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Cross-Species RNAi Rescue Platform in *Drosophila melanogaster*

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ABSTRACT

RNAi-mediated gene knockdown in *Drosophila melanogaster* is a powerful method to analyze loss-of-function phenotypes both in cell culture and *in vivo*. However, it has also become clear that false positives caused by off-target effects are prevalent, requiring careful validation of RNAi-induced phenotypes. The most rigorous proof that an RNAi-induced phenotype is due to loss of its intended target is to rescue the phenotype by a transgene impervious to RNAi. For large-scale validations in the mouse and *Caenorhabditis elegans*, this has been accomplished by using bacterial artificial chromosomes (BACs) of related species. However, in Drosophila, this approach is not feasible because transformation of large BACs is inefficient. We have therefore developed a general RNAi rescue approach for Drosophila that employs *Cre/loxP*-mediated recombination to rapidly retrofit existing fosmid clones into rescue constructs. Retrofitted fosmid clones carry a selection marker and a *phiC31 attB* site, which facilitates the production of transgenic animals. Here, we describe our approach and demonstrate proof-of-principle experiments showing that *D. pseudoobscura* fosmids can successfully rescue RNAi-induced phenotypes in *D. melanogaster*, both in cell culture and *in vivo*. Altogether, the tools and method that we have developed provide a gold standard for validation of Drosophila RNAi experiments.

RNAi-mediated gene knockdown, whereby an exogenous double stranded RNA (dsRNA) is used to trigger homology-dependent suppression of the target gene, is an effective loss-of-function method to interrogate gene function. The RNAi technology in *Drosophila melanogaster* is widely used for genomewide RNAi screens in cell culture (see review by Perrimon and Mathey-Prevot 2007a), and more recently has been extended to large scale *in vivo* studies (Dietzl *et al.* 2007; Ni *et al.* 2009; Mummery-Widmer *et al.* 2009). Gene knockdown by RNAi is achieved by the introduction of dsRNAs into cultured cells or by inducible overexpression of "hairpin" dsRNAs in transgenic flies. In the context of *in vivo* RNAi screening, the combination of a tissue-specific GAL4 driver with a GAL4-responsive hairpin dsRNA transgene allows knockdown of the target gene only in the desired cells, thus providing a powerful way of probing biological processes that have been so far difficult to investigate.

Analysis of the specificity of long dsRNAs in Drosophila cells has revealed that these reagents, depending on their sequences and levels of expression, can knock down genes others than the intended target (Kulkarni *et al.* 2006; Ma *et al.* 2006). This phenomenon is not specific to long dsRNAs and has also been commonly observed with 21-nt long siRNAs and shRNAs used in mammalian RNAi screens. In fact the rate of false positives associated with off-target effects observed in mammalian screens is usually higher than those observed with long dsRNAs (Echeverri and Perrimon 2006). Unwanted false positives created by off-target effects are a major problem in RNAi screens and require lengthy secondary validation tests (Echeverri and Perrimon 2006; Perrimon and Mathey-Prevot 2007b; Ramadan *et al.* 2007). Further, false positives associated with RNAi reagents are not limited to tissue culture experiments, as they have also been reported in the context of transgenic RNAi. For example, ~25% of the hairpins targeting nonessential genes cause lethality when driven by the constitutively expressed *Act5C-GAL4* driver (Dietzl *et al.* 2007; Ni *et al.* 2009).

A number of approaches can be used to validate the specificity of RNAi-induced phenotypes (Echeverri and Perrimon 2006). These include validation by multiple dsRNAs that target the same gene but that do not overlap in sequence, comparison of knockdown efficiencies of multiple dsRNAs and the phenotypic strengths, and rescue of the phenotype by either cDNAs or genomic DNAs. Rescue of RNAi phenotypes constitutes the gold standard in the field as it provides unambiguous proof that the targeted gene is indeed responsible for the phenotype observed. In Drosophila cell culture experiments, cDNAs that lack the original 3′-untranslated region (UTR) have been used to rescue

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phenotypes induced by dsRNAs targeting the 3′-UTR (Yokokura *et al.* 2004; Stielow *et al.* 2008). In mammalian cell culture experiments, cDNAs that have a silent point mutation in the region targeted by an siRNA are commonly used (Lassus *et al.* 2002). The intrinsic problem of these approaches, however, is that overexpression of cDNAs alone can evoke abnormal cellular responses on their own, complicating interpretation of the results. A cleaner method is based on cross-species rescue that uses genomic DNA from a different species whose sequence is divergent enough from the host species to make it refractory to the RNAi reagent directed against the host gene. This approach effectively addresses the issue of overexpression artifact, as the rescue transgene is expressed from its endogenous promoter, ensuring proper levels and precise spatiotemporal regulation of gene expression. Cross-species rescue methods that use bacterial artificial chromosome (BACs) retrofitted with an appropriate selection marker have been described for mammals and *C. elegans* (Kittler *et al.* 2005; Sarov *et al.* 2006). However, the BAC strategies are not realistic for large-scale studies, because transformation of BACs, which are typically larger than 100 kb, is inefficient, albeit not impossible, in Drosophila (Venken *et al.* 2006).

To provide a feasible way to validate large-scale RNAi screening results, we decided to develop a universal method for cross-species RNAi rescue in Drosophila. We chose to use fosmids, which are single-copy bacterial vectors with a cloning capacity of ~40 kb, rather than BACs because (1) transformation of plasmids around this size is relatively efficient (Venken *et al.* 2006) and (2) end-sequenced fosmid clones for 11 different Drosophila species generated by the Drosophila species genome project are now publicly available (Richards *et al.* 2005; Drosophila 12 Genomes Consortium 2007).

