Vector and parameters for targeted transgenic RNA interference in *Drosophila melanogaster*

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Supplementary text and figures:

SI Table 1: Positive gene set used to test the efficacy of Valium. Information on the hairpin lines generated against Epidermal Growth Factor Receptor (EGFR), Son of sevenless (Sos); sprouty (sty), decapentaplegic (dpp), cubitus interruptus (ci), armadillo (arm), domeless (dome), hopscotch (hop), Stat92, disc-large (dlg), and RacGap50C (RacGap). Two independent constructs were generated for most genes.

TABLE 1

Line	CG#/gene	Oligos	Size of fragment	Phenotype with en-Gal4 at 29°C
TR00039A.1	CG10079/EGFR	F: 5'-GTCTAGACTATGCAAGTTGCGCATTGT-3'; R: 5'-AGAATTCGGAGTCTGCACCAGGACATT -3'	443bp	growth and vein defects
TR00040A.1	CG10079/EGFR	F: 5'-GTCTAGAACAAGAGCAGGGATCGCTAA -3'; R: 5'-AGAATTCGGCAGTTATCTTGCTGCTCC-3'	424bp	growth and vein defects
TR00380A.1	CG7793/Sos	F: 5'-GTCTAGAGAAAACGAATCTGGAGCGAG-3'; R: 5'- AGAATTCCAACACGATATTGTCACCGC-3'	413bp	growth and vein defects
TR00606A.1	CG7793/Sos	F: 5'- GTCTAGACTGGATCTGGAACTCTCGCT-3'; R: 5'- AGAATTCTACTGGCAGTGCTATGCGTT-3'	435bp	growth and vein defects
TR00024A.1	CG1921/sty	F: 5'-GTCTAGAGTCTGAACCAGCCCATCATC-3'; R: 5'-AGAATTCATGGGCCAGTAGAACCACAG-3'	463bp	vein defects
TR00025A.1	CG1921/sty	F: 5'-GTCTAGAGCAGCTGTCTGAATCTGCAA-3'; R: 5'-AGAATTCAAGGTCAGGTGGTGGATCTG-3'	480bp	vein defects
TR00047A.1	CG9885/dpp	F: 5'-GTCTAGACCAGCACAGCATTAGCAAAA -3'; R: 5'-AGAATTCCCTCCTTGCTGTAGGTGGA-3'	441bp	veins missing
TR00048A.1	CG9885/dpp	F: 5'-GTCTAGAAACAATATGAATCCCGGCAA -3'; R: 5'-AGAATTCGGACTCTGCGCTCTCAAATC-3'	470bp	veins missing
TR00709A.1	CG2125/ci	F: 5'-GTCTAGAGAGCTCTTTGGGTGAACTGC-3'; R: 5'-AGAATTCTGTTGTGGTGTCATCGGATT-3'	402bp	crossvein missing
TR00710A.1	CG2125/ci	F: 5'-GTCTAGAAGCAGCCTTCATCGACATCT-3'; R: 5'-AGAATTCGTGTGGCTTTTCACCGGTAT-3'	446bp	crossvein missing
TR00681A.1	CG11579/arm	F: 5'-GTCTAGAGAAAGTGCTCTCCGTTTGCT -3'; R: 5'-AGAATTCCGTGATGGTGGATGCAATAG-3'	544bp	margin defects
TR00706A.1	CG14226/dome	F: 5'-GTCTAGACATCACTTCACCACGTCACC -3'; R: 5'-AGAATTCCTTACGCGGAATGTATCGGT-3'	566bp	extra vein
TR00703A.1	CG1594/hop	F: 5'-GTCTAGAAAAGTTGGCGCTTGCTAAAA -3'; R: 5'-AGAATTCGTTGAACACACGGATTGTGC-3'	507bp	extra vein
TR00704A.1	CG1594/hop	F: 5'-GTCTAGACGACGATGGCATGATGTTTA -3'; R: 5'- ACAATTGATAGCCGGGATCGCTAATTT-3'	442bp	extra vein
TR00701A.1	CG4257/Stat92	F: 5'-GTCTAGAAAGCTGCTTGCCCAAAACTA -3'; R: 5'-AGAATTCGTCGACGATAAAGGCAGAGC-3'	402bp	extra vein
TR00702A.1	CG4257/Stat92	F: 5'-GTCTAGATACGCGCAATACACAGATGG -3'; R: 5'-AGAATTCATCAATGGTCAGAGAACGCC-3'	442bp	extra vein
TR00031A.1	CG1730/dlg	F: 5'-GTCTAGAGAATGGCGATGATAGCTGGT -3'; R: 5'-AGAATTCATTGAGAAGCCCAGTCCCTT-3'	414bp	crumbled wings
TR00051A.1	CG13345/RacGap	F: 5'-GTCTAGATTGGCCTCTATCGATTGTCC -3'; R: 5'-AGAATTCCTGGGTGAAAACTTCCGTGT-3'	428bp	vein defects, crumbled wings

