Supplementary File	Title
Supplementary Figure 1	TSG experimental strategy.
Supplementary Figure 2	Identification of positive hybrid constructs by tissue culture assay.
Supplementary Figure 3	Additional examples of TSG.
Supplementary Figure 4	TSG strategy in genetic mosaic analysis.
Supplementary Table 1	Nucleotide sequences of hybrid partner DNA.
Supplementary Table 2	PCR primers for construction of hybrid cassettes and insertion of attB sites.
Supplementary Table 3	TSG fly stocks available.
Supplementary Table 4	Ratios of red/green twins to yellow clones in imaginal discs and brains.
Supplementary Table 5	Clone cell counts and doubling times.
Supplementary Note	**The Twin Spot Generator for differential *Drosophila* lineage analysis**

Online Methods

Generation of Hybrid Constructs

We constructed the different *GR* and *RG* hybrid cassettes from EGFP[8] and monomeric RFP[9] sequences purchased from Invitrogen. We generated chimaeric sequences (**Supplementary Table 1)** by PCR amplification with appropriate overlapping primers (**Supplementary Table 2**) of three DNA segments : a 5' sequence encoding the N-terminus of one fluoresent protein (EGFP or mRFP1); an identical αTub84B intron designed by Harrison and Perrimon[10] containing the yeast FRT site; and a 3' sequence encoding the C-terminus of the complementary fluorescent protein (mRFP or EGFP, respectively). We interrupted the coding sequences *in silico* by systematic insertion of the FRT-intron sequence at different positions until a theoretical splicing efficiency of greater than 93% was attained for the computer-generated splice junctions (http://www.fruitfly.org/seq_tools/splice.html).

We verified the construct sequences by standard sequencing reactions. We inserted the chimaeric *GR* and *RG* constructs, and a positive control construct, *GFP-intronFRT-GFP*, or *GG*, into the Gateway entry vector supplied in the pCR8/GW/TOPO TA Cloning Kit. We determined insert orientation by restriction enzyme digestion or sequence analysis of junction fragments. We transferred candidate hybrid and control sequences to a recipient Gateway Destination Vector AWM (Invitrogen) modified as below. See **Supplementary Table 2** for primer details.

Generation of the universal RMCE Destination Vector AWM-2attB

Because the TSG strategy requires the recombination sites to be allelic, we cloned *GR* and *RG* into a vector that would permit us to use the ϕC31 integrase for targeted genome transformation[11] coupled with Recombination-Mediated-Cassette-Exchange (RMCE)[12]. In RMCE, the ϕC31 integrase catalyzes the exchange of DNA flanked by inversely-oriented ϕC31 attB sites with that of genomic sequences flanked by inversely-oriented ϕC31 attP sites. We chose the Gateway Destination Vector, AWM, containing the Actin5C promoter and modified this *in vitro* cloning vector for use in *in vivo Drosophila* transformation by placing attB sites on either side of the selectable cloning cassette to create AWM-2attB, a universal RMCE Destination Vector.

We inserted inverted attB sites into the Gateway AWM vector in a 3-step process (see **Supplementary Fig. 1b**): First step, we added an MluI site to each extremity of the attB sequence during PCR amplification of the pCA4 vector with attB-specific primers (Microbix; see **Supplementary Table 2** for primer details). We digested with MluI the resulting PCR fragment and the AWM vector (unique MluI site at 5529) and joined the sequences by ligation. We determined the orientation of inserted attB sites by PCR analysis and restriction enzyme digestion with respect to an external unique PmeI site (position 5520 in AWM). Second step, we added BglI sites to primers at both ends: one, directly; and the second, as part of a 203-nucleotide sequence from the 3' end of the *ampicillin resistance* (*amp*^{R}) gene, since BglI digestion interrupted the *amp*^{R} gene at this point (position 6277). We digested this sequence and the vector from step 1 with BglI and ligated them. We selected for correct orientation of the second attB site by restored ampicillin resistance of the plasmid, and we subsequently verified this by PCR analysis and restriction enzyme digestion relative to an external unique DrdI site (position 7181 in AWM). Thus, the 203 nt-displaced 3' *amp*^{R} sequence is present twice in this vector, separated by the attB stretch. Third step: LR clonase reaction. The final integration vector AWM-2attB retains cloning capacity, accepting ORFs placed in Gateway entry clones, through standard single-site recombination at lambda attR/attL sites, replacing the Gateway cassette with the desired ORF (http://www.invitrogen.com/content/sfs/manuals/pcr8gwtopo_man.pdf).

