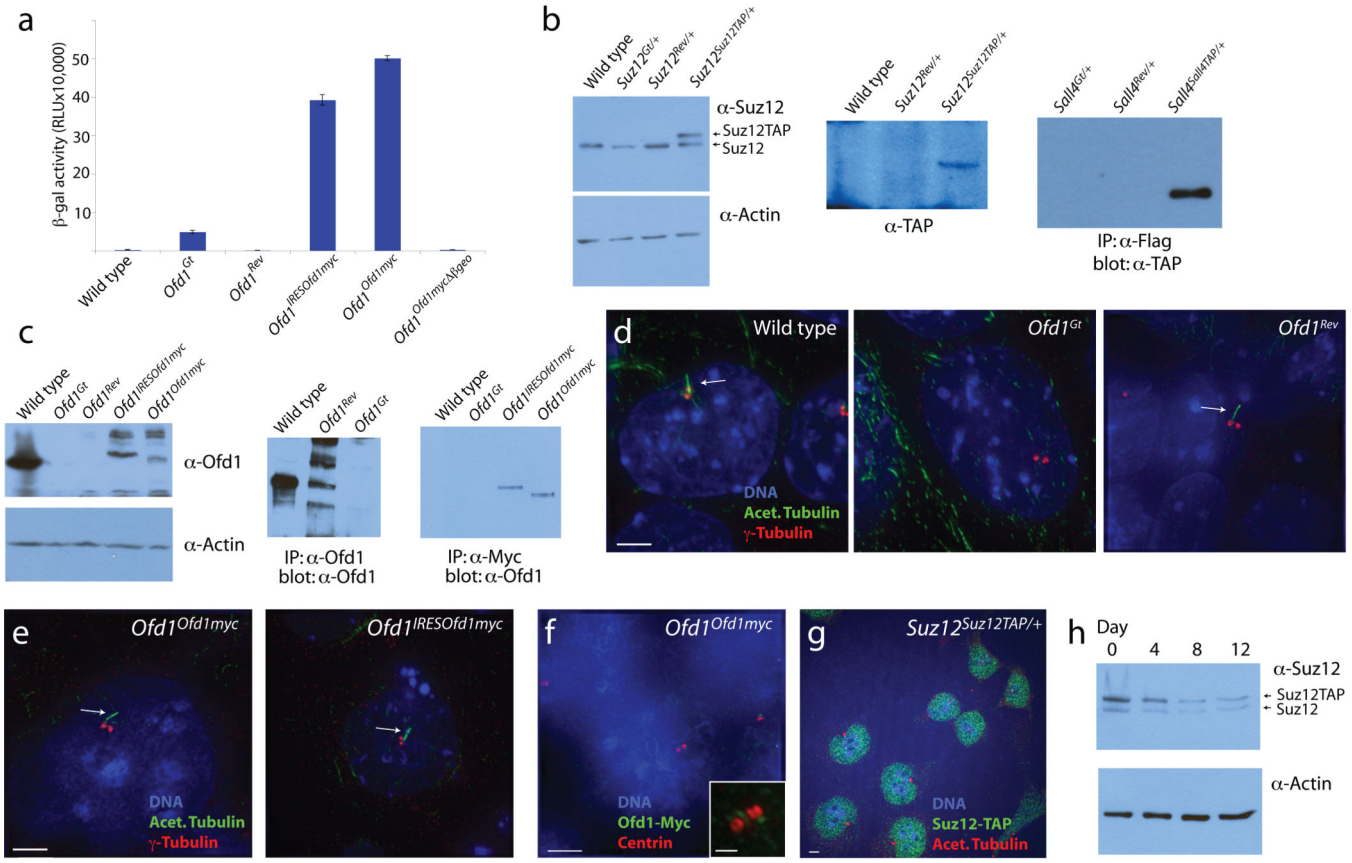

Figure 2. Efficient reversion of gene trap mutations and Floxin-mediated engineering of new alleles

(**a**) Plot of β-galactosidase activity in cell lines of the indicated genotypes. Error bars are standard deviations from 1–4 different experiments with a minimum of 3 replicates each. (**b**) Immunoblot showing Suz12 (left and middle panels, 20 μg protein per lane) and Sall4 (right panel, immunoprecipitated from 500 μg total protein for each cell line) protein levels in the cell lines of the indicated genotypes. (**c**) Immunoblots showing expression of full length Myc-tagged Ofd1 (left panel, 25 μg protein per lane; right panel, immunoprecipitate from 800 μg total protein for each cell line), and of Ofd1 in *Ofd1*^{Rev} cells (middle panel, immunoprecipitate from 4 mg total protein for each cell line). (**d, e**) Representative fluorescence micrographs of cell lines of the indicated genotypes. Cilia (acetylated Tubulin, green), centrosomes (γ-Tubulin, red), DNA (DAPI, blue). (**f**) Representative fluorescence micrograph of Ofd1-Myc localization. Ofd1 (Myc, green), centrosome (Centrin, red), DNA (DAPI, blue). (**g**) Representative fluorescence micrograph of Suz12-TAP localization. Suz12 (Flag, green), centrosome (Acetylated Tubulin, red), DNA (DAPI, blue). (**h**) Immunoblot showing wild type Suz12 and Suz12-TAP protein downregulation upon differentiation of *Suz12*$^{Suz12TAP/+}$ cells. 3 μg protein per lane. Scale bars 5 μm, with magnified inset scale bar 1 μm.

Table 1

Cell Line	% with correct reversion	Chromosome
Ofd1^{Rev}	62	X
Sall4$^{Rev/+}$	45	2
Suz12$^{Rev/+}$	74	11
Gli2$^{Rev/+}$	74	1
Tardbp$^{Rev/+}$	33	4
Sntb2$^{Rev/+}$	27	8
Pex14$^{Rev/+}$	24	4
Tet1$^{Rev/+}$	18	10
Average	**45**	

Table 2

Cell Line	% with correct Floxin
Ofd1^{IRESOf1myc}	92
Ofd1^{Of1myc}	73
Sall4$^{Sall4TAP/+}$	89
Suz12$^{Suz12TAP/+}$	91
Gli2$^{Gli2TAP/+}$	88
Tardbp$^{TardbpTAP/+}$	68
Sntb2$^{IRESeGFP/+}$	100
Pex14$^{IRESeGFP/+}$	89
Tet1$^{IRESeGFP/+}$	80
Average	**86**