10

RNAi in the Hedgehog Signaling Pathway: pFRiPE, a Vector for Temporally and Spatially Controlled RNAi in *Drosophila*

Eric Marois and Suzanne Eaton

Abstract

RNA interference (RNAi) has become an irreplaceable tool for reverse genetics in plants and animals. The universality and specificity of this phenomenon allows silencing of virtually any chosen gene to examine its involvement in biological processes. Many strategies exist to reduce the expression of a particular gene using RNAi. Some rely on delivering directly to cells the ~21-nucleotide long interfering double-stranded RNA (dsRNA) species that are central mediators of the silencing process. Others rely on the transgenic expression of longer dsRNA molecules, leaving it to the cellular machinery to process these hairpins into short active dsRNA.

In this chapter, we describe a transgenic method to deplete a chosen protein from a specific *Drosophila* tissue following induction of long dsRNA. It was used to uncover the role of lipidic particles in Hedgehog signaling by silencing lipophorin in the fat body ***(1)***, and we routinely use it to deplete specific proteins from wing imaginal disc subdomains ***(2)***. The method, certainly not restricted to the study of Hedgehog signaling, allows fast and efficient construction of a plasmid incorporating various *Drosophila* genetic tools to allow heat-shock-induced expression of dsRNA at the desired time and in the desired tissue. For protocols involving injection of in vitro synthesized dsRNA in embryos to study Hedgehog signaling, see for example ***(3)***. For genomic screens to identify Hedgehog pathway components in tissue culture cells by transfection of small interfering RNAs, *see* refs. ***(4,5)***.

Key Words: Inducible RNAi; tissue-specific RNAi; *Drosophila* wing imaginal disc; Gal4/UAS; FLP/FRT; pFRiPE.

1. Introduction

In 2000–2002, multiple reports indicated that it is possible to deplete a protein of choice from a tissue of choice by RNA interference (RNAi) in flies ***(6–14)***. All methods relied on the Gal4/UAS system ***(15,16)*** to tissue-specifically express

From: *Methods in Molecular Biology: Hedgehog Signaling Protocols*
Edited by: J. Horabin © Humana Press Inc., Totowa, NJ

long double-stranded RNA (dsRNA), usually from vectors expressing a gene fragment cloned in an inverted repeat configuration. Endogenous Dicer-2 is expected to cleave the resulting dsRNA into short interfering RNAs, which are packaged into the RISC complex that targets endogenous homologous mRNAs for degradation (for review on the RNAi process, *see* **ref.** ***[17]***). Unlike in *Caenorhabditis elegans*, RNAi does not spread systemically in *Drosophila*, which allows the useful possibility to compare cells subjected to RNAi with normal cells in neighboring tissue or to knock down gene function in one organ only. Following these initial successful reports, many laboratories made inverted repeat constructs to knock down their proteins of interest. It quickly became notorious that cloning inverted repeats of a gene fragment in a head-to-head configuration is difficult, even if using recombination-deficient *E. coli* strains, such as Stratagene's SURE2 competent cells that can stabilize recalcitrant DNA structures. In our and other's experience, initial *E. coli* transformant colonies are easily obtained but usually loose the inverted repeat during subsequent growth. In the minority of cases in which *E. coli* retained the repeats, transgenic flies generated from such constructs did display efficient RNAi and were genetically stable over many generations (***[18]***; E.M. and S.E., unpublished). To circumvent the repeat instability problem in *E. coli*, a spacer sequence, for example an intron, can be inserted between the repeats to suppress their loss ***(18)***. Some introns were even reported to improve the efficiency of particular RNAi constructs ***(11,19)***. In our experience, introns will allow RNAi when placed between certain repeats but will suppress RNAi if placed between others. In several independent cases, a given inverted repeat was more efficient at triggering RNAi when devoid of spacer than when cloned in an intron-containing vector (Marois and Eaton, unpublished). This may be due to improper intron splicing in certain contexts. We speculate that unspliced introns may participate in secondary structures with the first synthesized repeat, inhibiting efficient pairing of the second repeat to the first. After testing four different spacer introns with variable success depending on the gene targeted, we wanted to devise a vector strategy offering both spacer-mediated repeat stabilization and the more reproducible RNAi efficacy of inverted repeats without an intervening spacer.

pFRiPE, the RNAi vector we present here, contains a spacer that is excisable in *Drosophila* at the genomic level, using a recombination reaction based on the flip recombinase (FLP)/flipase recognition target sequence (FRT) system ***(20)***. While solving the problems associated with cloning head-to-head repeats, this allows the in vivo production of dsRNA without spacer. Cloning of the inverted repeats is facilitated by the use of the Gateway technology (Invitrogen Carlsbad, CA) which inserts two inverted repeats in a single in vitro recombination step ***(21)***. Furthermore, excision of the spacer by FLP recombinase (flipase) can

be controlled by heat shock, providing temporal control of dsRNA expression. Expression from pFRiPE (a pUAST derivative) is regulated by the GAL4/UAS system, conferring the advantages of RNAi induction in a spatially restricted fashion.

