



Genomes & Developmental Control

A simplified miRNA-based gene silencing method for *Drosophila melanogaster*

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ABSTRACT

MicroRNA-based RNA interference is commonly used to produce loss-of-function phenotypes in mammalian systems, but is used only sparingly in invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster*. Here, we evaluate this method in transgenic strains of *D. melanogaster* and cultured S2 cells. High throughput-ready expression vectors were developed that permit rapid cloning of synthetic hairpin RNAs. As proof of concept, this method was used for the efficient silencing of *dpp* gene activity in the adult wing, and the analysis of the general RNA Polymerase II (Pol II) elongation factor, *Nelf-E*.

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Introduction

Double-stranded RNAs (dsRNAs) are commonly used for gene silencing in *Drosophila*. However, these dsRNAs are typically quite long, >300-bp, and thereby produce dozens of unique ~21-nucleotide (nt) small interfering RNAs (siRNAs) (Elbashir et al., 2001). Sequence overlap of these RNAs with other regions of the genome, along with the unpredictable nature of the exact siRNAs derived from any given dsRNA, raises the likelihood of nonspecific, “off-target” phenotypes (Jackson and Linsley, 2004). Indeed, at least 40% of the dsRNAs in a widely used library might produce off-target effects in cultured *Drosophila* cell lines (Kulkarni et al., 2006). Nonetheless, considerable resources have been expended on this method for whole-genome assays (Dietzl et al., 2007).

siRNAs, defined as exogenous 21-nt silencing RNAs, can be expressed for targeted gene silencing assays through manipulation of the endogenous microRNA (miRNA) biogenesis pathway (Bartel, 2004). In this system, a customized siRNA sequence is swapped into a naturally occurring, imperfectly paired, ~70–90-base-pair (bp) stem-loop sequence (Chang et al., 2006). Cellular factors recognize the stem-loop as a miRNA precursor (pre-miRNA) and excise a

specific ~21-nt sequence (the miRNA/siRNA) from one or both arms of the stem. Like exogenously delivered siRNAs, the resulting small RNA(s) is loaded into an effector complex (RISC) that can induce endonucleolytic cleavage of complementary RNAs (Hutvagner and Zamore, 2002; Miyoshi et al., 2005). The small RNA preferentially loaded into RISC from a given duplex RNA, e.g. a pre-miRNA or a transfected siRNA, is known as the mi/siRNA, while the less-incorporated strand is the star (mi/siRNA*) sequence. The incorporation of the mi/siRNA or mi/siRNA* into RISC depends, in part, on the local free energy of the duplex's terminal base pairs (Khvorova et al., 2003; Schwarz et al., 2003). This property provides a means for controlling the exact sequence of an expressed siRNA, thereby diminishing nonspecific, off-target phenotypes. While several studies demonstrated the effectiveness of miRNA-based RNAi in mammalian systems, there have been just a few such studies in *Drosophila* (Chen et al., 2007; Huh et al., 2007).

Here, we present a versatile, miRNA-based system for the conditional silencing of gene activity in *Drosophila*. These newly developed expression vectors recapitulate the exact structure of the *Drosophila* pre-miRNA-1(miR-1) stem loop sequence, differing only by unique cloning sites within the stem itself. Synthetic oligonucleotides (oligos) are directly inserted into the pre-miR scaffold, thereby circumventing multiple PCR amplification and cloning steps. A subcloning vector was also developed for creating tandem hairpins, thereby permitting the expression of multiple siRNAs directed against different regions of a specific target mRNA. We have created separate one-step transgenesis vectors that are compatible

Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA; miRNA, microRNA; shmiR, short hairpin/miRNA; RISC, RNA induced silencing complex; *dpp*, decapentaplegic; *Nelf-e*, negative elongation factor e; GFP, green fluorescent protein; RFP, red fluorescent protein; M.C.S., multiple cloning site.

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with either $\Delta 2,3$ P-element transposase or phiC31 integrase (targeted integration) (Adams and Sekelsky, 2002; Groth et al., 2004). A web-based algorithm provides the exact design of the synthetic oligos for insertion into the pre-miR-1 scaffold. Both S2 tissue culture assays and targeted expression in transgenic larvae and adults were used to establish the effectiveness and specificity of the method. We discuss the results of targeted disruption of *dpp* and *Nelf-E* in wing imaginal disks, and compare these phenotypes with those produced by dsRNAs.

Materials and methods

Vector design

The original shmiR vector used in this study is based on the *D. melanogaster* pre-miR-1. A second-generation vector, pHB, includes ectopic cloning sites at the base of the pre-miR-1 sequence. Both types of vectors were constructed as mono or tri-cistronic synthetic genes by Celtek Bioscience (Nashville, TN), cloned into the KpnI and SacI restriction sites of pBluescript SK(+), and subsequently cloned into S2 cell expression or P-element transgenesis vectors as single or double hairpins by using appropriate restriction sites (see Supplemental File 1). Hairpins were inserted into pHB by directional ligation into the HindIII–BamHI cloning sites at the base of the pre-miR stem-loop.

The pNE2 vector was constructed in a modified pUAST backbone (Brand and Perrimon, 1993). EcoRI and XbaI cloning sites were removed by Klenow-mediated end fill. A mini-gene constructed as two annealed Ultramers (IDT) was inserted into this modified pUAST vector as a BglII–XhoI fragment. Hairpins were inserted into pNE2 by directional ligation into the NheI–EcoRI cloning sites at the base of the pre-miR stem-loop. pNE3 was constructed by end-filling a KpnI digested pNE2 vector, followed by linker-ligation of a BglII–KpnI–XbaI adaptor into the BglII–XbaI cloning site. This modification allows for directional insertion of a shmiR from pHB into pNE3, creating a tandem shmiR. *pattB*-NE3 is a variant of the phiC31 integrase-based *pattB*-UAST vector (Bischof et al., 2007). It was modified to include the same shmiR insertion and multi-cloning site as pNE3 (see Supplemental File 2 for complete restriction maps). A suggested method for creating two or more tandem shmiRs in the pHB, pNE2, or pNE3 vectors is presented in Supplemental File 3.