S2. Materials and Methods

Construction of Valium. The SV40 polyadenylation signal sequence of the *Drosophila* transformation vector pUAST (Brand and Perrimon, 1993) was amplified using the specific primers SV40-SacII (5'-GCCGCGGGATCTTTGTGAAGGAACCTTAC-3') and SV40-SacI (5'-GGAGCTCTGGAACCAGACATGATAAGATAC-3'), sequenced and subcloned into pBluescript (Stratagene). The *ftz* intron (Read and Manley, 1992; Rio, 1988) was amplified using genomic DNA as the template and the specific primers ftz-XbaI-MfeI (5'-CTCTAGACAATTGTTGGCATCAGGTAGGCATCA-3') and ftz-SacII (5'-ACCGCGGCTCTAGTTCTTTGCAATCTGTA-3'). After sequencing, the correct fragment was subcloned into SV40-pBluescript. Loxp1 and Loxp2 oligonucleotides (Loxp1-HindIII: 5'- AGCTTATAACTTCGTATAATGTATGCTATACGAAGTTATCTGCA-3'; Loxp1-PstI: 5'- GATAACTTCGTATAGCATACATTATACGAAGTTATA -3') (Loxp2-NcoI: 5'- GACCATGGATAACTTCGTATAATGTATGCTATACGAAGTTATG-3'; Loxp2-BamH1: 5'- GATCCATAACTTCGTATAGCATACATTATACGAAGTTATCCATGGTCTGCA -3') were denatured at 95°C and after annealing were subcloned into ftz-SV40-pBluescript. One 5XUAS cassette of pUAST was amplified by the specific primers (UAS-PstI (5'-TCTGCAGGCAGGTCGGAGTACTGTCC-3'); UAS-NcoI (5'-TCCATGGCTCCGCTCGGAGGACAGTA-3'), and another 5XUAS cassette was amplified by primers UAS-BamHI (5'-TGGATCCGCAGGTCGGAGTACTGTCCT-3') and UAS-SalI (5'-AGTCGACCTCGCTCGGAGGACAGTA-3'). Both were confirmed by sequencing and then subcloned individually into Loxp1-Loxp2-ftz-SV40-pBluescript. The HSP70 promoter was amplified from pUAST using the specific primers HSP70-SalI (5'-GGTCGACAGCGAGCGCCGGAGTATAAAT-3') and HSP70-EcoRI-BglII-XbaI (5'-GTCTAGAGCAGATCTGCGAATTCCCAATTCCCTATTC-3'). The correct PCR product was subcloned into Loxp1-5XUAS-Loxp2-5XUAS-ftz-SV40-pBluescript. Two DNA fragments, one containing the Hind III, EcoRV, ClaI, XhoI and KpnI restriction sites, and another containing the EcoRI, NotI, SpeI, NheI, XbaI, NdeI, BglII and MfeI sites were cloned into Loxp1-5XUAS-Loxp2-5XUAS-HSP70-ftz-SV40-pBluescript. The attB fragment in pCa4 (Microbix Biosystems) was amplified using attB-Xho (5'-CTCGAGGCTGCATCCAACGCGTTGG-3') and attB-KpnI (5'-GGTACCGAATTAGGCCTTCTAGTGGAT-3'). Subsequently, the correct PCR product was introduced into Loxp1-5XUAS-Loxp2-5XUAS-HSP70-ftz-SV40-pBluescript. The 1.8kb DNA fragment which contains both the regulatory and coding regions of *vermilion* was cut by HindIII from pYC1.8 (kindly provided by Lillie Searles), and then subcloned into AttB-Loxp1-5XUAS-Loxp2-5XUAS-HSP70-ftz-SV40-pBluescript. A small *white* intron from pWIZ (Lee and Carthew, 2003) was cut by NheI/SpeI and then subcloned into the restriction sites of AttB-Loxp1-5XUAS-Loxp2-5XUAS-HSP70-ftz-SV40-pBluescript. Finally, the EcoRI/MfeI restriction sites in *vermilion* and the SV40 polyadenylation tail were destroyed by specific mutations using the QuickChange site-directed mutagenesis kit (Stratagene). The mutant plasmid was generated with two pairs of mutagenesis primers (EcoRI-F: 5'-CTCCGAGTTGCGAATCGAGTTCCGCGCCTCCTCGTC-3'; EcoRI-R: 5'-GACGAGGAGGCGCGGAACTCGATTCGCAACTCGGAG-3'; MfeI-F1: 5'-GCATAGCCAAACATTGACGAATTGGATACCCTGCCGATTG-3'; MfeI1-R: 5'-CAATCGGCAGGGTATCCAATTCGTCAATGTTTGGCTATGC-3').