RNAi-mediated gene knockdown using pFRiPE provides several advantages when compared with generating null mutant clones. Firstly, gene knockdown can be induced within 48 h in large fields of cells without a requirement for cell division. In contrast, mutant cells may fail to divide, be eliminated or over the longer term produce compensation mechanisms that can blur result interpretation. Secondly, Gal4 control of RNAi allows reproducible targeting of an entire tissue compartment (for example, all Hedgehog-secreting cells), whereas cell clones generate largely unpredictable mosaics.

The organization of pFRiPE is outlined in **Fig. 1**. Inverted repeats are inserted by Gateway cassette replacement on either side of an excision cassette flanked by FRT recombination sites (**Fig. 1A**). Before excision, Gal4 transcribes an *HcRed* gene present within the excision cassette. The *HcRed* sequence is followed by a long transcription terminator to prevent premature RNAi activation (*see* **Note 1**). Upon spacer excision, an RNA inverted repeat is produced, of which the center of symmetry is a single remaining FRT site (**Fig. 1B**). Because the FRT site itself is an almost perfect palindrome, only three nucleotide mismatches are found at the center of symmetry (**Fig. 1C**).

In **Section 2.**, we will outline (i) the design of the gene fragment that will trigger RNAi, initially to be cloned in a Gateway entry vector; (ii) the preparation of recombination-ready pFRiPE vector; (iii) the recombination reaction that generates the desired construct by mixing the entry clone with pFRiPE; (iv) the selection of appropriate constructs for transgenic fly generation; and (v) some aspects of using pFRiPE transgenic flies.

2. Materials

1. Petri dishes containing LB medium with: kanamycin 30–50 μg/mL; ampicillin 100 μg/mL; ampicillin 100 μg/mL + chloramphenicol 40 μg/mL.
2. DB3.1 competent cells.
3. DH5α competent cells.
4. pENTR plasmid (Invitrogen; for example, pENTR1A, pENTR2B, or pENTR3C).
5. Restriction enzymes: *Bam*HI (or *Acc*65I/*Kpn*I), *Xho*I (or *Eco*RV, or *Not*I), *Eco*RI (or *Stu*I or *Kas*I), *Nhe*I (or *Aat*II).
6. Gateway LR enzyme mix (Invitrogen).
7. DNA purification columns (Qiagen Hilden, Germany).
8. TE: 10 m*M* Tris–HCl (pH 8) and 10 m*M* EDTA.
9. Oligonucleotides:
   - EM15 GCAGGCTCTTTAAAGGAACCAA
   - EM16 GCTGGGTCTAGATATCTCGAG