Synthetic 71-base-pair oligonucleotides

71-nt oligos (Elim Biopharmaceuticals or IDT) were annealed at a final concentration of 50 μ M in 1 \times annealing buffer (75 mM KCl, 20 mM Tris [pH 8.0]), boiled for ~2 min, and then cooled to room temperature for ~30 min. Annealed oligos were diluted 1:100 in 1 \times annealing buffer, and then ligated to ~500 ng of the appropriate linearized vector for ~15 min at room temperature using T4 DNA ligase (New England Biolabs). All inserts were sequenced and assessed for proper folding of the pre-miR stem loop via mFold (Mathews et al., 1999; Zuker, 2003).

siRNA sequence design

siRNAs directed against *dpp* recognize all *dpp* mRNA isoforms. siRNAs targeting mRNAs encoding pGL3-based Luciferase (Promega) and the mCherry red fluorescent protein were designed using the Dharmacon “siDesign” center (Shaner et al., 2004). *Nelf-E* siRNAs were designed using the Ambion “siRNA target finder” algorithm. The target-specificity of each 21-nt sequence was determined using parameters described by Khvorova and colleagues (Birmingham et al., 2007). Further, miR-1, like most miRNAs, begins with a 5' uracil (Du and Zamore, 2005). In accordance with this generality, all siRNAs designed for use with these vectors contain a 5'-U. The complete sequences of all of the siRNAs used in this study are presented in Supplemental File 1.

Online hairpin design tool

A Perl-CGI script was written to automate the design of insert oligos compatible with our vectors, based on a user-defined siRNA sequence. This interactive web page provides the shmiR sequence required to produce a given 21-nt siRNA, using either the pHB or pNE expression vector. Information is also provided for the predicted secondary structure of the top (sense) strand hairpin produced with UNAFold (formerly mFold) (Dimitrov and Zuker, 2004). The structure of the resultant shmiR incorporates specific mismatched bases (at nts 2 and 11, as measured from the siRNA's 5'-most base) to mimic the native *D. melanogaster* pre-miR-1 structure.

It has recently been found that siRNAs containing the same seed residues (nts 2–8) as those present within known miRNAs can induce systematic off-target phenotypes in cultured cell lines (Birmingham et al., 2006; Jackson et al., 2006). Therefore, each input 21-mer is cross-referenced against a database with all annotated *D. melanogaster* miRNA sequences, as of June 2008 (Griffiths-Jones, 2004). An alert will be displayed if the user-defined 21-mer contains a seed sequence overlapping a known miRNA.

S2 cell culture and transient transfection assays

S2 cells were plated at a density of 5×10^5 cells/well in 24-well plates. Synthetic shmiRs were inserted into a pAc5.1-B-based expression vector (Invitrogen), and used to transfect plated cells the following day with Effectene (Qiagen), as suggested by the manufacturer. Cell lysates were collected 48 h after transfection, and then analyzed using a dual-Luciferase assay kit (Promega). For mCherry RNAi, the shmiR vector was transfected at a ~4-fold excess over the target vector, and fluorescence was assayed at 48 and 72 h following transfection. shmiRs directed against mCherry, as well as the mCherry ORF were cloned into pAc5.1, as above.

An ~500-bp dsRNA directed against the firefly Luciferase coding sequence was amplified by PCR using primers that contain a T7 RNA polymerase promoter sequence—primer sequences are published in Supplemental File 1. RNA was transcribed using MEGAscript T7 (Ambion) and processed for transfection as described (Forstemann et al., 2005).

AGO immunoprecipitation

Cells were transfected with 450 ng shmiR-Luc 1HB and 50 ng mCherry, as described above. After 48 h, cells were collected by centrifugation, washed 3 \times with PBS [pH 7.4] (Gibco), and resuspended in 15 μ l of lysis buffer per mL of cell culture media, as described (Forstemann et al., 2005). Cell debris was removed following centrifugation, producing lysate with a final protein concentration of ~2 μ g/ μ l. Monoclonal antibodies against *Drosophila* AGO1 or AGO2 were bound to sheep anti-mouse-Ig conjugated Dynabeads (Dyna) by rocking at 4 $^{\circ}$ C overnight (Miyoshi et al., 2005). Antibody-conjugated beads were collected on a magnetized stage and washed 3 \times in PBS. 80 μ l of lysate was added to the beads per IP, and this mixture was incubated on ice for 4 h, with occasional shaking. Following IP, beads were collected on a magnetized stage, the supernatants were removed, and then washed 3 \times with cold lysis buffer. Beads were resuspended in 100 μ l of lysis buffer and bound proteins were removed by boiling for 5 min. Both the supernatant and recovered solutions were added directly to 900 μ l of Trizol (Invitrogen) and 200 μ l of chloroform. RNA was extracted and precipitated with 1 V/V isopropanol and 20 μ g glycogen (Roche).

Fly stocks

P-element-mediated transformations of yw strains with the pUAST-based shmiR vectors were done using traditional methods,

either in-house or by BestGene Inc. (Chino Hills, CA). At least 3 independent lines were recovered and assayed for each UAS-shmiR construct, except for UAS-shmiR-*dpp1*. The following Bloomington Stock Center lines were used in this study: A9-GAL4 (stock #8716), *sd*-GAL4 (stock #8609), daughterless-GAL4 (stock #5460), *dpp*^{shv}, also known as *dpp*^{s1} (stock #397), *dpp*^{d5} (stock #2071), and UAS-*Nelf-e*-IR/dsRNA (stock #6788) (Enerly et al., 2002). UAS-*dpp*-dsRNA lines “47A.1” and “48A.1”—also identified as TR00047A.1 and TR00048A.1, respectively—were kindly provided by Norbert Perrimon (Ni et al., 2008).

Wing mounting

Adult flies were recovered 1–3 days after eclosion, and stored for 24 h or more in absolute ethanol. Wings were dissected in ethanol, mounted immediately in CMCP-10 (Polysciences Inc.), and baked overnight in a desktop convection oven.