MATERIALS AND METHODS

Plasmid construction: *TKVBL-w+* and *TKVBL-GVB*, *plac-Cre* were constructed in an *R6K* vector, *pGPS1.1* (New England Biolabs) by standard molecular biology techniques. Briefly, *pGPS1.1* was PCR amplified with primers containing a *loxP* site and the PCR product was self-ligated to produce *TKL*. *attB* and *oriV* were PCR amplified from a BAC cloning vector, *attB-P[acman]-ApR-F-2-5-attB* (Venken *et al.* 2006) and inserted into *TKL* to produce *TKVBL*. The mini-white gene was excised from *pUAST* (Brand and Perrimon 1993) and inserted into *TKVBL* to produce *TKVBL-w+*. A Venus expression cassette comprising the *Act5C* promoter, *Venus* and the SV40 polyadenylation signal was excised from our custom *Venus* expression vector (S. Kondo, unpublished results) and cloned into *pCa4B2G* (Markstein *et al.* 2008) between the *gypsy* elements. The resulting *gypsy-Venus-gypsy* sequence was excised and inserted into *TKVBL* to produce *TKVBL-GAV*. The *blasiticidin* resistance gene cassette was excised from *pCoBlast* (Invitrogen) and cloned into *TKVBL-GAV* to produce *TKVBL-GVB*. *plac-Cre* was constructed by assembling the *lac* promoter and the *cre* gene by overlap extension PCR and cloning the PCR product into *pGPS1.1*. The *lac* promoter was amplified from *pBluescript II SK(+)* (Stratagene). The *cre* gene was amplified from *pCAGGS-Cre* (Araki *et al.* 1997). During the cloning processes, a *pir+* bacterial strain *EC100D pir-116* (Epicentre Technologies) was used for transformation.

TKVBL-w+, *TKVBL-GVB*, and *plac-Cre* are kanamycin resistant. Since these vectors carry only *R6K* as a replication origin, they cannot be amplified in regular bacterial strains, such as *DH5α* and *XL1-Blue*. For transformation and plasmid purification, *EC100D pir-116* was used. The sequences of *TKVBL-w+*, *TKVBL-GVB*, and *plac-Cre* have been deposited into GenBank (accession nos. FJ804475–FJ804477).

Fosmid clones: The following fosmid clones of *D. pseudoobscura* were obtained from BACPAC Resources Center at Children's Hospital Oakland Research Institute: CH1226-60K5 (no ORF), CH1226-68E13 (*diap1*), CH1226-44M20 (*dronc*), CH1226-71A5 (*Aurora B*), CH1226-72D18 (*ptc*), and CH1226-43A10 (*lz*).

Retrofitting fosmid clones: Bacterial stocks carrying a fosmid clone were streaked on LB plates (12.5 μg/ml chloramphenicol) and incubated overnight at 37°. The bacteria were made electrocompetent and transformed with the retrofitting and helper vectors according to the following protocol. A single colony was inoculated into 1 ml of LB (12.5 μg/ml chloramphenicol) and incubated with shaking (220 rpm) overnight at 37°. One hundred microliters of the overnight culture was transferred to 5 ml of fresh LB (12.5 μg/ml chloramphenicol) in a 50-ml plastic tube and incubated with shaking (220 rpm) at 37° for 3 hr. The bacteria were pelleted by centrifugation at 3000 rpm for 5 min at 4° in a precooled centrifuge. The supernatant was aspirated and the pellet was resuspended in 5 ml of ice-cold 10% glycerol. The bacteria were pelleted and resuspended in water two more times by the same procedure except that the final bacterial pellet was resuspended in 50 μl of ice-cold 10% glycerol. This bacterial suspension was used as electrocompetent cells. 0.1 μg each of the retrofitting vector (*TKVBL-w*$^{+}$ or *TKVBL-GVB*) and the *plac-Cre* helper vector was added to 25 μl of the electrocompetent bacteria. Electroporation was performed in a 1-mm gap cuvette (47727-640, VWR Scientific) with a 1.8-kV electric pulse using *Escherichia coli* Pulser (Bio-Rad). After electroporation, the bacteria were transferred to 1 ml of LB containing 0.1 mm IPTG and incubated with shaking (220 rpm) for 1 hr at 37° in a 15-ml plastic tube to induce the expression of *Cre* from the *lac* promoter of *plac-Cre*. They were subsequently plated on an LB plate (12.5 μg/ml chloramphenicol and 50 μg/ml kanamycin). The next day, a single colony was inoculated into 5 ml of LB (12.5 μg/ml chloramphenicol and 50 μg/ml kanamycin) in a 50-ml plastic tube and incubated with shaking (220 rpm) overnight at 37°. The retrofitted fosmid DNA was purified from the bacteria following standard procedure. To obtain large quantities of DNA, the retrofitted fosmid DNA was electroporated into *E. coli* EPI300 (Epicentre Biotechnologies), in which the copy number of a plasmid with an *oriV* replication origin can be induced to amplify up to 100 copies per cell. Transformed bacteria were plated on an LB plate (12.5 μg/ml chloramphenicol and 50 μg/ml kanamycin) and incubated overnight at 37°. A single colony was inoculated into 5 ml of LB (12.5 μg/ml chloramphenicol and 50 μg/ml kanamycin) in a 50-ml plastic tube and incubated with shaking (220 rpm) overnight at 37°. Copy-number induction was done using CopyControl Induction Solution (Epicentre Biotechnologies) according to the manufacturer's instruction on a 50-ml scale. Fosmid DNA was purified from the 50-ml culture by the QIAGEN plasmid kit. The typical DNA yield was between 50 μg and 100 μg.

Cell culture and transfection: S2R+ cells were maintained in Schneider's medium (Invitrogen) supplemented with

10% FBS and penicillin–streptomycin. Stable transfection of fosmid DNA was performed using the Effectene kit (QIAGEN) as follows. Cells were harvested from a confluent flask and diluted to 2.0×10^6 cells/ml. One microgram of fosmid DNA was diluted in 100 μl of Buffer EC (provided with the kit). Eight microliters of the Enhancer solution (provided with the kit) was added and incubated for 2 min at room temperature. Ten microliters of Effectene reagent was added and incubated for 5 min. The Effectene–DNA complex was transferred to 1 well of the 12-well plate. Seven hundred fifty microliters of the cell suspension was added to the well. Two days after transfection, the medium was changed to medium containing 25 μg/ml blasticidin S (Invitrogen). Stably transfected cells were selected by incubation in blasticidin S-containing media for 4 weeks. During this incubation period, the media was replaced every 4–5 days.