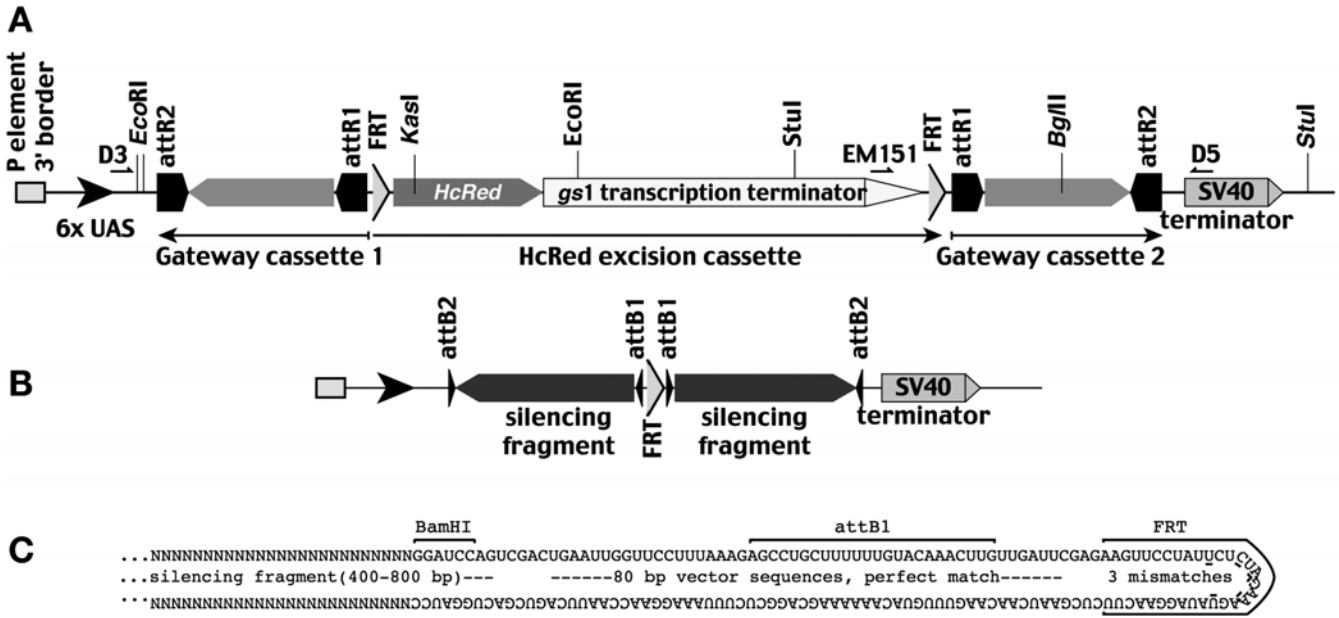

Fig. 1. Scheme of pFRiPE (scale is approximate). (**A**) pFRiPE before insertion of a silencing fragment. The new DNA sequences inserted into pUAST to construct pFRiPE are shown. Gateway cassettes 1 and 2 (flanked by attR1 and attR2 recombination sites) are in inverted orientation with respect to one another, resulting in corresponding orientation of the silencing fragment after the recombination reaction. *Bgl*II restriction site in Gateway cassette 2 serves for vector linearization prior to recombination. Tandem FRT sites provide flipase-mediated excisibility of the HcRed gene and the accompanying transcription terminator. D3, D5, and EM151 primer binding sites are shown. (**B**) RNAi construct generated from pFRiPE after in vitro LR recombination (which replaced the Gateway cassettes with silencing fragment) and after in vivo flipase-mediated HcRed cassette excision. A single remaining FRT site separates the repeats. (**C**) Nucleotide sequence of the center of symmetry of the inverted RNA repeat expressed from construct shown in **B**. pFRiPE sequence can be found at GenBank (Accession number DQ305103).

- D5 GGTAGTTTGTCCAATTATGTCACACC
- D3 CAACTGCAACTACTGAAATCTGCC
- EM151 GACAAGCGGCAATAAACGGGTA

3. Methods

3.1. Design of the Gene Fragment to Clone into pENTR

1. Run the entire mRNA sequence of the gene to be silenced in a program screening the sequence for potential genes that could be cross-silenced. BLAST is not sufficient, because every possible 21 nucleotide sequence from the target gene should be checked for potential targeting of other genes. We find the online program Deqor ***(22)*** (http://deqor.mpi-cbg.de/Deqor/deqor.html) very useful for this purpose (*see* **Note 2**). Upon running Deqor, gene regions devoid of 21 nucleotide stretches homologous to potential off-target genes can be selected. In addition, select a gene region with the following characteristics.
   – Size between 400 and 800 bp long (other sizes may function but were not tested);
   – Absence of either *Nhe*I or *Aat*II restriction sites (one of these enzymes will be used to linearize the pENTR plasmid prior to the Gateway recombination reaction, *see* **Note 3**);
   – Absence of the restriction sites incorporated in the oligonucleotides used to amplify this fragment for subsequent cloning (preferably *Xho*I and *Bam*HI, *see* **step 2** in this subsection);
   – Selected in any region of the mRNA (5′UTR, 3′UTR or coding sequence, depending on available possible controls, *see* **Section 3.5.**). If chosen to contain part of the coding sequence, care should be taken to avoid methionine-coding triplets in the fragment (*see* **Note 4**). If that is impossible, the fragment should be cloned as an antisense first repeat (*see* **Note 5**), i.e., as a *Bam*HI/*Xho*I fragment.
2. Design primers with *Xho*I and *Bam*HI sites at the extremities to amplify the chosen region. Add three extra nucleotides 5′ to the restriction site to ensure efficient restriction digestion of the PCR product. In case no appropriate gene region can be found devoid of *Xho*I and/or *Bam*HI sites, *Eco*RV or *Not*I can be used instead of *Xho*I; *Acc*65I (*Kpn*I) or *Sal*I instead of *Bam*HI. If the construct is desired "sense first", the PCR primers should be designed to amplify an *Xho*I/*Bam*HI fragment, i.e., 5′ primer contains the *Xho*I and 3′ primer contains the *Bam*HI site. If the construct is to be "antisense first", 5′ primer contains the *Bam*HI and 3′ primer contains the *Xho*I site.
3. PCR amplify the gene fragment from total cDNA (or from an individual cDNA clone if one is available) and clone the resulting PCR product into a pENTR plasmid using the chosen restriction sites. Cloning PCR products into pENTR1A, 2B or 3C is very efficient as long as the 3:1 insert:vector molar ratio is approximately respected. pENTR plasmids lose the *ccdB* gene upon successful ligation (*ccdB* is toxic to DH5α *E. coli*, but not to DB3.1, XL1-Blue or other F plasmid-containing *E. coli* strains) which ensures that virtually 100% of the obtained colonies are positive when transforming DH5α.