Larval RNA and Northern blot assays

GAL4 and UAS transgenic strains were mated as follows. Three males carrying a specific UAS shmiR construct were mated with three virgin females carrying a particular GAL4 expression vector. RNA was extracted from 10–20 wandering larvae or 0–1 day pupae in ~700 μ l Trizol and 140 μ l chloroform, followed by precipitation of the

aqueous phase with 3V/V absolute ethanol and 20 μ g glycogen. The final RNA concentration was determined using a Nanodrop ND-1000 spectrophotometer. ~15 μ g of total RNA per lane RNA was fractionated on 20% PAGE (SequaGel System, National Diagnostics) using a Hoefer Scientific SE250 Mighty Small Gel Electrophoresis unit in 0.5 \times TBE. RNA size markers (IDT) were designed to minimize 2° structures, the oligonucleotides were pooled, end labeled with γ -32P-ATP (MP Biochemicals) end-labeled using T4 Polynucleotide kinase (NEB), and unincorporated label was removed using an Illustra Microspin G-25 spin column (GE Healthcare). ~2 fmoles of the size markers were used for each Northern blot. RNA was transferred onto a Hybond N+ membrane (Amersham Biosciences), pre-wetted with 0.5 \times TBE in a TransBlot-SD semi-dry apparatus (Bio-Rad) at constant 20 V for one hour. Following transfer, the membrane was UV crosslinked in a Stratalinker (Stratagene), pre-hybridized in ULTRAhyb Oligo (Ambion), and treated according to manufacturer's recommendations. The membrane and ~50 pmoles of γ -32P-ATP end-labeled deoxyoligonucleotides complementary to the appropriate small RNA were incubated at ~40 °C in rolling 50-ml conical tubes overnight. Membranes were washed 3 \times 20 min in 2 \times SSC supplemented with 0.2% SDS, exposed on a Fuji phosphorimager plate and analyzed on a Typhoon TRIO Phosphorimager (Amersham Biosciences). Probes were stripped by boiling in RNase-free water supplemented with 0.1% SDS for 5 min, followed by immediate pre-hybridization. Probe and marker sequences are published in Supplemental File 1.

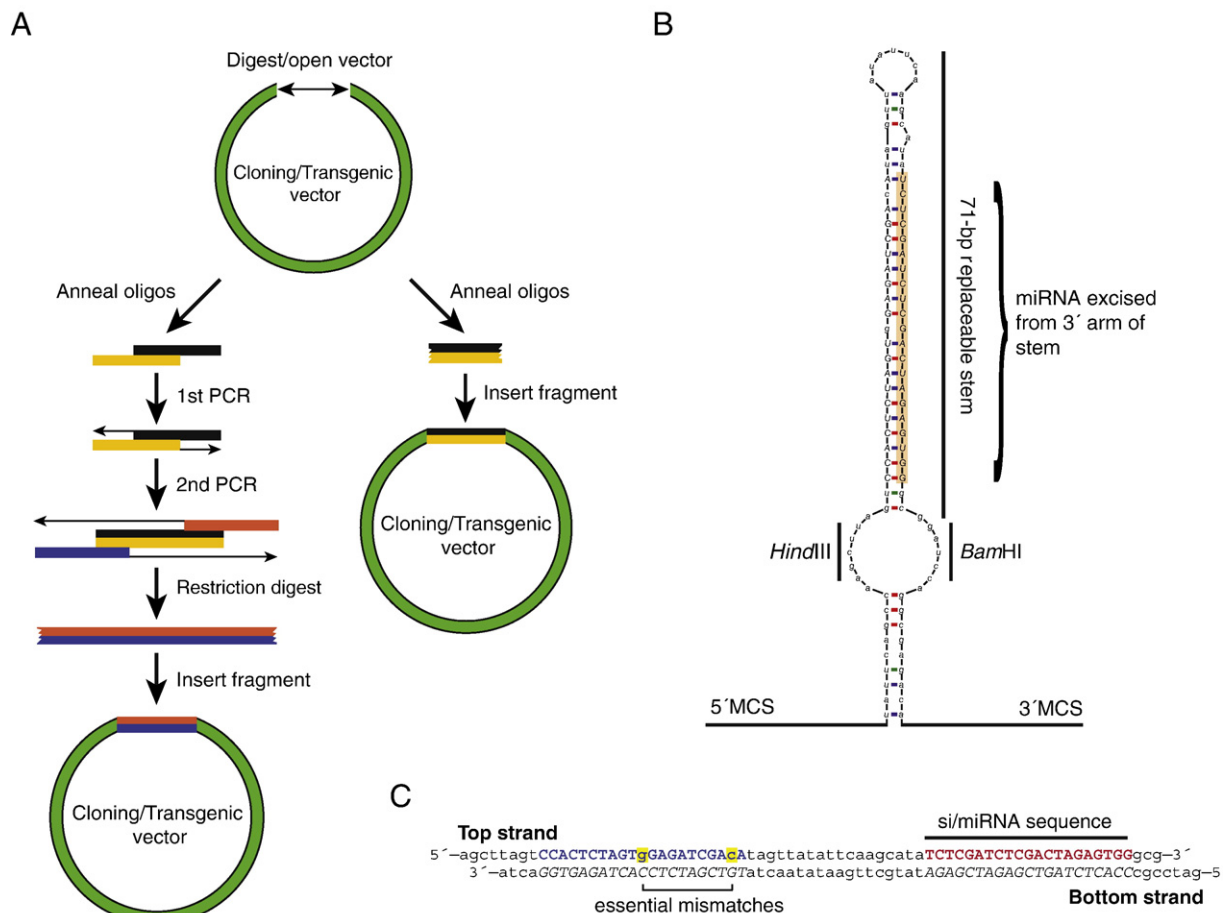


Fig. 1. Schematic of miRNA-based RNAi vector-shmiRs. (A) Standard method for cloning miRNA-based RNAi vectors, left, is compared to miRNA-based vectors described in this study, right. (B) Structure of shmiR-*dpp2* in pHb scaffold. Annealed 71-nt DNA oligos are inserted into the open-loop region with the specified restriction sequences, HindIII–BamHI in this example. Resultant siRNAs will be excised, like *miR-1*, from the 3' arm of the stem-loop (orange highlighted region). This construct is flanked by 5' and 3' cloning sites (M.C.S.), enabling the creation of polycistronic hairpins. (C) shmiR-*dpp2* knockdown oligos inserted into the HindIII–BamHI site of pHb. The siRNA sequence is highlighted in red, while the blue nucleotides represent the presumptive star sequence of the *dpp2* siRNA. Highlighted in yellow are two specific mismatched bases necessary to maintain the endogenous pre-*miR-1* stem-loop structure.

qPCR

~10 transgenic males carrying UAS-shmiR-*dpp*2HB or UAS-NLS-GFP were mated with ~10 transgenic females carrying daughterless-GAL4 at 25 °C. 2nd and 3rd instar larvae were collected as they emerged from the fly food and total RNA was extracted from 4–8 individuals per genotype, as described earlier. cDNA was prepared using the Quantitect RT-PCR kit (Qiagen) from 1 µg of total RNA. qPCR was performed using Applied Biosystems SYBR Green PCR Master Mix and analyzed on an

Applied Biosystems 7300 Real-Time PCR system. Sequences for the *RP49* and *dpp* primers are published in Supplemental File 1.