RNAi: RNAi experiments were performed essentially as described in Clemens *et al.* (2000) with the following modifications. First, 0.25 μg of dsRNAs was placed into each well of the 384-well plates. Suspended cells ($0.5–1.0 \times 10^4$) in serum-free Schneider's medium were added to each well. After 60-min incubation at 25°, 20 μl of Schneider's medium containing 20% FBS and 50 μg/μl blasticidin S was added to each well. Cells were incubated at 25° for indicated times before analysis. The following DRSC dsRNAs (http://flyrnai.org/) were used: *Aurora B* (DRSC23324) and *dronc* (DRSC11231). The template DNA for synthesizing *diap1* dsRNA was amplified from Drosophila genomic DNA with the following primers: TAATACGACTCA CTATAGGGGTGCTGGCCGAGGAGAAGG, TAATACGACTC ACTATAGGGACCGCATGGCAGGAATGCC.

Fly stocks and genetics: *lz*^{L}, *ptc*^{9}, *th*^{4}, *Df(2R)Exel7098*, *Df(3L)st-f13* were obtained from the Bloomington Drosophila Stock Center (BDSC). *dronc*$^{\Delta 8}$ and *GMR-GAL4 UAS-diap1-RNAi* have been described previously (Huh *et al.* 2004; Kondo *et al.* 2006). Transgenic flies were generated by injecting fosmid DNA into *nos-phiC31*; *attP40* eggs (Bischof *et al.* 2007). *attP40* is a *phiC31* second-chromosome landing site in which a transgene is not susceptible to silencing (Markstein *et al.* 2008). The *nos-phiC31*; *attP40* line is available from BDSC. Injections were performed by Genetic Services (Boston). Standard genetic crosses were used to obtain flies of indicated genotypes.

RESULTS

Development of retrofitting vectors: We developed a protocol to retrofit fosmid clones with desired selection markers for establishing stable cell lines and generating transgenic flies. The fosmid vector used to create the genomic libraries of various Drosophila species (Richards *et al.* 2005; Drosophila 12 Genomes Consortium 2007) contains a unique *loxP* site that can accept a new DNA cassette by *Cre/loxP*-mediated recombination. Several methods to retrofit BAC clones by *Cre/loxP*-mediated recombination have been described (Wang *et al.* 2001; Magin-Lachmann *et al.* 2003). We took a similar approach to retrofit fosmid clones by *in vivo* recombination in bacteria. We designed two retrofitting vectors, *TKVBL-GVB* and *TKVBL-w*$^{+}$, which carry selection markers for cell culture and fly transgenesis, respectively (Figure 1A). Retrofitting of a fosmid clone is done by simultaneous transformation of bacteria carrying the fosmid clone with a retrofitting vector and the *Cre* expression vector *plac-Cre* (Figure 1B). The retrofitting vectors have a single *loxP* site that recombines with the *loxP* site of a target fosmid clone upon expression of *Cre* in bacterial cells. Importantly, the retrofitting vectors and the *Cre* expression vector do not amplify in regular strains of *E. coli*, since their R6K conditional replication origin requires *Pi* initiator protein in the host cell. Thus, the retrofitting vector and the *Cre* expression vector are lost as the host bacteria propagate after transformation.

TKVBL-GVB contains Venus YFP for direct visualization and a *blasticidin S* resistance gene for antibiotic selection of transfected cells. Our initial transfection experiments of retrofitted fosmid clones revealed that the large genomic fragment in a fosmid clone often has a strong silencing activity against the expression of Venus (data not shown). Therefore, we included a *gypsy* insulator in *TKVBL-GVB* to prevent silencing of the marker genes (Markstein *et al.* 2008). The retrofitting vector for transgenesis, *TKVBL-w*$^{+}$, contains a mini-*white* gene for eye-color selection of transgenic flies and a *phiC31 attB* site to allow efficient site-specific integration of large-sized plasmids into the genome (Venken *et al.* 2006), which is extremely inefficient by conventional *P*-element transformation (Haenlin *et al.* 1985). The retrofitting vectors also contain a conditional origin of replication, *oriV*, which can be induced to amplify the copy number of otherwise single-copy fosmid vectors, thus allowing us to obtain large quantities of DNA (Wild *et al.* 2002).

We found the retrofitting reactions to be extremely accurate. Although we have performed more than 30 retrofitting reactions, we have not encountered a single erroneous clone. The retrofitting vectors are applicable to any low-copy vector with a *loxP* site. Indeed, we have successfully retrofitted several Drosophila BAC clones with a *loxP* site (data not shown).

Selection of species appropriate for RNAi rescue: Cross-species RNAi rescue requires selection of an appropriate "matched pair" composed of a dsRNA for gene knockdown and a rescue construct containing an orthologous gene from a different species. It has been shown previously that a 19-bp match in a dsRNA is sufficient for gene suppression (Kulkarni *et al.* 2006). Thus, the dsRNA and the orthologous gene should, at the minimum, not have identical stretches of nucleotide sequences ≥19-bp long. However, the amino acid sequences encoded by the genes from host and donor species should be as similar as possible, as differences may prevent the donor protein from functioning properly in the host species. In the present study, we chose *D. pseudoobscura*, which is relatively closely related to *D. melanogaster*, but at the same time is divergent enough at the DNA sequence level to test our approach (Figure 2, A and B). The more closely related Drosophila species, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, and *D. ananassae* are usually not suitable for RNAi rescue due to high conservation of DNA sequences in the coding regions.