Generation of 5XUAS hairpin derivative. The pentamer of UAS was flanked by two minimal *loxP* sites which each contain an 8bp asymmetric core sequence and two 13bp inverted repeats. By crossing a 10XUAS line with a line containing the Cre enzyme (Oberstein et al., 2005), one pentamer of UAS can be easily removed. Specifically, virgin females of genotype *y w, P[y+Crey]1b; D/TM3, Sb* were crossed to males of genotype *y v/Y; 10XUAS hairpin/10X-UAS hairpin*. In the F1 generation, males of genotype *y w, P[Crey]1B/Y; 10XUAS-hairpin/TM3*, Sb were isolated and crossed en masse to virgin females of genotype *y v; Sb/TM3, Ser*. Among the resulting progeny, males of genotype *y v/Y; putative 5XUAS-hairpin/TM3, Ser* were isolated and mated individually to virgin females of genotype *y v; Sb/TM3, Ser*. Sib progeny of genotype *y v; putative 5XUAS hairpin/TM3, Ser* from this cross were mated inter se to establish homozygous stocks of genotype *y v; 5XUAS hairpin/5XUAS hairpin*. To verify the loss of one of the 5XUAS cassettes from individual lines, genomic DNA from single males was amplified by PCR using primers UAS-F (5'-AGAAGGCCTAATTCGGTACC-3') and UAS-R (5'-GCGCCTCTATTTATACTCCG-3'). Using these primers, the presence of both 5XUAS cassettes will generate a band of ~400bp, while the presence of only one of the cassettes will generate a band of ~200bp.