Results

Design and rationale of miRNA-based vectors

Previous attempts to use miRNA-based siRNA expression vectors {termed short hairpin RNAs or (sh)miRs} relied on the manual design

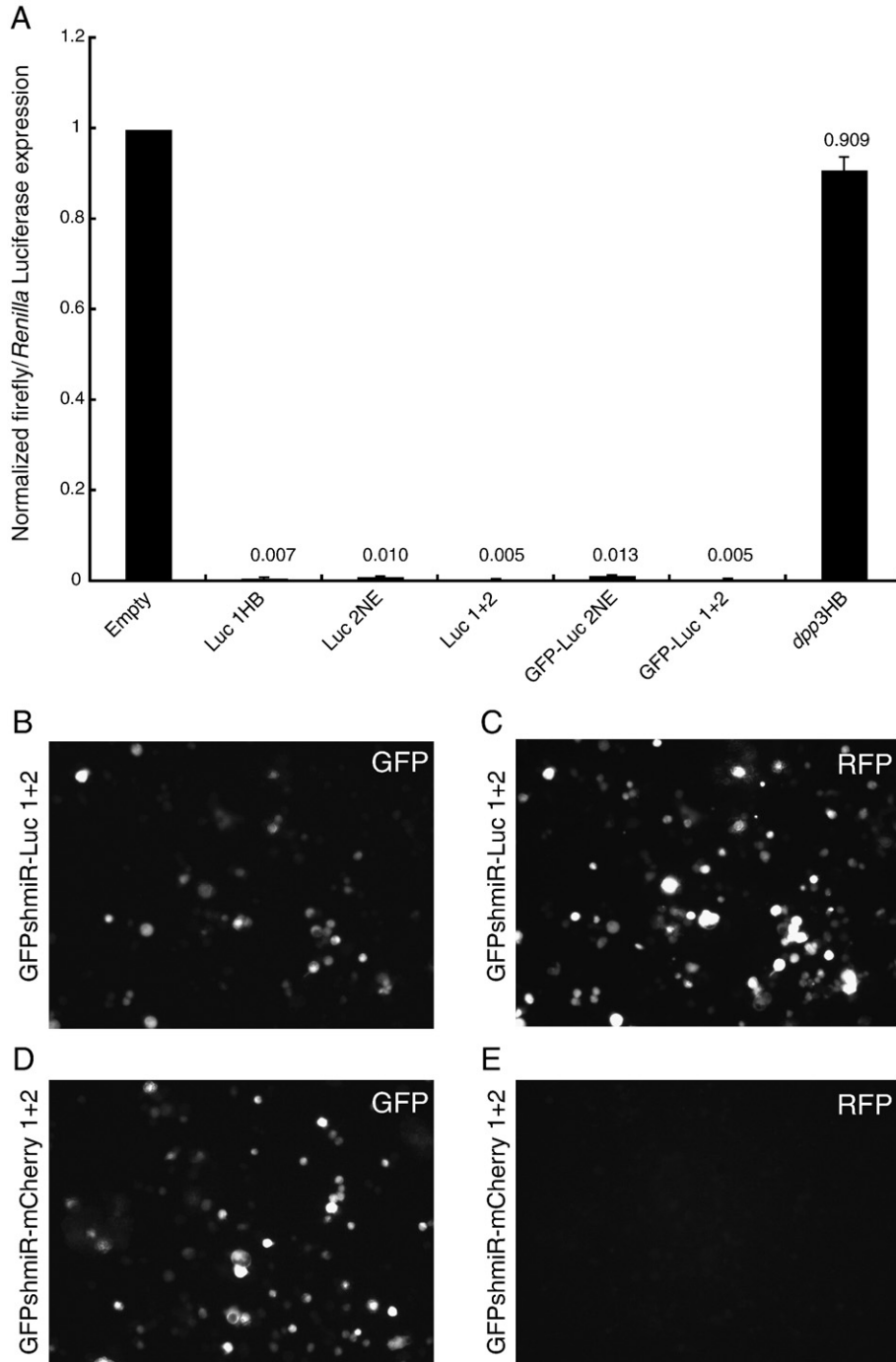


Fig. 2. Pre-miR-1-based shmiRs effectively silence a reporter gene *in vitro*. (A) Normalized ratios of firefly (target) to *Renilla* (control) Luciferase co-transfected with indicated constructs are presented, values are displayed above appropriate bars. Empty refers to an empty expression vector, and Luc 1HB and Luc 2NE refer to shmiRs targeting separate regions of the pGL3-derived firefly Luciferase gene, cloned using the pHB or pNE scaffold, respectively. Luc 1+2 is a bicistronic construct with shmiRs-Luc 1HB and Luc 2NE in tandem. GFP-shmiRs contain aforementioned shmiRs in the 3' UTR of EGFP, and *dpp*2HB expresses a *dpp*-targeting shmiR not expected to interact with the firefly Luciferase transcript. (B, C) RNAi control transfection using GFP-shmiR-Luc 1+2 from (A): EGFP expression (B) and mCherry/RFP expression (C). (D, E) Targeted RNAi transfection using GFP-shmiR-mCherry 1+2 (pHB and pNE shmiR tandem): EGFP expression (D) and mCherry/RFP expression (E).

of an appropriate hairpin sequence, synthesis of ~80-bp oligos, followed by one or two rounds of PCR amplification, and digestion with a restriction enzyme to produce the final expression vector (Fig. 1A, left) (Chen et al., 2007). We sought to streamline this process for high-throughput applications by creating a one-step cloning system (Fig. 1A, right).