RNAi rescue in cultured Drosophila cells: To test the efficacy of the fosmid-based rescue method in cultured

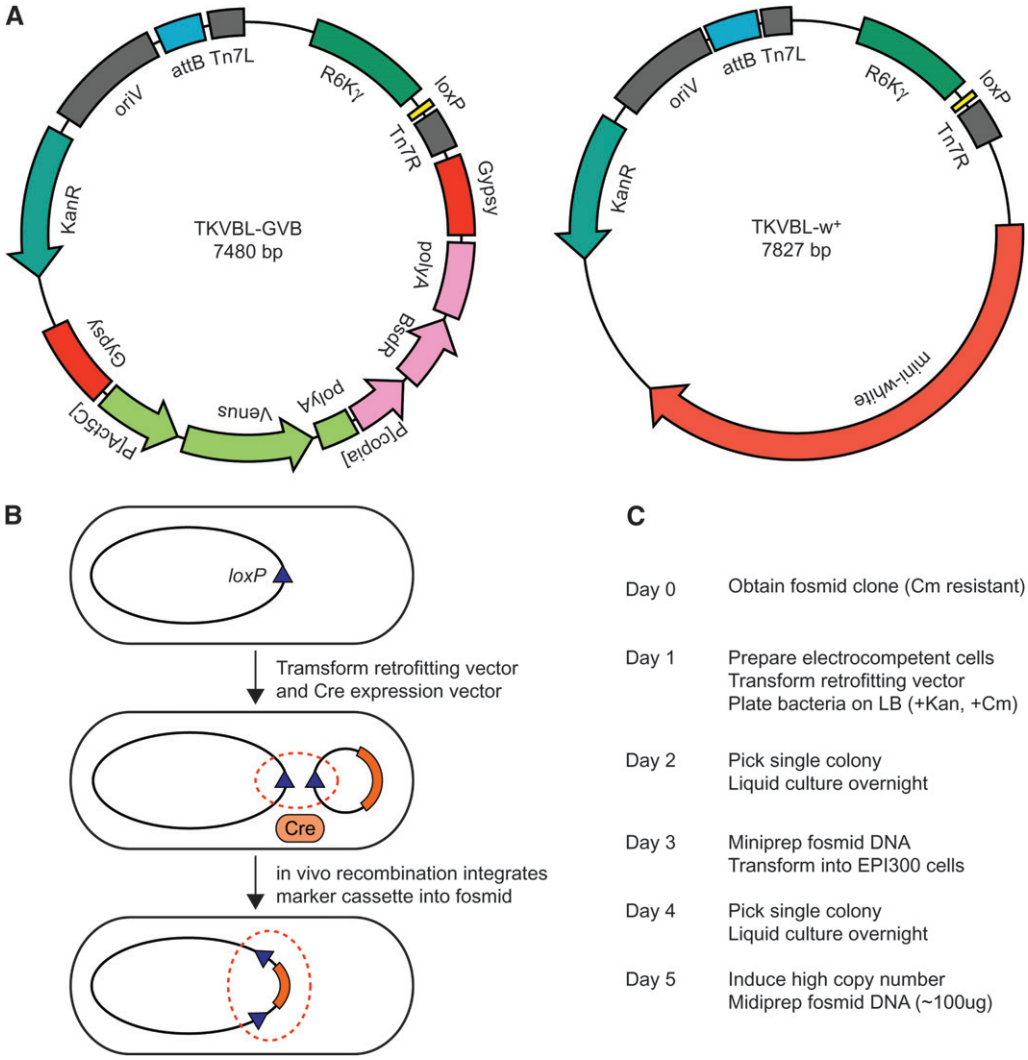

FIGURE 1.—Vectors and procedure for *in vivo* retrofitting of fosmid DNAs. (A) Map of the retrofitting vectors. Site-specific integration of retrofitting vector is mediated by recombination between the *loxP* site of each retrofitting vector and the *loxP* site of a target plasimd. The retrofitting vectors also include Tn7 transposon ends, which provide an alternative means for site-specific integration. The Tn7-based approach has not been tested. (B) Scheme of the retrofitting reaction. (C) Timeline of the retrofitting procedures. Tn7L, Tn7 transposon left inverted repeat; Tn7R, Tn7 transposon right inverted repeat; polyA, polyadenylation signal; BsdR, blasticidin resistance gene; P[copia], copia promoter; P[Act5C], Act5C promoter; KanR, kanamycin resistance gene; Cm, chloramphenicol; and Kan, kanamycin.

cells, we chose three genes that give specific and strong phenotypes when depleted by RNAi: *Aurora B* (*ial* in FlyBase), a kinase essential for cytokinesis (EGGERT *et al.* 2004); *diap1*, a ubiquitin-ligase constitutively antagonizing activation of caspases to prevent precocious apoptosis (HAY *et al.* 1995); and *dronc*, the initiator caspase required for apoptosis (KONDO *et al.* 2006). By searching fosmid clone end sequences at the NCBI Trace Archive, we identified multiple *D. pseudoobscura* clones that contain the ortholog of either of the three genes. For each gene, we selected clones that contain upstream and downstream sequences up to the adjacent genes with the hope of including most, if not all, of the transcriptional regulatory elements (Figure 2C). These fosmids were retrofitted with *TKVBL-GVB* to add the *Venus* and *blasticidin S* resistance genes. Then, we generated polyclonal S2R+ cells stably transfected with the retrofitted fosmids by blasticidin S selection.

