Letter to the Editor

Modified vectors for the two-step directional cloning of inverted repeats for RNA interference in *Drosophila*

Post-transcriptional gene silencing has become the fastest and most frequently used approach to reduce gene function in cell culture. In an organism without an interferon response, such as *Drosophila melanogaster*, RNA interference (RNAi) can be evoked using long, double-stranded RNAs (1). In order to achieve a gene knockdown at a stage not amenable to dsRNA injection into early embryos, transgenic approaches have been used in *Drosophila* (2,3). Commonly, a 500 to 700 bp cDNA product is cloned as an inverted repeat with or without a spacer and expressed under the control of UAS-Gal4 sequences (4). To allow tissue-specific and/or temporally controlled expression of the RNAi transgene, the bimodular UAS-Gal4 system can be used, bypassing any potential toxicity of such transgenes.

Inverted repeats tend to be inherently difficult to clone in *Escherichia coli* due to recombination-mediated DNA repair that occurs during DNA replication (5). Lee and Carthew (6) designed pWIZ, a pUAST-based (4) fly transformation vector in which the two inverted repeats are separated by an intron of the *white* gene. After transcription, the intron is spliced out and the mRNA forms a hairpin in vivo. The cloning of inverted repeats into pWIZ is very difficult, even when *sbcC*$^{-}$ *E. coli* strains reducing recombination repair are used. A smaller shuttle vector in which the repeat/intron cassette is pre-assembled is easier to use, but requires at least 3 or 4 sub-cloning steps (7). Zhu and Stein used a strategy allowing directional cloning of inverted repeats in the germline UAS expression vector pUASp (8,9). pWIZ was also adapted for the GATEWAY in vitro recombineering system (Invitrogen, Carlsbad, CA, USA) (10). This elegant system, however, requires the purchase of expensive, special reagents.

In an attempt to simplify the generation of genomic RNAi constructs, we modified pWIZ to improve the cloning efficiency of dsRNA probes. The biggest problem during the cloning of inverted repeats is to obtain the inverted versus the direct orientation of the dsRNA target fragment during the second round of cloning. We therefore constructed pWIZdir by changing the polylinkers of pWIZ to allow the consecutive, directional insertion of a single PCR product using pairs of compatible restriction enzyme sets: *Spe*I/*Avr*II and *Nhe*I/*Xba*I adjacent to the *white* intron, and *Bgl*II and *Bam*HI upstream and downstream, respectively (Figure 1). The directionality of the second cloning step greatly increases the frequency of obtaining correctly inverted repeats and obviates the need for the intermediate use of a shuttle plasmid. We have made eight dsRNAi constructs and obtained between 16% and 90% correctly inserted second-round cloning products, a significant improvement over previous attempts (not shown). All eight constructs were successfully used to generate transgenic fly lines. Examples of eye and wing phenotypes obtained in vivo are shown in Figure 2.

Typical cloning procedure. Target sequences are chosen using the Heidelberg eRNAi prediction site (www.dkfz.de/signaling2/e-rnai). Fragments should lack either a *Bgl*II, *Bam*HI, or *Bcl*I site, and should not contain either a *Xba*I, *Avr*II, *Nhe*I, or *Spe*I site. Preferentially, we attach 3 to 5 random nucleotides followed by an *Avr*II and *Bam*HI site at the 5′ ends of the forward and reverse primers, respectively. This strategy results in the first insert having the antisense orientation, avoiding the expression of any potential protein fragment (Figure 1B).

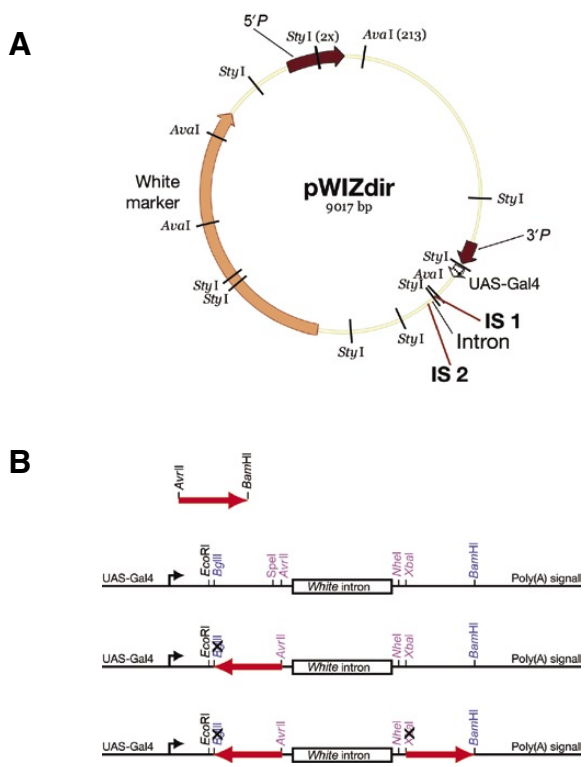

Figure 1. Schematic representation of pWIZdir and the directional cloning strategy. (A) Map of pWIZ-dir. IS 1 and IS 2 designate the insertion sites of the products separated by the *white* intron. 5′*P* and 3′*P* are the *P*-element terminal repeats. *Ava*I and *Sty*I are typically used to assess the integrity of the final construct containing the inverted repeats. (B) Scheme of the directional cloning strategy. Large red arrows correspond to the PCR product. Colors of indicated restriction sites correspond to the overhang compatibility group. Crossed out sites are lost due to the directional cloning strategy.