The newly developed expression vectors are based on the *Drosophila melanogaster* pre-miR-1 stem-loop sequence since it contains all of the features predicted to be required for efficient processing of a mature miRNA (Fig. 1B). This includes an open secondary structure at the penultimate 5' position of the mature miR-1 sequence, which ensures efficient loading into RISC (Khvorova et al., 2003; Schwarz et al., 2003). A similar mismatch at position 11 is thought to direct the small RNA into the miRNA, not RNAi, biogenesis pathway (Tomari et al., 2007). In order to facilitate the rapid cloning of siRNAs into the miR-1 scaffold, specific mutations were introduced in the pre-miR-1 stem loop sequence that maintain the structure of the predicted pre-miR, including a fixed 7-nt unpaired region implicated in optimal miRNA processing (Han et al., 2006). For example, these mutations allow directional cloning of 71-bp synthetic oligos into HindIII–BamHI restriction sites (vector “pHB”, Figs. 1B and C). The stem-loop sequence is flanked by multiple cloning sites (M.C.S.), thereby facilitating the construction of polycistronic shmiRs, which are particularly potent in disrupting gene activity in mammalian systems (restriction maps of each

vector are published as Supplemental File 2) (Sun et al., 2006). We have also prepared derivatives of this vector—pNE2, pNE3, and pattB-NE3—that also contain the pre-miR-1 scaffold for direct cloning of 71-nt synthetic oligos harboring unique NheI–EcoRI cloning sites into derivatives of the previously described pUAST or pattB-UAST P-element transformation vectors (Bischof et al., 2007; Brand and Perrimon, 1993). This approach facilitates the creation of transgenic strains for targeted expression assays using the GAL4-UAS expression system (Phelps and Brand, 1998). An accompanying web-tool provides optimal hairpin design for either the pHB or pNE-based vectors (<http://flybuzz.berkeley.edu/cgi-bin/constructhairpin.cgi>).

Gene silencing assays in cultured S2 cells

In order to assess the effectiveness of the miRNA-based RNAi system, shmiRs were designed that target the firefly Luciferase RNA (Fig. 2A). We placed shmiRs in a mono- or bi-cistronic configuration in either a “naked” vector or in the 3' UTR of EGFP (hereafter referred to as GFP-shmiRX, with X identifying the target gene). The shmiR sequences were placed under the control of a strong Pol II promoter in order to mediate constitutive expression in transfected S2 cells. The GFP-shmiR expression vectors allow simple monitoring of transfection efficiency, and also permit FACS-based sorting of cells expressing shmiR constructs. Separate siRNAs derived from the pHB and pNE vectors, individually or linked as part of a bi-cistronic

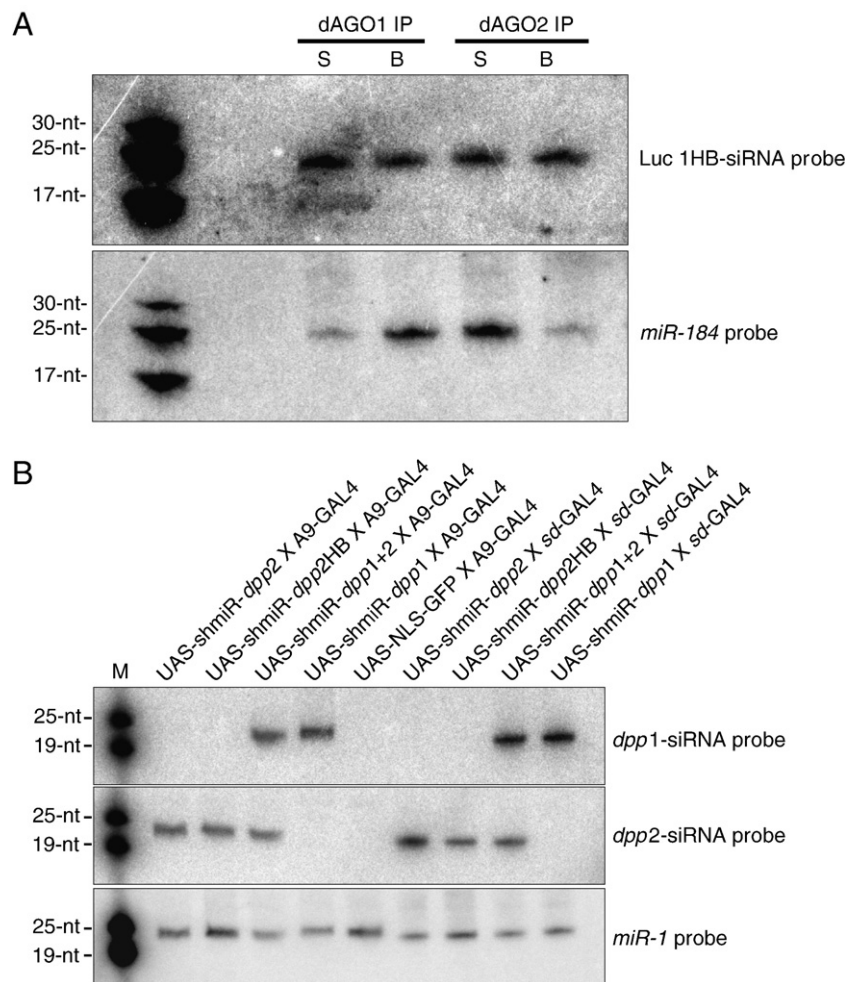


Fig. 3. Processing of small RNAs from endogenous miR-1 or pHB-based vectors *in vitro* and *in vivo*. (A) Northern blot for a shmiR-derived siRNA (Luc 1HB) or endogenous miRNA (miR-184) following AGO1 or AGO2 immunoprecipitation of S2 cell cytoplasmic extract. Unbound fraction is marked as “S” and antibody bound fraction is marked as “B”. 30, 25, and 17-nt RNAs were used as size markers. (B) Northern blot for siRNAs derived from transgenic strains of *Drosophila* upon UAS-GAL4 mediated expression of indicated *dpp* shmiRs or NLS-GFP transgenes, top. Each lane contains ~15 µg of total RNA extracted from climbing third instar larvae and 0–1 day-old pupae. 19 and 25-nt RNAs were used as markers.

construct (connecting one pHB-shmiR to a separate pNE-shmiR), provided >95% knockdown of firefly Luciferase (Fig. 2A). Overall, we observed peak silencing efficiency when shmiRs were expressed in tandem, consistent with previous reports from mammalian cell culture experiments (Sun et al., 2006). Moreover, shmiRs expressed individually or in tandem retained high efficiency when placed in the 3' UTR of EGFP. Overall efficiency of the shmiRs were comparable to analogous knockdown conditions using an ~500-bp dsRNA direct against Luciferase—published as Supplemental File 4.