To ask if the transgenes can indeed rescue RNAi-induced phenotypes, we treated the stably transfected cells with the dsRNA against each gene. We chose dsRNAs that lack contiguous sequence matches of ≥19 bp in the *D. pseudoobscura* counterpart, to avoid possible suppression of the rescue transgene (Figure 2B). RNAi against *Aurora B* results in a highly specific and easily discernible phenotype characterized by drastic enlargement of the cell and nuclear size due to inhibition of cytokinesis (Figure 3A). When S2R+ cells harboring the *D. pseudoobscura Aurora B* transgene were treated with a dsRNA targeting *D. melanogaster Aurora B*, the phenotype was completely rescued allowing the cells to conduct normal cell divisions. By contrast, cytokinesis of the control cells stably transfected with an unrelated fosmid was effectively blocked by *Aurora B* RNAi, ruling out the possibility that cells gain general resistance to RNAi after stable transfection of retrofitted fosmids. Likewise, the *dronc* and *diap1* RNAi phenotypes were completely suppressed by the *D. pseudoobscura* rescue fosmids (Figure 3, B and C). RT–PCR analysis confirmed that the *D. pseudoobscura* genes were expressed in S2R+ cells and that they were refractory to RNAi against *D. melanogaster* genes (Figure 3D, data not shown). These results demonstrate that *D. pseudoobscura* genes carried on fosmid clones are expressed in heterologous *D.*

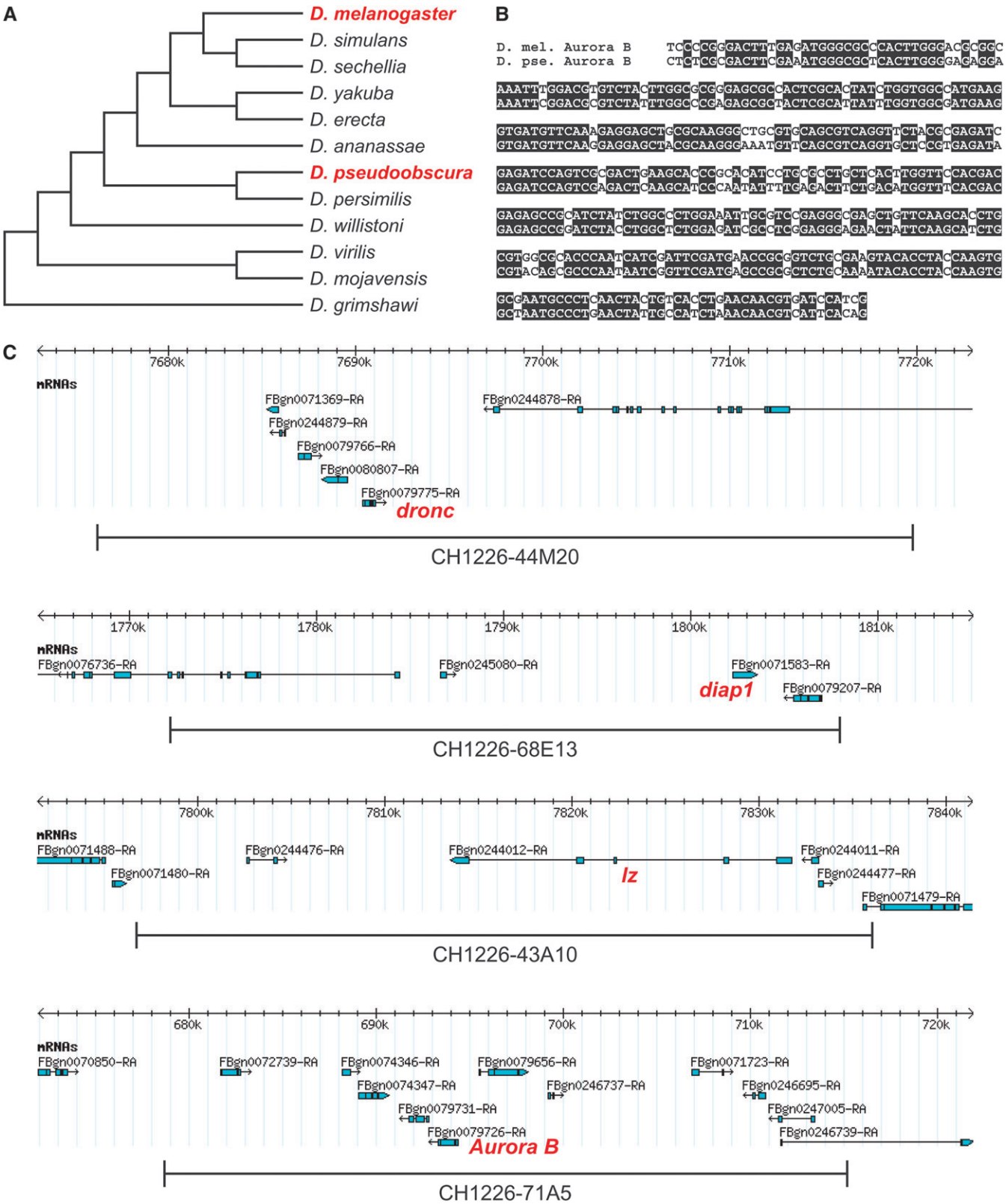

FIGURE 2.—dsRNAs and fosmid clones used in rescue experiments. (A) Phylogenetic tree of the 12 Drosophila species whose genome has been fully sequenced. In the present study, *D. pseudoobscura* is used because it is the closest species whose DNA sequences are sufficiently divergent with respect to *D. melanogaster*. (B) Alignment of *Aurora B* genes of *D. melanogaster* and *D. pseudoobscura*. The 384-bp region targeted by the dsRNA used in Figure 3 is shown. (C) The gene contents of the fosmid clones used in Figures 3 and 4.