Hairpin design and construction. Primers for hairpins were designed using the DRSC amplicon design tool that we refer to as SnapDragon (http://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl <http://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl>). To construct the hairpins, we first identified regions of 400-600bp that are free of EcoR1 (or MfeI) and XbaI (or SpeI, AvrII) and that are free of 19bp predicted off targets. If this was not possible, then regions with either no 20 or 21bp matches to other genes were used. Blast analyses were performed on genomic and cDNA sequences. In most cases the hairpin sequences were selected to be within a single exon. However, when this was not possible, we used post-spliced transcript sequence for the design and ensured that splice sites AG/GTRAGT, TTTYYYYTNCAG/RT (R for A or G; Y for C or T; and N for any base) were not present. We then added XbaI (or SpeI, AvrII) cutting sites to the 5' end of the forward primer and EcoRI (or MfeI) to the 5' end of the reverse primer. PCR products were cut by EcoRI (or MfeI) and XbaI (or SpeI, AvrII). Following gel purification, the DNA fragment was sequentially subcloned twice into XbaI, MfeI, EcoRI and SpeI sites of Valium. Since digests with either EcoR1 and Mfe1, or Spe1, AvrII and Xba1 result in compatible cohesive ends, a single PCR product can be cloned in both orientations in the Valium multiple cloning site (MCS) that contains EcoR1, Spe1, AvrII, Xba1 and Mfe1 restriction sites.

Establishment of transgenic RNAi hairpin lines. Lines were established either in our lab or at Genetic Services, Inc (GSI, http://www.geneticservices.com). In the Perrimon lab, construct DNA and capped integrase mRNA were co-injected into embryos of genotype *y v; attP2/attP2*. We used the attP2 site since it been shown to be optimal for the induction of UAS-constructs with several Gal4 drivers (Markstein et al., submitted). The capped integrase mRNA was prepared as previously described (Groth et al., 2004). Briefly, the pET11□C31-polyA plasmid was cut by BamH1, digested by proteinase K, then extracted with phenol-chloroform, precipitated, and finally resuspended in RNAse-

free water. 1 ug of linear DNA was used to transcribe the integrase RNA using the mMESSAGE mMACHINE T7 transcription system (Ambion). The resulting mRNA was resuspended in RNAase-free injection buffer and then mixed with the hairpin-construct before injection. Surviving Go individuals were individually crossed to *y v; Sb/TM3, Ser* and *y+ v+* progeny from these crosses were individually back-crossed to *y v; Sb/TM3, Ser* to establish homozygous stocks. About 25% transformants were usually recovered using this approach.

At GSI, construct DNA was injected into a *y w, nanos integrase; attP2/attP2* stock. Surviving Go males were then individually crossed to *y v; attP2/attP2* virgin females, and *y+v+* males in the progeny were selected and individually back-crossed to *y v; Sb/TM3, Ser* virgin females to establish homozygous stocks.

Due to the presence of background lethal(s) on some of the attP2 chromosomes about 5% of the transformed lines cannot be homozygosed. These lines were discarded and the DNA reinjected. Once homozygous lines were established, flies were checked by PCR for the presence of the hairpin constructs. The presence of the two inverted DNA repeats in transgenic flies was confirmed by two PCR reactions using the following oligos: HSP-forward: 5'-CGCAGCTGAACAAGCTAAAC -3'; *gene X*-forward; and *ftz*-reverse: 5'-TAATCGTGTGTGATGCCTACC -3'.

***Notch* hairpins.** We used two different hairpin constructs in this study. For *Notch hairpin 1*, a 402bp fragment was amplified using: F: 5'-GTCTAGAATTGTCCCAGTGGCTTTACG-3'; R: 5'-AGAATTCCGCAATTCTGACCCTGAAAT-3'. For *Notch hairpin 2*, a 406bp fragment was amplified with F: 5'-GTCTAGAATCGGATCTATTGGCACAGG-3'; R: 5'-AGAATTCGTTCACCGCTTCGGTATTGT-3'.

Stocks. The following stocks were used: *w; C96-Gal4* (homozygous viable on the 3rd chromosome (Gustafson and Boulianne, 1996); *w; en-Gal4 UAS-GFP* (homozygous viable on the 2nd chromosome, from our lab); *y w nanos-Integrase phiC31* was obtained from F. Karch (Bischof et al., 2007) and *w; UAS-Dcr2* (homozygous on the 2nd) from B. Dickson (Dietzl et al., 2007).