4. Sequence a few pENTR plasmids (or PCR products amplified from single pENTR *E. coli* colonies) to select one that does not contain PCR mutations. For both colony PCR screening and sequencing, use primers EM15 and EM16 (*see* **Section 2.**).
5. Miniprep the chosen pENTR plasmid using a standard alkaline lysis protocol (resuspend DNA in 50 µL TE).

3.2. Preparation of pFRiPE

1. Upon receipt of pFRiPE plasmid DNA, transform an aliquot into *E. coli* strain DB3.1 (other strains are not appropriate). Select on ampicillin 100 µg/mL + chloramphenicol 40 µg/mL. Chloramphenicol selection ensures that the two inverted Gateway cassettes each containing a chloramphenicol-resistance gene will not be lost by the bacteria. Chloramphenicol resistance will be lost in positive clones after the Gateway recombination reaction.
2. Purify pFRiPE by plasmid miniprep (resuspend in 50 µL TE buffer). It is important to apply chloramphenicol selection during *E. coli* growth.
3. Linearize 20 µL of the pFRiPE miniprep with *Bgl*II in a total volume of 100 µL. This step is necessary for the recombination reaction to occur efficiently.
4. Purify the linear DNA (for example with a Qiagen spin column). Measure DNA concentration and check the plasmid on an agarose gel (should be a band of 15,857 bp).

3.3. LR Reaction and Selection of Positive Final RNAi Constructs

1. Digest 6 µL of the pENTR construct miniprep with either *Nhe*I or *Aat*II (should not cut inside the silencing fragment) in a total volume of 20 µL. Heat inactivate the enzyme. Run 15 µL of the digest on an agarose gel to estimate DNA concentration and to check that the enzyme was active: a single linear band should be visible around 3 kb. If two bands are seen, an *Nhe*I or *Aat*II site was present in the gene fragment: the converse enzyme must be used. Save the remainder of the reaction for the next step.
2. Perform LR reaction in a total volume of 5 µL (1 µL Invitrogen LR enzyme mix, 1 µL LR buffer, up to 3 µL DNA) with about 75 ng *Bgl*II-linear pFRiPE and 45 ng *Nhe*I-linear ENTR plasmid (this approximates a 1:3 pFRiPE: pENTR molar ratio). Overnight incubation at room temperature yields best results. Caution: the reaction will fail if a large excess of ENTR plasmid is used.
   During this step, the attL1/2 recombination sites flanking the gene fragment in pENTR will directionally recombine with the attR1/2 sites flanking each Gateway *ccdB*-chloramphenicol resistance cassette in pFRiPE, resulting in fragment exchange and the formation of 25 nucleotide-long, attB1/2 sites. For more information about the Gateway system, see "Gateway cloning" on the Invitrogen web site (http://www.invitrogen.com/).
3. Add 1 µL proteinase K from the LR mix package, incubate 10 min at 37°C to digest the recombination enzymes.
4. Transform the whole reaction into chemically competent *E. coli* strain DH5α (*ccdB*-sensitive). Plate on ampicillin. Normally, several hundred colonies are obtained. If less than 100 colonies are obtained, something probably went wrong (too much pENTR plasmid used or one of the two plasmids was not linear).

5. Screen colonies by PCR with primer pair D5/EM151 (*see* **Fig. 1** for binding sites). Positive colonies yield a band of: size of the gene fragment + 431. If the LR reaction worked well, virtually all colonies are positive.
6. Miniprep several colonies (6 to 10 per construct: only 50% will be in the desired orientation, *see* **Note 6**). Check for correct orientation of the flip-out *HcRed* cassette using *Eco*RI or *Stu*I or *Kas*I, whichever of these enzymes do not cut inside the gene fragment of interest. The correct clones are those that yield a smaller *Eco*RI or *Stu*I insert or a larger *Kas*I insert. Discard minipreps that yield an insert of the wrong size (the flip-out cassette is reversed in those). The exact sizes of the correct inserts are:
   *Eco*RI: size of the gene fragment + 957
   *Kas*I: size of the gene fragment + 5303
   *Stu*I: size of the gene fragment + 1814

   Plasmid DNA from at least three correct clones (to reach sufficient DNA amount) can be pooled and cleaned using a Qiagen column followed by one chloroform extraction and injected into fly embryos to produce transgenic flies. If insufficient amounts of DNA are recovered, perform a larger-scale DNA purification from the correct *E. coli* colonies.