Tissue culture assay

We tested the ability of the *GR* and *RG* inserts in AWM-2attB to recombine after their transfection into *Drosophila* S2R+ cells in culture. Cells were cultured in Schneider's insect medium (Invitrogen), 10% Fetal Bovine Serum (SAFC), and Penicillin-Streptomycin (Gibco). We set up a series of transfections using AWM-2attB plasmids containing the *GR* and *RG* reciprocal hybrid cassettes as well as the control plasmid *GG*. In addition, an *Actin5C-GAL4* driver plasmid and a *UAS-FLP* target plasmid were also co-transfected to constitutively produce active FLP. Transfection of plasmids was performed using Effectene reagents (Qiagen) as described at www.flyrnai.org. Results are shown in **Supplementary Figure 2**. In this assay we generated doubly-marked (GFP + RPF) *Drosophila* cells that glowed yellow demonstrating FLP-dependent mitotic exchange in S2R+ cells and providing preliminary evidence that *GR* and *RG* would function as predicted in TSG. Signals were apparent after 24 hours and increased in intensity over three-five days when the cells were imaged.
It should be noted that the hybrid constructs exist as plasmids in this assay with no possibility of extensive alignment as in homologous fly chromosomes; however, even if exchange is quite inefficient, since the fundamental change is at the DNA level, the fluorescent protein pool is amplified and regenerated in the cell by constitutive *Actin5C*-driven expression of the restored functional RNA.

Creation of transgenic lines

Target strains: The TSG protocol also called for *Drosophila* lines carrying genomic targets for RMCE. Here, we applied P-element transformation technology to *w* (white-eyed) flies in order to integrate target cassettes consisting of two inversely-oriented attP sites flanking the *miniwhite* gene[12], which codes for red eye color in the fly. We identified transformed flies by their colored eyes, and verified the presence of the attP sites by reverse PCR analyses. We screened lines derived from these flies for proximity of the target cassettes to the centromere. We chose this criterion because induction of MR close to centromeres maximizes the number of genes lying distal to the site of recombination and, therefore, the number of genes available for potential use in genetic mosaic analysis. One strain carrying a pUASTP2 target cassette at cytological position 82F7 was described previously[12]. We generated target cassette insertions at positions 38F2, 43F9, and 77C4 using *P[attP.w+.attP]*[14] via standard P-element-mediated germline transformation[15,16]. Together these four lines render about 80% of all autosomal genes potentially available for mosaic genetic analysis by TSG. Note that the pUASTP2 target cassette is juxtaposed to UAS and TATA sequences, while the *P[attP.w+.attP]* target cassettes have these sequences removed. All four strains carrying target cassettes are healthy as homozygous stocks. In order to facilitate further injections into these strains, we have introduced an X-chromosome carrying the ϕC31 integrase gene under the control of the *nanos* promotor into all four target strains[17].

TSG fly lines containing hybrid constructs: We used RMCE[12] to replace the target cassettes in the line carrying the insert at 82F7 with the *GR* and *RG* sequences, by co-injecting embryos with mRNA encoding the ϕC31 integrase and an AWM-2attB plasmid carrying either *GR* or *RG*. Successful insertions were tracked by loss of the red eye marker (*w+)* carried by the outgoing target cassettes, and we isolated putative TSG-competent flies through a screen for white-eyed flies in the F1 generation, after crosses to *y w* flies with the appropriate balancers. We balanced candidate TSG chromosomes over autosomal balancer chromosomes *CyO* and *TM3* and then made them homozygous. We confirmed the presence and orientation of the hybrid GR and *RG* constructs in each candidate strain by reverse PCR and sequence analysis. All white-eyed flies that were tested indeed carried an integrated hybrid construct (18/18 white-eyed F1 classes). We made the chromosome carrying the hybrid construct homozygous in subsequent standard genetic manipulations, and an X-chromosome was crossed in carrying

the *hs-FLP* gene[18]. One to three independent TSG lines were isolated for each *GR* and *RG* hybrid construct insertion.
See **Supplementary Table 3** for list of strains available.