Transfected cells were examined for the production of active siRNAs from the GFP-shmiR fusion RNAs (Figs. 2B–E). Here, tandem shmiRs targeting mCherry RNA were inserted in the 3'UTR of EGFP, and then co-expressed with mCherry. Indeed, all cells co-expressing GFP-shmiR-mCherry and the mCherry target RNA had nearly undetectable levels of red fluorescence (Fig. 2E), while those cells co-expressing the mCherry target RNA and a control expression vector (GFP-shmiR-Luciferase) exhibited strong red fluorescence (Fig. 2C). Thus, the siRNAs directed against mCherry RNA produce specific and quantitative loss of mCherry protein synthesis.

In order to assess whether the shmiR-derived siRNAs were entering into the miRNA or RNAi pathways, antibodies directed against AGO1 and AGO2 were used to immunoprecipitate (IP) RNA/protein complexes from S2 cells transfected with the shmiR-Luc 1HB-siRNA expression vector (Fig. 3A). As expected, the control miRNA, *miR-184*, was enriched in the AGO1-IP sample, but not in the AGO2-IP. In contrast, shmiR-derived products are partitioned almost equally between AGO1 and AGO2. Thus, it would appear that the shmiR-derived RNAs are loaded into both the AGO1-RISC and AGO2-RISC

complexes, suggesting engagement of both the siRNA and miRNA pathways of gene silencing.

Gene silencing assays in transgenic imaginal disks

Both the pHB version of the pre-*miR-1* scaffold (shmiR-*dpp*XHB, where X identifies a unique siRNA directed against the *dpp* mRNAs in the pHB pre-miR), as well as an unmodified version lacking the cloning sites (shmiR-*dpp*X), was used to express *dpp*-specific siRNAs *in vivo* in transgenic larvae and adults. *dpp* was selected as a target since it functions in a highly dosage-sensitive manner to produce a spectrum of well-defined mutant phenotypes in adult tissues, thereby providing an immediate measure of the effectiveness of siRNAs in suppressing *dpp* activity (Segal and Gelbart, 1985; Spencer et al., 1982). All synthetic hairpins were introduced into a modified version of pUAST, a *Drosophila* P-element expression vector containing five tandem UAS-response elements for the yeast GAL4 activator (Brand and Perrimon, 1993). We also compared the *dpp* mutant phenotypes obtained with mono- or bi-cistronic shmiR-*dpp* expression vectors. shmiRs were expressed in the *Drosophila* wing disc using A9-GAL4, which produces moderate levels of expression along the dorsal surface of the wing imaginal disc, or *sd*-GAL4, which directs strong expression in the developing wing pouch (Klein et al., 1997; Marquez et al., 2001). A9-GAL4 is located on the *Drosophila* X-chromosome, and consequently, stronger mutant phenotypes are observed in males as compared with females.

Two distinct regions of the *dpp* mRNA were targeted by the *dpp1*- and *dpp2*-siRNAs, respectively. Northern blot assays were performed with RNA extracted from transgenic stains expressing a unique shmiR