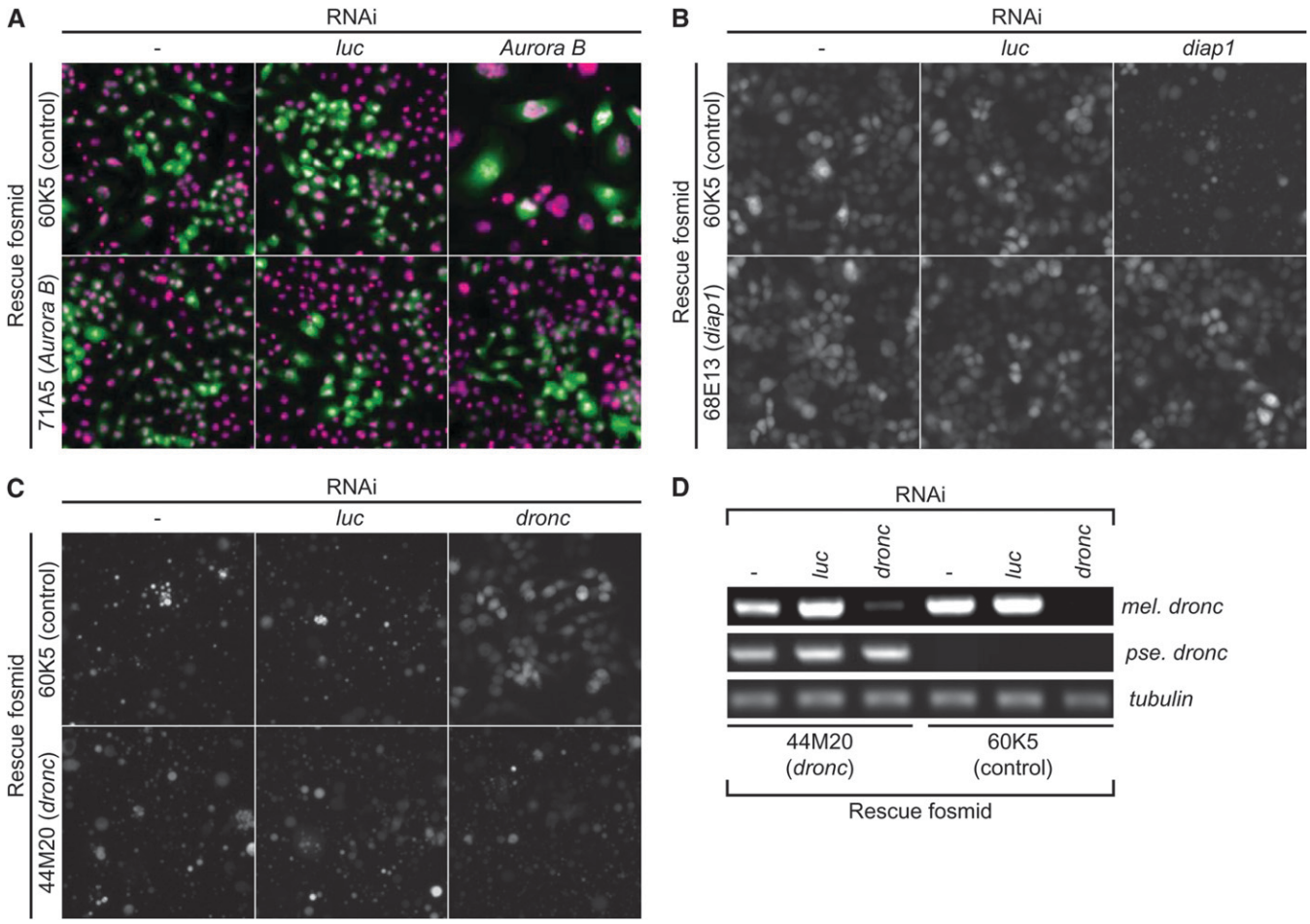

Figure 3.—RNAi rescue in S2R+ cells. (A–C) S2R+ cells stably transfected with a control fosmid (60K5) and rescue fosmids (71A5, *Aurora B*; 68E13, *diap1*; and 44M20, *dronc*) were either untreated or treated with a dsRNA against luciferase or a test dsRNA. Stably transfected S2R+ cells express *Venus* (green in A and *white* in B and C). The level of GFP expression is highly variable between cells due to the polyclonality of the cell line. Cells were either fixed and stained with DAPI [magenta in A or imaged live (B and C)]. *Aurora B* RNAi causes enlargement of the nuclei due to cytokinesis defect (upper right in A). Widespread apoptosis induced by *diap1* RNAi is characterized by small blebs and rounded-up cells (upper right in B), whereas live cells are flat and firmly attached to the dish surface (the other panels in B). In C, all the cells were treated with a *diap1* dsRNA to induce apoptosis. Simultaneous RNAi knockdown of the initiator caspase *dronc* blocks this apoptosis (upper right panel in C). All of the three RNAi phenotypes were fully recued by the respective rescue fosmids (lower right in A–C). (D) RT–PCR analysis of the cells stably transfected with 60K5 (control) or 44M20 (*dronc*) fosmids. The cells were treated or untreated as in C and total RNAs were extracted after 3 days. RT–PCR of *alpha-tubulin* was used as a control. *D. pseudoobscura dronc* is abundantly expressed in S2R+ cells, and the dsRNA against *dronc* suppresses endogenous *D. melanogaster dronc*, while it does not perturb the expression of the *D. pseudoobscura dronc* transgene.

melanogaster S2R+ cells and that they can functionally complement their *D. melanogaster* counterparts to rescue RNAi phenotypes.

RNAi rescue in transgenic flies: Next, we examined whether RNAi phenotypes induced by overexpression of a long hairpin RNA in transgenic flies could be rescued by *D. pseudoobscura* fosmids. *In vivo* RNAi rescue presents a new level of challenge, since the transgene has to recapitulate the complex spatiotemporal regulation of gene expression. To get a general idea of how effectively cross-species rescue works *in vivo*, we first tested whether *D. pseudoobscura* fosmids could rescue *D. melanogaster* mutants. For these experiments, we chose two developmentally regulated genes *lozenge* (*lz*) and *patched* (*ptc*) and two ubiquitously expressed genes involved in apoptosis, *diap1* and *dronc*. *ptc*, *diap1*, and *dronc* mutants are embryonic lethal, while *lz* mutants are viable with a severely deformed eye structure. The *lz* and *ptc* loci are almost 40 kb in size, suggesting that they have a number of enhancer elements that produce dynamic spatiotemporal expression pattern during development. *lz*, the Drosophila homolog of AML1, plays an important function in the development of hemocytes and eye development (Daga *et al.* 1996). Null mutants of *lz* are viable but have severely disorganized eye structures. *ptc* encodes a membrane receptor for Hedgehog and is required for various developmental processes during both embryogenesis and imaginal tissue development (Hooper and Scott 1989). In all four cases, *D. pseudoobscura* fosmids successfully rescued