Imaging
We dissected tissues in PBS and fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature. We washed and permeablized them in PBST (0.1% Triton X-100) for 10 minutes (3X). For **Fig. 2a-c**, with no antibody staining, we mounted larvae in Vectashield (Vector Laboratories, H-1000) ; for **Fig. 2d** with antibody staining, we incubated L2 larvae in a cocktail of primary antibodies diluted in 0.3% PBST overnight at room temperature. Primary antibodies: sheep anti-GFP (1/1000, Biogenesis), rabbit anti-DsRed (1/1000, Clontech), and rat anti-DECad (1:25, DSHB). Brains were washed (5 min, PBS, 3x) and incubated in secondary antibodies diluted in 0.3% PBST for 3 hr. Secondary antibodies (Molecular Probes): donkey anti-sheep Alexa488 (1/1000), donkey anti-rabbit Alexa555 (1/1000), goat anti-rat Alexa647 (1/200). After washing overnight, brains were mounted in Vectashield. For **Fig. 3a**, L3 larval imaginal discs were stained for two hours at RT with mouse anti-GFP (1:500, Invitrogen) and rabbit anti-DsRed (1:500, Clontech), followed by 4ºC overnight staining with secondary antibodies, goat anti-mouse Alexa488 and goat anti-rabbit Alexa568 (1:200 each, Molecular Probes) followed by DAPI (1 μg/ml in PBS) for five minutes. For **Fig. 2e-i and Fig. 3b** sheep anti-GFP (1/1000, Biogenesis), rabbit anti-DsRed (1/500, Clontech), and mouse anti-histone (Chemicon, 1/1000) were followed with secondaries, donkey anti-sheep Alexa488, goat anti-rabbit Alexa568, and goat anti-mouse Alexa647 (1/200 each, Molecular Probes). After washing, discs For **Fig. 2e-i and Fig. 3** were mounted in fluoromount-G (Southern Biotech). For Fig. **2a,b** and **d**, images were collected on a LeicaTCS SP2 AOBS confocal microscope system and processed with Leica confocal software imported into Adobe Photoshop7.0; for **Fig. 2c**, Nikon C1 confocal, Metamorph for imaging; for **Fig. 2e-i**, and **3**, BIO-RAD Radiance2000 confocal, Photoshop7.0 for imaging.

References

14. Bateman, J. R., & Wu, C. T. *Genetics* **180**, 1763-1766 (2008).
15. Rubin, G. M., & Spradling, A. C. *Science* **218**, 348-353 (1982).
16. Spradling, A. C., & Rubin, G. M. *Science* **218**, 341-347 (1982).
17. Bischof, J., Maeda, R. K., Hediger, M., Karch, F., & Basler, K. *Proc. Natl. Acad. Sci. USA* **104**, 3312-3317 (2007).
18. Chou, T. B., & Perrimon, N. *Genetics* **144**, 1673-1679 (1996).

AOP
This technique, adapted from Mosaic Analysis with Double Markers in mice, relies on mitotic recombination to reconstitute sequences encoding EGFP or mRFP. After cell division each daughter cell contains one fluorescent marker, causing a green and a red twin spot that can be traced through development.

Issue
This technique, adapted from Mosaic Analysis with Double Markers in mice, relies on mitotic recombination to reconstitute sequences encoding EGFP or mRFP. After cell division each daughter cell contains one fluorescent marker, causing a green and a red twin spot that can be traced through development.