3.4. Sequencing the Final RNAi Constructs

Sequencing into either repeat is blocked due to single-molecule duplex formation during the annealing steps of the sequencing reaction, so that the sequence readout stops abruptly at the nucleotide where the repeat starts. This is already a sign that the plasmid does contain an inverted repeat. Sequencing can be made possible by restriction digestion of the DNA to be sequenced with an enzyme cutting both between the repeats (i.e., inside the flip-out cassette) and inside the vector backbone. This places the repeats on separate DNA fragments and prevents single-molecule duplex formation during sequencing. Enzymes that can be used for this purpose are again *Eco*RI, *Kas*I, or *Stu*I, as long as the chosen enzyme does not also cut inside the gene fragment. The restricted DNA must be purified before sequencing. A faster approach is to perform the colony PCR of **Step 3.3.5.** incorporating a third primer in the PCR: EM151, D5, and D3. Sequence the reaction product with EM151. At the end of the readable sequence of the cloned gene fragment, two sequences should overlap, representing the normal EM151/D5 PCR product plus the aberrant EM151/D3 PCR product. This sequence overlap proves that the gene fragment is present twice and in inverted orientations.

3.5. Using Transgenic pFRiPE Fly Lines and Checking for Decrease in Gene Expression Levels

Once transgenic pFRiPE fly lines are obtained (*see* **Note 7**), they are crossed with the desired Gal4 driver lines also containing the heat-shock *Flipase* transgene (*see* **Note 8**). We usually induce dsRNA expression 48 h before larvae reach

the desired developmental stage (*see* **Note 9**), to allow sufficient time for protein depletion (but the optimal incubation time to reach sufficient depletion depends on each protein's turnover rate and should be determined empirically). RNAi is induced by incubating food vials for 1 h and 30 min in a warm water bath (37.2°C). This treatment leads to flipase-mediated FRT cassette excision in nearly 100% of cells. Occasionally, a small number of cells do not excise the cassette. Clones derived from these cells are identified by persistent HcRed fluorescence in the Gal4-expressing domain (*see* **Note 10**). RNAi does not occur in these clones. If desired, the flip-out cassette can be excised in the germline and new fly lines will be generated that express dsRNA under GAL4 control in the absence of heat shock (*see* **Note 11**). The latter procedure, combined with the use of temperature sensitive Gal80, can be extremely useful in experiments where sequential transgene induction is desired (*see* **Note 12**).

There is no perfect method to quantify the decrease in gene expression levels, because the amount will depend on the Gal4 driver used, will be restricted to the Gal4-expressing cells, and few methods are absolutely quantitative.

- *In situ* RNA hybridization (with a probe binding to the RNA message outside of the chosen silencing fragment) can provide an estimate of the reduction of mRNA levels in expressing tissue when compared with neighboring nonexpressing tissue. Reduction in RNA levels does not necessarily correlate with protein levels since some proteins may have a long half-life.
- RT-PCR, simpler to apply than in situ hybridization, has the same limitations and requires Gal4 expression in all the cells from which the RNA is prepared. If RT-PCR is performed, PCR primers should be designed outside the silencing fragment. For oligodT-primed cDNA, the RT-PCR primers should be chosen close to the 3′ end of the cDNA for good PCR efficiency.
- Antibody staining followed by confocal microscopy provides a more satisfying assessment, as protein rather than mRNA is visualized and the occurrence of possible noninduced cell clones can be directly observed.
- Western blotting can quantitatively assess the reduction in protein levels, in dissected organs where 100% of the cells express the Gal4 driver or in whole animals expressing dsRNA ubiquitously (for example, using tubulin-Gal4).
- If no antibodies to the protein of interest are available, but tagged constructs do exist (GFP or other tags), the efficiency of the RNAi construct can be assessed by Western blotting or confocal imaging to visualize reduction of tagged protein (provided the chosen silencing fragment is present in the tagged construct). In cases where the tagged protein is only available as a UAS construct, RNAi and tagged protein will be expressed in the same cells. However, it is still possible to obtain an internal control of RNAi efficiency by subjecting the flies to a mild heat shock. Only some cells will excise the HcRed cassette and activate RNAi. Resulting excision clones should show decreased levels of the tagged protein when compared with unexcised surrounding tissue in the Gal4-expressing domain.

– Ultimately, the phenotype of adult flies provides an indication of the efficiency of gene knock-down, but gene redundancy can obscure well-functioning RNAi constructs.