Wing preparation and scoring. Flies were dehydrated in 100% EtOH overnight and then mounted in Hoyer's medium with lactic acid and photographed with a Zeiss Axiophot. At least 50 individual wings were scored in each experiment.

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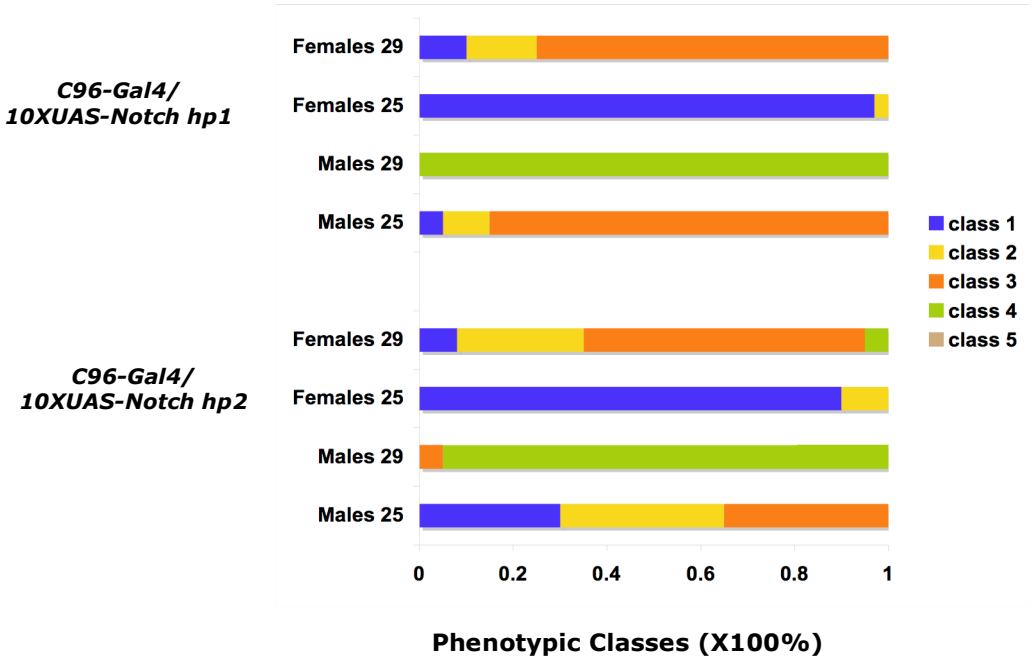
S3. Supplementary Figure 1:
Two different *Notch* hairpin constructs behave similarly. The wing phenotypes *C96-Gal4/+; 10XUAS-Notch-hp1/+* and *C96-Gal4/+; 10XUAS-Notch-hp2/+* were scored at different temperatures.

Supplementary FIGURE 1



Supplementary Note

The various genotypes are:

- *C96-Gal4, 5XUAS-Notch hp2: C96-Gal4/+; 5XUAS-Notch-hp2/+*
- *C96-Gal4, 10XUAS-Notch hp2: C96-Gal4/+; 10XUAS-Notch-hp2/+*
- *C96-Gal4, (10XUAS-Notch hp2)X2: C96-Gal4/+; 10XUAS-Notch-hp2/10XUAS-Notch-hp2*
- *(C96-Gal4)X2, 10XUAS-Notch hp2: C96-Gal4/ C96-Gal4, 10XUAS-Notch hp2/+*
- *(C96-Gal4)X2, (10XUAS-Notch hp2)X2: C96-Gal4/ C96-Gal4, 10XUAS-Notch hp2/10XUAS-Notch hp2*
- *C96-Gal4, 10XUAS-Notch hp2, 10XUAS-GFP: C96-Gal4/+; 10XUAS-Notch-hp2/10XUAS-GFP*
- *C96-Gal4, UAS-Dcr2, 10XUAS-Notch hp2: C96-Gal4/UAS-GFP; 10XUAS-Notch-hp2/+*