All restriction digests are performed in 20 µl reactions using 5 µl (about 2.5 µg) of a typical midiprep or 10 µl of a miniprep and 1 µL of each enzyme for 2 h to overnight. Gel purifications are done using the Qiaquick gel-elution kit (Qiagen, Valencia, CA, USA) and DNAs are eluted in 30 µl elution buffer. Ligations are performed in 10 µl volumes containing 7 µl insert, 1 µL vector, 1 µl 10× ligation buffer, and 1 µl ligase (NEB, Ipswich, MA, USA) for 2 h to overnight at room temperature.

The PCR product is ethanol-precipitated according to standard procedures (11) and digested with *Avr*II and *Bam*HI in buffer 2 (NEB), gel-purified, and ligated into pWIZdir digested (and dephosphorylated) with *Bgl*II and *Avr*II. After transformation, minipreps are analyzed for the presence of the insert with *Eco*RI/*Avr*II (buffer 4, NEB).

A positive miniprep is then sequenced with pUAST_for (CTGAAATCTGCCAAGAAGT) to confirm the identity of the PCR product. 10 µl of the miniprep are digested with *Xba*I/*Bam*HI and ethanol-precipitated. After dephosphorylation, the second-round vector is gel-purified and ligated to the same PCR product used in the first round of cloning. The ligation is transformed into chemically competent (12) Sure cells (Stratagene, La Jolla, CA, USA). Recovery of the heatshock and growth of colonies, as well as the following liquid cultures for minipreps are grown at 30°C instead of 37°C. Positive minipreps are identified by digestion with *Bam*HI/*Nhe*I. Positive clones are characterized by restriction analysis and compared with the parental pWIZdir to avoid clones with deletions in the insert or vector backbone. We typically test again for the presence of both inserts and assess the integrity of the vector backbone with *Ava*I and *Sty*I (Figure 1A).

Vector construction. To generate pWIZdir, the *Bam*HI sites flanking the UAS cassette of pWIZ (6) were destroyed by opening, blunt-ending, and religating the fragments. Sequencing confirmed the absence of *Bam*HI sites. Annealed oligos Wiz_dir_for and Wiz_dir_rev (CTAGCTC TAGAATATCAATTGGGATCCA; CT AGTGGATCCCAATTGATATTCTA GAG) were subsequently ligated into the *Nhe*I/*Xba*I sites of pWIZΔBam to produce pWIZdir (Figure 1A). We also generated a RNAi-based vector in pUASp (pUASPdir), an expression vector that, unlike pUAST, enables Gal4-mediated expression in the germline, as well as in somatic tissues (9). To assemble pUASPdir, pWIZdir was amplified by PCR with oligos UASP_WIZ_for and UASP_WIZ_rev (ATATAGGTACCGAATTCGTTAAC AGATCTGCGG; TATATCCCGGGG GATCCCAATTGATATTCTAGAG), the product digested with *Kpn*I and *Sma*I and inserted into the *Kpn*I/*Xba*I (blunt) sites of pUASp. Although we have not tested this vector in vivo, it is available to individuals who are interested in using it. Compiled sequences of pWIZdir and pUASPdir can be obtained from GenBank (accession nos. EU178814 and EU178815, respectively).

In summary, we generated a new vector useful to knockdown gene functions in transgenic *Drosophila melanogaster*. In contrast to pWIZ, pWIZdir allows efficient, directional cloning of the hairpin stems. This greatly increases the frequency of successfully cloning the required inverted repeats without use of expensive reagents. Furthermore, our cloning strategy eliminates the need to determine the orientation of each fragment, a task often complicated by the lack of appropriate, asymmetric restriction sites in such short inserts. In addition to its use as a fly transformation vector, pWIZdir could also be used to knock down genes in cell culture in combination with a co-transfected Gal4 expressing plasmid.