Once a pFRiPE construct triggering a phenotype has been obtained, controls need to be performed to ensure that this phenotype results from knock-down of the intended gene rather than off-target gene silencing or other artifacts. Here are possible approaches:

– Generate different pFRiPE constructs against the same gene. If different constructs derived from different regions of the cDNA show identical phenotypes, the probability of off-target effects becomes very low.
– Rescue the RNAi effect by transgenic expression of the same protein. This approach will work best if the rescuing construct does not share sequences with the silencing fragment (e.g., silencing fragment was chosen in the untranslated regions of the mRNA and these were not incorporated within the rescuing construct). Other types of rescue may be considered; for example metabolic (if knocking down an enzyme synthesizing an essential compound, this compound could be provided in the food) or with transgenic expression of a gene of conserved function from a different organism, as long as DNA homology to the silencing construct is low enough to avoid RNAi.
– To rule out artifacts due to potential transcriptional silencing of genes adjacent to the target gene or other integration site effects, several independent insertion lines should be tested for each pFRiPE construct. To rule out potential artifacts due to expression of peptides from the first repeat, flies that were not heat-shocked but carry the pFRiPE construct and Gal4 driver should be used as negative controls.

How well does "friping" work? To date we have constructed 19 pFRiPE RNAi constructs. For 12 of these constructs, molecular data (in situ hybridization, antibody staining, and loss of GFP fusion fluorescence) indicate that seven triggered efficient RNAi, while five were inefficient. Three additional constructs produced a strong phenotype indicative of RNAi, but specificity has not yet been confirmed. In the four remaining cases, reduction in RNA and protein levels was not tested; therefore, the absence of phenotype is due either to construct inefficiency, to gene redundancy or to gene dispensability. In the case of the *arrow* gene, a first pFRiPE construct clearly did not function. We made a second construct choosing a different region from the gene, which did trigger very efficient RNAi. Failures are therefore not necessarily because a gene was "immune" to RNAi, but because the chosen inverted repeat did not trigger RNAi efficiently. We have not detected a correlation between inherent characteristics of long inverted repeats and RNAi efficiency. If friping only a few genes, it is therefore recommended to try several fragments to maximize chances of success.

4. Notes

1. In an earlier generation of our RNAi vectors, the FRT-HcRed-FRT cassette was cloned immediately after the pUAST UAS sequence and only contained the short SV40 transcription terminator (amplified along with the *HcRed* gene from pHcRed1-N1 [Clontech]). The SV40 terminator was sufficient to abrogate expression from downstream-cloned protein-coding genes, but was insufficient for preventing RNAi from downstream-cloned, intron-separated inverted repeats. Therefore, in subsequent vectors including pFRiPE, we added a much longer terminator (~2.5 kb) from the glutamine-synthetase (*gs1*) gene 3′ region. The length of genomic sequence was meant to ensure that RNA polymerase would have sufficient time to fall off from template DNA before reaching the second inverted repeat. The *gs1* terminator appears to perform as intended in our current experiments.
2. The Deqor program was designed primarily to select 21 nucleotide siRNA for in vitro synthesis and injection into embryos. When running the program, one can ignore the siRNA output and use the rest of the displayed information to choose a sequence devoid of cross-silencers. When designing a silencing fragment to perform RNAi in *Drosophila*, the *Drosophila* transcriptome should be employed as the database for fragment scanning (check the corresponding box).
3. The Gateway LR reaction is only efficient (in an inverted repeat context) if pFRiPE and pENTR are both linear before mixing. pFRiPE must be linearized inside one of the two Gateway cassettes (so that fragment exchange by recombination recircularizes the plasmid). This is done with *Bgl*II. One can purify a large amount of *Bgl*II-linearized pFRiPE in advance, for use in all recombination reactions to be performed. pENTR is linearized with *Nhe*I or *Aat*II (whichever is not present in the gene fragment of interest).
4. If ATG triplets encoding a methionine in the original cDNA are present, a peptide from the gene of interest might be expressed prior to RNAi induction if the first repeat is cloned in the sense orientation. This should be avoided, as side-effects due to expressing fragments of endogenous proteins may exist. Therefore, the silencing fragment should be selected in a region of the gene that does not encode any methionine, or be cloned "antisense first". ATG triplets may however be present in the fragment, as long as they are not in frame with respect to the original coding sequence (though this may result in benign nonsense peptide expression). An unrelated consequence of the presence of ATG triplets within the first repeat is a possible decrease in the expression level of the HcRed gene contained in the excision cassette, resulting in weaker or absent red fluorescence in GAL4-expressing tissue even before RNAi induction. Indeed, ATG triplets upstream of the methionine codon of HcRed might be interpreted as translational starts by the biosynthetic machinery. If these ATGs are in frame with respect to *HcRed*, this can result in a peptide fusion to HcRed (which may preserve red fluorescence). If ATGs are not in frame with respect to HcRed, less frequent translation of HcRed (decreasing red fluorescence) might ensue. HcRed fluorescence is not related to the subsequent efficiency of RNAi, but can be useful to visualize possible cell clones that failed to excise the cassette.