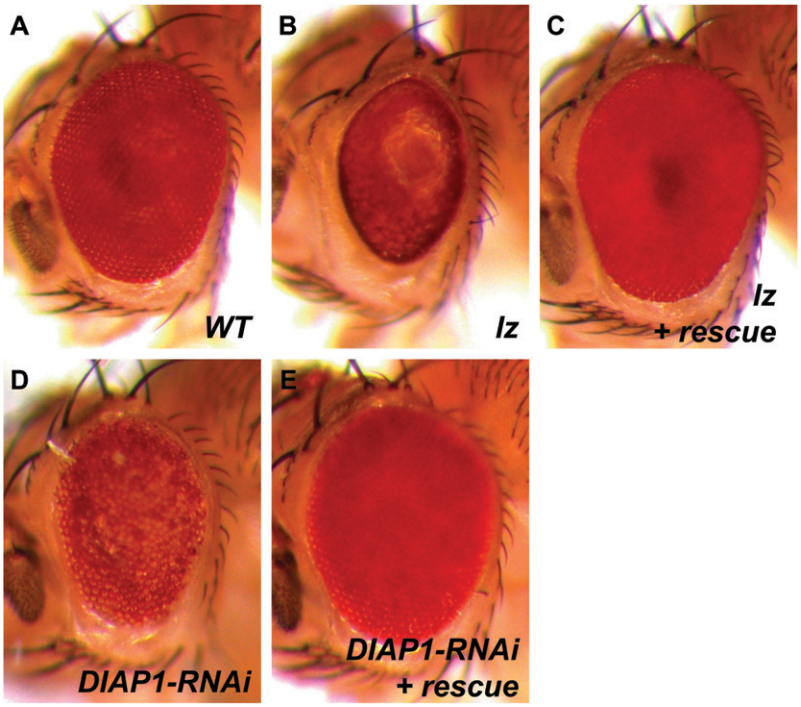

FIGURE 4.—Rescue of mutant and RNAi-induced phenotypes. (A) Wild-type, (B) *lz*^{1}*/Y*, (C) *lz*^{1}*/Y*; *43A10-attP40*, (D) *GMR-GAL4 UAS-DIAP1-RNAi/+*, and (E) *GMR-GAL4 UAS-DIAP1-RNAi/ 68E13-attP40*. The rough-eye phenotypes of *lz* mutants (B) and *DIAP-RNAi* transgenics (C) are completely suppressed by the respective rescue fosmids (D and E).

the viability and developmental abnormalities associated with genetic mutations in these genes (Figure 4, A–C, and data not shown). Importantly, although *P*-element transposition of large DNA fragments is known to be very inefficient (HAENLIN *et al.* 1985), the use of targeted integration by *phiC31* recombination dramatically improved the transformation efficiency (VENKEN *et al.* 2006), allowing us to obtain transgenic flies for all of the four fosmids that were injected.

To expand the *in vivo* fosmid rescue method to RNAi-induced phenotypes, we generated transgenic flies overexpressing a dsRNA directed against *diap1* specifically in the developing eye (HUH *et al.* 2004). These flies show a strong rough-eye phenotype due to widespread apoptosis during eye development. When they were crossed to flies harboring *D. pseudoobscura diap1*, the rough-eye phenotype induced by *diap1*-RNAi was completely suppressed (Figure 4, D and E), indicating that *D. pseudoobscura* fosmids can effectively be used to rescue both *D. melanogaster* mutants and transgenic RNAi-induced phenotypes.

Web-based tool and resources: In addition to using *D. pseudoobscura* fosmids to rescue *D. melanogaster* RNAi-induced phenotypes, genomic DNAs from other species can obviously be used. To facilitate the search of the optimal fosmid clones to be used in rescue experiments we have constructed a Web-based tool (Figure 5, http://www.flyrnai.org/cgi-bin/RNAi_find_rescue_compl.pl). Upon entry of a *D. melanogaster* gene symbol, CG identifier, or FlyBase identifier, the tool displays a genome map of the orthologous region of a specified sibling species along with fosmid clones that map to the region, allowing users to visually select a fosmid clone that includes the gene of interest. Since our goal was to tailor this Web site to screeners that use the Drosophila RNAi Screening Center (DRSC) RNAi libraries, the tool also displays a list of the DRSC dsRNAs and the number of nucleotide identity matches to the genes from each selected sibling species, with an adjustable threshold for nucleotide match sensitivity. This allows users to select dsRNAs that are unlikely to suppress the rescue transgene by off-target effects. In addition, the protein primary sequence similarity between the *D. melanogaster* gene and the orthologous gene from each sibling species is displayed.

Regarding the availability of the fosmid libraries, end-sequenced fosmid clones of *D. pseudoobscura* are available from BACPAC Resources Center (http://bacpac.chori.org), *D. virilis*, *D. sechellia*, *D. simulans*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. willistoni*, *D. mojavensis*, and *D. grimshawi* from the Drosophila Genomics Resource Center (DGRC, https://dgrc.cgb.indiana.edu/). To facilitate the access of fosmid reagents to DRSC screeners, we obtained the end-sequenced fosmid library of *D. persimilis*, which is a closely related species of *D. pseudoobscura*, from the DGRC. The library comprises ~70,000 clones, equivalent to a 20-fold coverage of the genome. As shown in Figure 5, the fosmid clone density is sufficiently high such that each locus is covered by >10 clones. *D. persimilis* fosmid clones are available from the DRSC upon request (http://www.flyrnai.org).