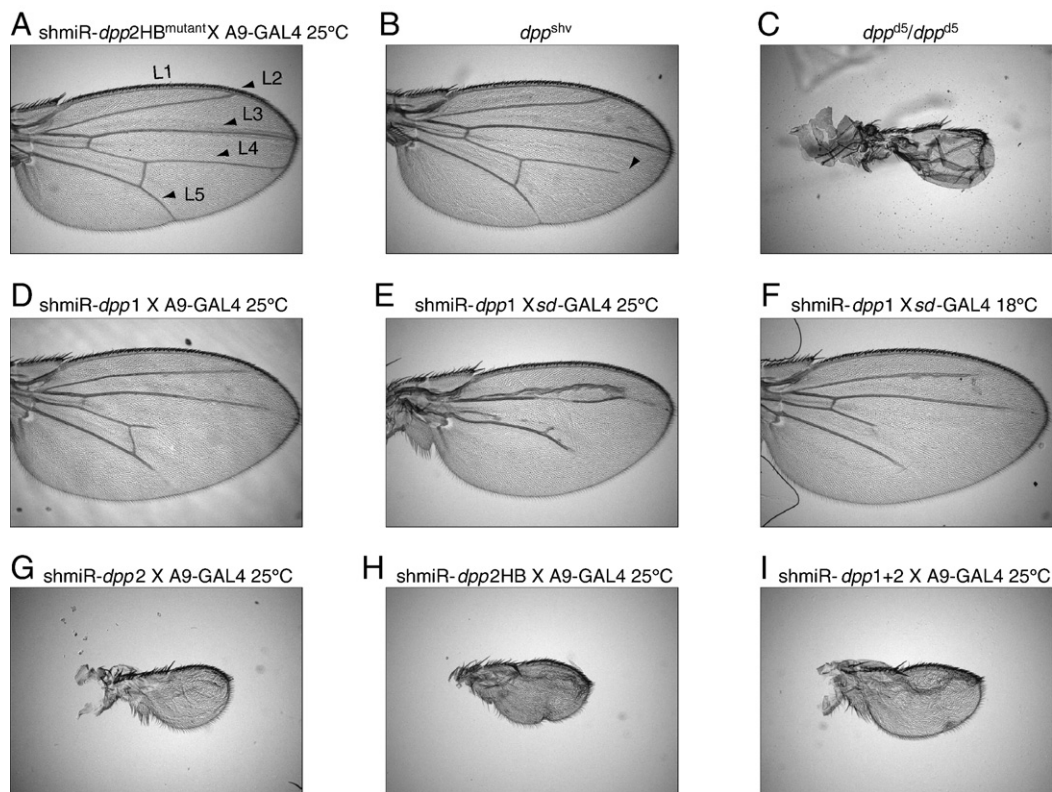


Fig. 4. Phenocopy of classical *dpp* wing mutations via GAL4-mediated expression of miRNA-based RNAi vectors. Only wings from male UAS/GAL4 flies are displayed in appropriate panels. (A) Ectopic expression of a control shmiR-*dpp2HB*^{mutant}—in the wing by A9-GAL4. Flies were reared at 29 °C to induce any potential GAL4 or non-specific shmiR effects at the highest level of expression. Arrowheads identify L1–L5 wing veins. (B) Classical, weak *dpp*^{shv} phenotype, with arrowhead indicating shortened L4 wing vein. (C) Severe *dpp*^{d5}/*dpp*^{d5} phenotype, showing small wings with no vein structures. (D) Weak *dpp* knockdown phenotype with *dpp* siRNA 1 in endogenous *miR-1*-based vector. (E) *dpp* knockdown phenotype obtained with the stronger *sd*-GAL4 driver. (F) Same as panel E except that a weaker phenotype is obtained at the lower temperature of 18 °C. (G) Phenocopy of strong *dpp* mutation with *dpp* siRNA 2, derived from an unmodified *miR-1* vector. (H) *dpp* knockdown phenotype with *dpp* siRNA 2 in a pHB-based vector. (I) *dpp* knockdown phenotype with bi-cistronic endogenous *miR-1*-based hairpins containing *dpp* siRNAs 1 and 2. See Supplemental Fig. 6 for additional characterization of *dpp2*-siRNA-induced phenotypes.

construct to determine the size and expression levels of the predicted siRNAs (Fig. 3B). For each construct, we observe the expected ~21-nt mature siRNA. All shmiR-*dpp1* and *dpp2* variants were efficiently and specifically processed, since larger precursor products (pre-miRNAs) were not detected. Moreover, insertion of an exogenous siRNA into the pre-miR-1 scaffold does not affect the inherent miR-1/miR-1* duplex asymmetry. This is shown by the different levels of *dpp2* and *dpp2** siRNAs produced by the shmiR-*dpp2* expression vector (see Supplemental File 5).

To assess potential non-specific effects, we created a mutant form of the *dpp2HB* shmiR (shmiR-*dpp2HB*^{mutant}) that contains <15 bp with any region of the *D. melanogaster* genome (Fig. 4A; see Supplemental File 1). The shmiR-*dpp2HB*^{mutant} produced no obvious mutant phenotypes in adult wings over a range of experimental conditions. Efficient expression of the mature *dpp2HB*^{mutant} siRNA is shown in Supplemental File 5. The directed expression of a nuclear-localized GFP (NLS-GFP) transgene also produced no wing phenotype (see Fig. 5B).

Both the *dpp1* and *dpp2* shmiRs recapitulated an allelic series of *dpp* mutants in adult wings, including progressive loss of wing vein tissue and reduction of tissue size (Fig. 4). shmiR-*dpp1* expressing flies induced a moderate *dpp* phenotype, resembling the shortened wing veins of the classical *dpp*^{shv} mutant (Figs. 4B, D–F) (Segal and Gelbart, 1985). Overall stronger phenotypes were observed for *dpp2* siRNAs derived from either pHB or the unmodified pre-miR-1 expression vectors (Figs. 4G, H; compare with C).

The dual-hairpin scaffold containing tandem *dpp1* and *dpp2* shmiRs triggered consistently more severe phenotypes among independent transgenic strains (Fig. 4I), although similar phenotypes were observed in strains with the strongest expression of *dpp2* alone (Figs. 4G, H). The transgenic lines expressing the highest levels of either single or bicistronic hairpins produced adult flies with a virtual loss of the wings and halteres, similar to the complete loss of *dpp* activity, exemplified by *dpp*^{d5} homozygotes (Fig. 4C) (Spencer et al., 1982). The specificity of these phenotypes suggests efficient and specific disruptions in *dpp* activity. Position effects are observed, as predicted for randomly integrated transgenes (see Supplemental File 6).

To assess whether the *dpp2* siRNAs cause degradation of *dpp* mRNA, larvae were produced from the mating of transgenic strains carrying UAS-shmiR-*dpp2HB* and the ubiquitous *daughterless*-GAL4

(da-GAL4) driver. Larvae are translucent and sluggish, similar to mutations in *glass bottom boat*, which encodes a TGF β signaling molecule related to *dpp* (data not shown, and Khalsa et al., 1998). RNA was extracted from these larvae and used for qPCR assays. There is a $\geq 75\%$ reduction in the normal levels of *dpp* mRNA as compared with larvae expressing GFP—published as Supplemental File 7.

The efficiency of shmiR and dsRNAs were compared using UAS-*dpp*-dsRNA transgenic lines (Ni et al., 2008). Two different dsRNAs directed against the *dpp* mRNA were expressed in wing imaginal disks using the A9-GAL4 driver. One of them, line 48A.1, overlaps the same region of the *dpp* mRNA as the shmiR-*dpp1*. Neither dsRNA produced a very strong phenotype. Females completely lacked a phenotype, while males displayed a weak *dpp*^{shv}-like phenotype (evidenced by a thinning L4 wing vein), even at the highest levels of expression (29 °C) (see Supplemental File 8). This result suggests that, shmiRs promote much stronger attenuation of *dpp*+ gene activity as compared with dsRNAs.

Wing phenotypes were also observed upon expression of mono- or bi-cistronic shmiRs directed against *Nelf-e* (Fig. 5). The NELF-E protein is part of a complex that binds to nascent transcripts associated with paused Pol II (Narita et al., 2003; Yamaguchi et al., 1999). Recent studies have shown that a large number of developmental control genes are paused in the early *Drosophila* embryo (Wang et al., 2007; Zeitlinger et al., 2007). Reduced *Nelf-e* activity might cause ectopic expression or hyperactivation of genes that are normally paused in the wing imaginal disk (Aida et al., 2006). Indeed, shmiR-*Nelf-e* expression vectors cause the formation of ectopic wing veins, quite distinct from the loss of veins caused by reductions in *dpp* activity (Figs. 5D–F). This phenotype might reflect ectopic activation of *dpp* and/or EGF signaling components (Marquez et al., 2001). As seen for *dpp* shmiRs, consistently more severe phenotypes were observed with tandem *Nelf-e* shmiRs (Fig. 5F).