5. If cloning the first repeat in the antisense orientation, one might be concerned that antisense-mediated gene silencing might occur prior to desired RNAi induction. We have not observed this so far with our constructs, probably because antisense gene silencing is inherently very inefficient. Antisense RNA molecules have to anneal to endogenous RNA cognates from which they are physically separated, whereas inverted repeats readily self anneal.
6. One drawback of the Gateway LR reaction (in the inverted repeat configuration) is that 50% of the plasmid clones resulting from the double-LR reaction display a flip-out HcRed cassette that is reversed with respect to UAS orientation. This happens when the attL1 site in one ENTR plasmid recombines with the attR1 site of Gateway cassette A, but the attL2 site in the same ENTR molecule recombines with the attR2 site of Gateway cassette B instead of A. Then a second ENTR plasmid recombines with the remaining crossed attR1 and attR2 sites and inverts the cassette. Therefore, after the LR reaction, *E. coli* colonies should be screened for correct orientation of the flip-out cassette. An RNAi construct with a reverted flip-out cassette might still function but (a) HcRed will not be expressed and (b) the reversed transcription terminator is unlikely to function properly, giving rise to a small risk of observing RNAi before heat shock. PCR cannot be used to determine cassette orientation (using a primer inside the flip-out cassette and a pUAST primer, such as the EM151/D5′ combination), because this yields the expected product even for clones in the wrong orientation (this phenomenon is due to primer extension into a repeat during each PCR cycle, the resulting product then serving as a primer for the other repeat in the next PCR cycle. Amazingly, the yield of these aberrant PCRs is as high as the normal reaction). Instead, a few clones must be mini-prepped and their orientation checked by restriction digestion.
7. We recommend selecting transgenic lines containing single genomic insertions of pFRiPE. If multiple insertions are present (especially on the same chromosome), one cannot exclude potential chromosomal deletions/translocations mediated by recombination between FRT sites at different insertion loci. Practically, several independent insertion lines with paler eyes should be established. Dark-eyed transgenic flies should be avoided as these are likely to be multiple insertions, or the multiple insertions separated by recombination.
8. In our experiments, we have used flies containing the P{hsFLP}22 insertion on the X chromosome in combination with various Gal4 drivers. With this insertion, excision only occurred after heat shock in most tissues (for example, wing imaginal discs). However, a subset of fat body cells (~50%) excised the FRT cassette without heat shock. Therefore, RNAi will occur before heat shock in these cells if expression is driven by a GAL4 driver active in the fat body. Other hs-FLP insertion lines might provide better regulated heat inducibility in this tissue, but have not been tested.
9. We have not studied RNAi in adult flies, but "friping" may be possible in the adult stage as well. For efficient heat-shock induction of the RNAi, we suggest heat-shocking food vials containing pupae on the walls of the tube 24–48 h prior to eclosion, or adults in the absence of food since flies tend to drown in the food during heat shock.

10. Most HcRed fluorescence produced before cassette excision will decay within 24 h of excision. Therefore, only unexcised clones will still fluoresce red when excited with laser light between 545 and 633 nm (excitation peak: 596 nm). Thus, antibody stains may be performed with secondary antibodies emitting wavelengths that overlap with the HcRed emission spectrum (e.g., Cy3, Cy5). If antibody staining is required prior to HcRed cassette excision, a green fluorescent secondary antibody should be used to avoid HcRed bleed through into the antibody signal. However, in many pFRiPE constructs, red fluorescence is faint enough even before cassette excision not to bleed through into the Cy3 or Cy5 channel, provided that primary antibodies yielding strong signals are used.
11. Some pFRiPE constructs (or transgenic construct integration sites) appear to yield more frequent unexcised clones than others. For example, a pFRiPE construct against *arrow* typically yields —two to six clones in the dorsal compartment of the imaginal disc wing pouch (driven with *apterous*-Gal4), which is still a minor proportion of the expressing cells.

    If the HcRed cassette in an existing pFRiPE RNAi construct appears to be excised inefficiently, derivative fly lines can be generated that do not contain the cassette any more. RNAi is then independent of heat shock-mediated excision, but can be made inducible by introducing temperature-sensitive Gal80 ***(23)*** in the Gal4 driver line.

    Generate excision lines by excising the *HcRed* cassette in the germ line. Heat-shock food vials containing larvae expressing both hs-FLP and the pFRiPE construct, twice in a two- or three-day interval. Cross the emerging flies to a balancer stock and establish several independent stocks containing the pFRiPE construct (now presumably missing the *HcRed* cassette). Test each for the presence/absence of the *HcRed* cassette (by PCR or cross with a Gal4 driver).
12. In a $Gal80^{ts}$ + Gal4 driver genetic background, combining a pFRiPE derivative excision line with a different pUAST transgene containing a flip-out cassette (for example, the FRT-HcRed-FRT cassette) is an excellent method to achieve sequential transgene inductions: RNAi is first induced by inactivating Gal80 (flies are shifted from 18 to 29°C), later the second transgene is induced by heat shock-mediated *HcRed* cassette excision. This can serve to study the effect of a particular perturbation in the absence of a given protein. For an example, *see* ref. ***(2)***.