Concluding remarks: Here, we have described a streamlined method to create large genomic rescue constructs from fosmid clones of different Drosophila species for rescuing RNAi phenotypes, and demonstrated that the *D. pseudoobscura* fosmids rescue RNAi phenotypes effectively in both tissue cell culture and in whole animals. Because of the prevailing off-target effects

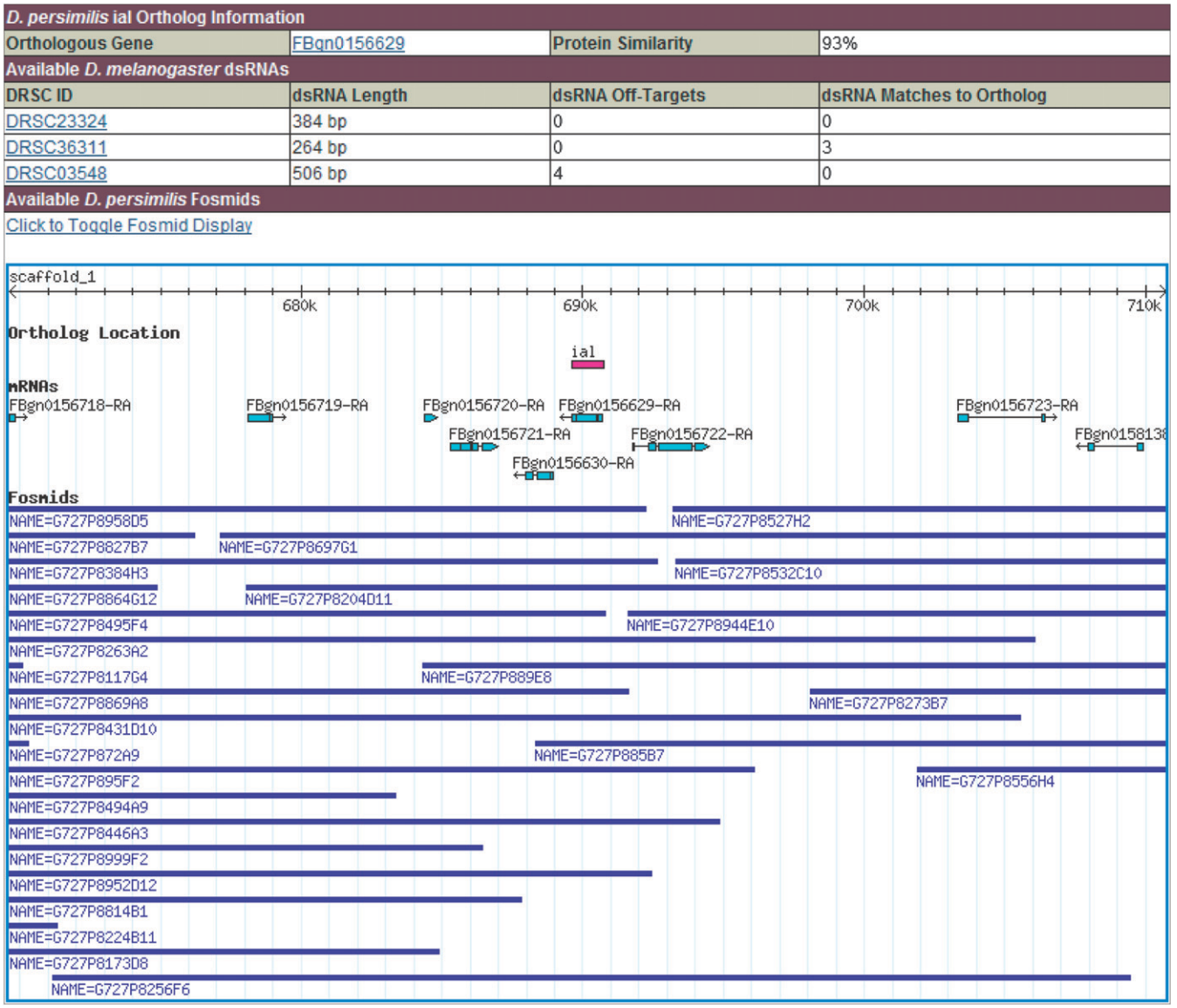

Figure 5.—Web-based tool for finding Drosophila species fosmids. The tool displays a genome map with tiling fosmid clones and information on the species ortholog and available DRSC dsRNAs. (See RESULTS for details.)

in RNAi experiments (Kulkarni *et al.* 2006; Ma *et al.* 2006), this cross-species rescue method should become the gold standard in validation of RNAi phenotypes.

Another approach for generating rescue transgenes is to build an entire genomic DNA library in a fosmid vector that contains appropriate markers and a *phiC31 attB* site. A *D. pseudoobscura* genomic DNA library was recently constructed in a fosmid vector with an *attB* site (Ejsmont *et al.* 2009). The advantage of this approach is that the fosmid clones can be directly used for transformation of flies. However, the clones of this library cannot be used for RNAi rescue in cell culture, since the vector contains no selection markers. Further, libraries for other species are currently not available. In these respects, our retrofitting method provides a quick and flexible solution, making effective use of the existing resources.

In the present study, we showed that *D. pseudoobscura* genes can functionally complement *D. melanogaster* genes. Some genes show extremely high homology between these two species and the use of *D. pseudoobscura* fosmid clones are not suitable for RNAi rescue. In such cases, it is necessary to use fosmid clones of more distantly related species. Since our method is applicable to any fosmid vector with a *loxP* site, one can simply choose an appropriate species and generate a rescue construct. Further, to facilitate identification of suitable fosmid clones from multiple different species, we designed a Web-based tool that can simultaneously search three different species, *D. pseudoobscura*, *D. persimilis*, and *D. virilis*.

In addition to their use for RNAi rescue, retrofitted fosmids of Drosophila species should be useful for evolutionary biology studies that aim to identify geno-

mic elements that modify species-specific features. For example, species-specific pigmentation patterns of the body and the wing are known to arise from differential expression patterns of genes involved in melanin biogenesis (Gompel *et al.* 2005; Jeong *et al.* 2008). Further, differences in the expression cycle of the *period* gene lead to distinct circadian rhythms in *D. melanogaster* and *D. pseudoobscura* (Petersen *et al.* 1988). Thus, we anticipate that our retrofitted fosmid method, as it provides a simple way to create transgenic animals carrying a large genomic DNA fragment of another species, will allow the systematic identification of genomic regions that modify species-specific features.

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