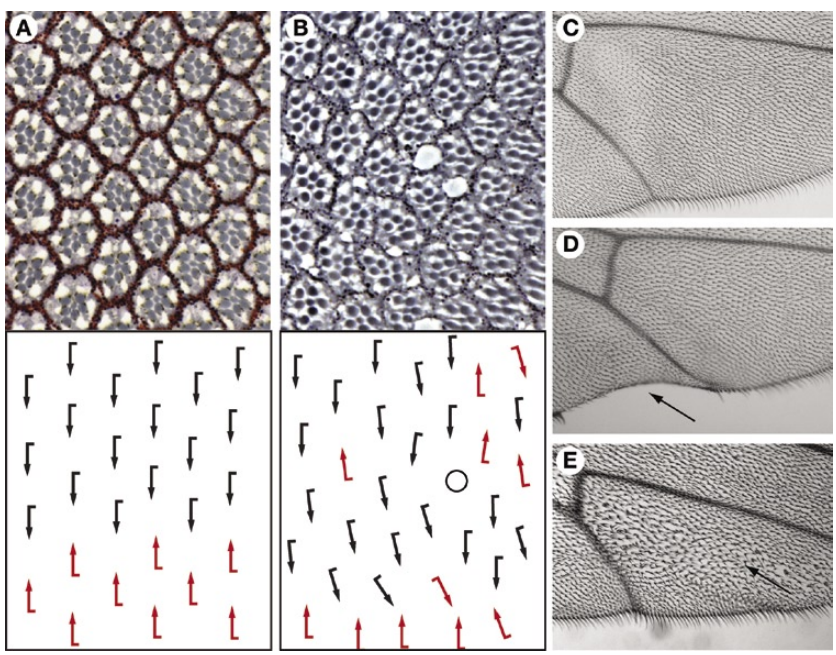

Figure 2. Examples of in vivo phenotypes generated by pWIZdir transgenes. (A and B) Tangential sections of adult eyes with schematic representation of the orientation of ommatidia. (A) In a wild-type eye, dorsal (black arrows) and ventral ommatidia (red arrows) are precisely oriented and separated from each other. (B) This planar cell polarity (PCP) arrangement can be disturbed by expressing a dsRNA directed against CG7236 from pWIZdir under the control of *eyless*-Gal4 (for review about PCP establishment see Reference 13). (C-E) A high magnification of a part of an adult wing. (C) A wild-type wing has sensory bristles all around its margin and a single actin hair protruding distally from each cell within the wing blade. (D) Expressing dsRNAs directed against CG7177 under the control of *scalloped*-Gal4 leads to wing margin defects. (E) Knocking down CG7236 using *engrailed*-Gal4 leads to defects in actin hair formation (multiple wing hairs).

Andreas Jenny and Marek Mlodzik
Mount Sinai School of Medicine
Dept. of Developmental and
Regenerative Biology
New York, NY, USA
Andreas Jenny's present address is:
Albert Einstein College of Medicine

Dept. of Developmental and
Molecular Biology
Bronx, NY, USA
e-mail: ajenny@aecom.yu.edu

ACKNOWLEDGEMENTS

We thank Richard Carthew for pWIZ and Katya Serysheva for help with sectioning eyes. We thank Steve Cohen for sd*Gal4 and* en*Gal4 strains and Uwe Walldorf for* ey*Gal4. We are grateful to David DelAlamo, Matias Simons, and Elisa Wurmbach for critically reading the manuscript. We thank the reviewers for bringing the publications of Zhu and Stein (8) and Kondo et al. (10) to our attention. The work has been supported by the National Institutes of Health (grant no. R03 DC007055 to A.J. and grant no. RO1 GM062917 to M.M.).*

REFERENCES

1. **Sandy, P., A. Ventura, and T. Jacks.** 2005. Mammalian RNAi: a practical guide. BioTechniques *39*:215-224.
2. **Dietzl, G., D. Chen, F. Schnorrer, K.C. Su, Y. Barinova, M. Fellner, B. Gasser, K. Kinsey, et al.** 2007. A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. Nature *448*:151-156.
3. **Kennerdell, J.R. and R.W. Carthew.** 2000. Heritable gene silencing in *Drosophila* using double-stranded RNA. Nat. Biotechnol. *18*:896-898.
4. **Brand, A.H. and N. Perrimon.** 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*:401-415.
5. **Cromie, G.A., C.B. Millar, K.H. Schmidt, and D.R. Leach.** 2000. Palindromes as substrates for multiple pathways of recombination in *Escherichia coli*. Genetics *154*:513-522.
6. **Lee, Y.S. and R.W. Carthew.** 2003. Making a better RNAi vector for *Drosophila*: use of intron spacers. Methods *30*:322-329.
7. **Bao, S. and R. Cagan.** 2006. Fast cloning inverted repeats for RNA interference. RNA *12*:2020-2024.
8. **Zhu, X. and D. Stein.** 2004. RNAi-mediated inhibition of gene function in the follicle cell layer of the *Drosophila* ovary. Genesis *40*:101-108.
9. **Rorth, P.** 1998. Gal4 in the *Drosophila* female germline. Mech. Dev. *78*:113-118.
10. **Kondo, T., S. Inagaki, K. Yasuda, and Y. Kageyama.** 2006. Rapid construction of *Drosophila* RNAi transgenes using pRISE, a P-element-mediated transformation vector exploiting an *in vitro* recombination system. Genes Genet. Syst. *81*:129-134.
11. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual. CSH Laboratory Press, Cold Spring Harbor, NY.
12. **Inoue, H., H. Nojima, and H. Okayama.** 1990. High efficiency transformation of *Escherichia coli* with plasmids. Gene *96*:23-28.
13. **Jenny, A. and M. Mlodzik.** 2006. Planar cell polarity signaling: a common mechanism for cellular polarization. Mt. Sinai J. Med. *73*:738-750.