Acknowledgments

This work was supported by an A. von Humboldt fellowship to E. M. and by a grant from the Deutsche Forschungsgemeinschaft (EA 4/2-1).

References

1. Panáková, D., Sprong, H., Marois, E., Thiele, C., and Eaton, S. (2005) Lipoprotein particles are required for Hedgehog and Wingless signaling. *Nature* **435,** 58–65.
2. Marois, E., Mahmoud, A., and Eaton, S. (2006) The endocytic pathway and formation of the Wingless morphogen gradient. *Development* **133,** 307–317.

3. Desbordes, S. C. and Sanson, B. (2003) The glypican Dally-like is required for Hedgehog signaling in the embryonic epidermis of Drosophila. *Development* **130,** 6245–6255.
4. Lum, L., Yao, S., Mozer, B., et al. (2003) Identification of Hedgehog pathway components by RNAi in Drosophila cultured cells. *Science* **299,** 2039–2045.
5. Nybakken, K., Vokes, S. A., Lin, T. Y., McMahon, A. P., and Perrimon, N. (2005) A genome-wide RNA interference screen in Drosophila melanogaster cells for new components of the Hh signaling pathway. *Nature Genet*. **37,** 1323–1332.
6. Kennerdell, J. R. and Carthew, R. W. (2000) Heritable gene silencing in Drosophila using double-stranded RNA. *Nature Biotechnol*. **18,** 896–898.
7. Martinek, S. and Young, M. W. (2000) Specific genetic interference with behavioral rythms in Drosophila by expression of inverted repeats. *Genetics* **156,** 1717–1725.
8. Fortier, E. and Belote, J. M. (2000) Temperature-dependent gene silencing by an expressed inverted repeat in Drosophila. *Genesis* **26,** 240–244.
9. Billuart, P., Winter, C. G., Maresh, A., Zhao, X., and Luo, L. (2001) Regulating axon branch stability: the role of p190 RhoGAP in repressing a retraction signal pathway. *Cell* **107,** 195–207.
10. Piccin, A., Salameh, A., Benna, C., et al. (2001) Efficient and heritable functional knock-out of an adult phenotype in Drosophila using a GAL4-driven hairpin RNA incorporating a heterologous spacer. *Nucleic Acids Res*. **29,** e55.
11. Reichhart, J. M., Ligoxygakis, P., Naitza, S., Woerfel, G., Imler, J. L., and Gubb, D. (2002) Splice-activated UAS hairpin vector gives complete RNAi knockout of single or double target transcripts in Drosophila melanogaster. *Genesis* **34,** 160–164.
12. Enerly, E., Larsson, J., and Lambertsson, A. (2002) Reverse genetics in Drosophila: From sequence to phenotype using UAS-RNAi transgenic flies. *Genesis* **34,** 152–155.
13. Kalidas, S. S. D. (2002) Novel genomic cDNA hybrids produce effective RNA interference in adult Drosophila. *Neuron* **33,** 177–184.
14. Giordano, E., Rendina, R., Peluso, I., and Furia, M. (2002) RNAi triggered by symmetrically transcribed transgenes in Drosophila melanogaster. *Genetics* **160,** 637–648.
15. Brand, A. H. and Perrimon, N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118,** 401–415.
16. Duffy, J. B. (2002) GAL4 system in Drosophila: a fly geneticist's Swiss army knife. *Genesis* **34,** 1–15.
17. Filipowicz, W. (2005) RNAi: the nuts and bolts of the RISC machine. *Cell* **122,** 17–20.
18. Lee, Y. S. and Carthew, R. W. (2003) Making a better RNAi vector for Drosophila: use of intron spacers. *Methods* **30,** 322–329.
19. Smith, N. A., Singh, S. P., Wang, M. B., Stoutjesdijk, P. A., Green, A. G., and Waterhouse, P. M. (2000) Total silencing by intron-spliced hairpin RNAs. *Nature* **407,** 319–320.
20. Golic, K. (1991) Site-specific recombination between homologous chromosomes in Drosophila. *Science* **252,** 958–961.

21. Wesley, S. V., Helliwell, C. A., Smith, N. A., et al. (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *The Plant J*. **27,** 581–590.
22. Henschel, A., Buchholz, F., and Habermann, B. (2004) DEQOR: a web-based tool for the design and quality control of siRNAs. *Nucleic Acids Res*. **32,** W113–W120.
23. McGuire, S. E., Le, P. T., Osborn, A. J., Matsumoto, K., and Davis, R. L. (2003) Spatiotemporal rescue of memory dysfunction in Drosophila. *Science* **302,** 1765–1768.