Discussion

We have described the development and use of a series of novel expression vectors that permit targeted silencing of defined genes. The method depends on the expression of specific siRNAs via synthetic 71-nt stem-loop sequences within a modified pre-miR-1 scaffold. The resulting ~21-nt silencing RNAs are pre-defined and

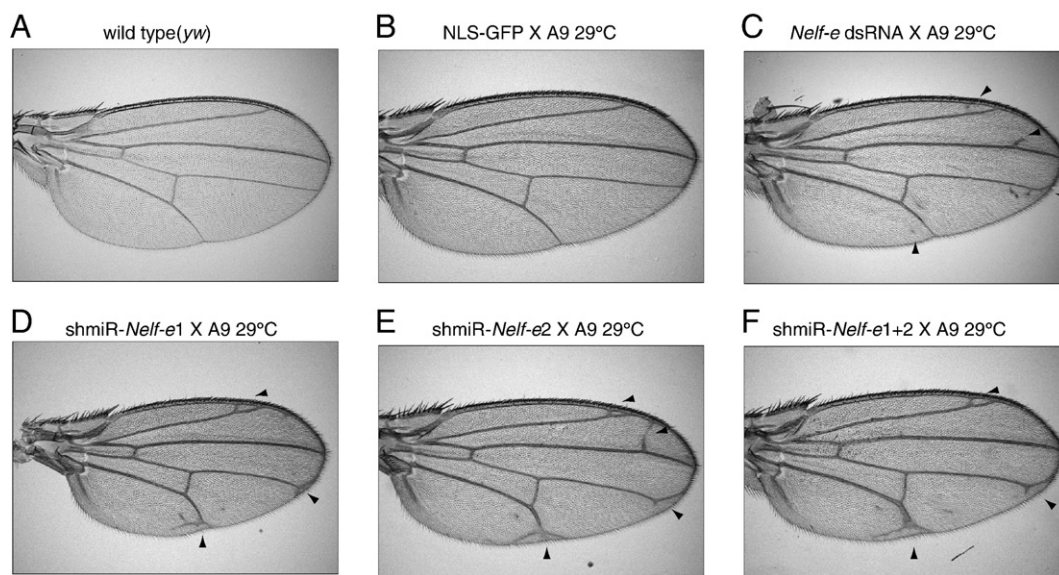


Fig. 5. *Nelf-e* knockdown phenotypes in the adult *Drosophila* wing. (A) Wild type adult wing from yw stock. (B) Ectopic expression of a NLS-GFP under the control of A9-GAL4. Flies were reared at 29 °C to induce any non-specific phenotypes due to general transgene overexpression. (C) Ectopic expression of a GAL4-responsive, ~800-bp dsRNA containing an inverted repeat that overlaps the *Nelf-e* ORF. Aberrant wing vein formation is marked by black arrowheads in panels C–F. (D) shmiR expression targeting region 1 of the *Nelf-e* transcript. (E) shmiR expression targeting region 2 of the *Nelf-e* transcript. (F) Bicistronic shmiR expression targeting both regions 1 and 2 of the *Nelf-e* transcript.

work through known molecular mechanisms. In contrast, the use of long dsRNAs creates the potential for unexpected siRNAs with unknown targeting activities. The direct comparison of different shmiRs and dsRNAs directed against *dpp* mRNAs suggests that the defined siRNAs produce much more efficient disruptions in *dpp+* gene activity. Moreover, tandem shmiRs producing two different siRNAs yield consistently stronger disruptions in gene activity, consistent with recent studies in mammalian systems (Sun et al., 2006).

We have also described P-element-based shmiR expression vectors that can be used with either the conventional $\Delta 2,3$ transposase or the recently developed phiC31 integrase system (Brand and Perrimon, 1993; Groth et al., 2004). The phiC31 system utilizes site-specific insertion of transgenes, effectively eliminating line-to-line variation between constructs. However, it is sometimes of interest to generate an allelic series for a particular gene (Hemann et al., 2003). Such a series is presented for *dpp* in Supplemental File 6. Less phenotypic variation is observed among independent lines using the tandem shmiR expression vectors, indicating that the single shmiR vectors should be used for the creation of an allelic series.

All of the shmiR expression vectors incorporate a single-step cloning method. Moreover, these vectors do not require the specialized strains of bacteria often used for the cloning of inverted repeats (Bao and Cagan, 2006). Instead, synthetic 71-nt shmiRs are directly inserted into the vectors, which could be done at a high throughput scale. In contrast, dsRNAs require template DNA preparation, primer design and synthesis, PCR amplification of the target region, and at least two additional cloning steps in order to create the final expression plasmid (Bao and Cagan, 2006).

At present, predictions of off-target effects by potential siRNAs produced from long-dsRNAs invoke a ≥ 19 -bp complementarity cut-off (Dietzl et al., 2007; Kulkarni et al., 2006). This criterion may be rather stringent and significantly underestimate off-target effects. Indeed, only 11 of 21- contiguous nucleotides were needed for detectable target RNA cleavage in *Drosophila* extracts, and only 15 total bps were necessary for off-target effects in human tissue culture experiments (Haley et al., 2003; Jackson et al., 2003; Martinez and Tuschl, 2004). The present method employs specific siRNAs that are designed to minimize off-target activities. Moreover, an integrated database containing all annotated *Drosophila* miRNAs was incorporated into the shmiR design webtool. All input siRNAs are tested for the presence of a seed sequence matching that of a known miRNA. Eliminating such seed sequence overlap has been shown to reduce off-target effects for a given siRNA (Birmingham et al., 2006; Jackson et al., 2006).

In summary, we have demonstrated the effectiveness of the miRNA-based RNAi system for silencing defined genes in S2 cells and transgenic flies. Since this method employs precise 21-nt siRNAs, it is less likely to produce non-specific, off-target effects than more commonly used long dsRNAs. The newly developed expression vectors permit one-step cloning through the use of inexpensive synthetic oligos, facilitating creation of whole-genome libraries for systematic gene disruption assays. The present system is based on current knowledge of miRNA processing and siRNA design. Since this field is advancing at a rapid pace, it is likely that simple modifications in the current method will yield even more efficient gene silencing in future years.

Author contributions

B.H. designed experiments. B.H. and V.T. carried out experiments. D.H. generated the automated shmiR design tool. B.H. and M.L. wrote the paper.

Competing interest statement

The authors declare that they have no competing financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.06.015.

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