The Transgenic RNAi Project at Harvard Medical School: Resources and Validation

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ABSTRACT To facilitate large-scale functional studies in *Drosophila*, the *Drosophila* Transgenic RNAi Project (TRiP) at Harvard Medical School (HMS) was established along with several goals: developing efficient vectors for RNAi that work in all tissues, generating a genome-scale collection of RNAi stocks with input from the community, distributing the lines as they are generated through existing stock centers, validating as many lines as possible using RT–qPCR and phenotypic analyses, and developing tools and web resources for identifying RNAi lines and retrieving existing information on their quality. With these goals in mind, here we describe in detail the various tools we developed and the status of the collection, which is currently composed of 11,491 lines and covering 71% of *Drosophila* genes. Data on the characterization of the lines either by RT–qPCR or phenotype is available on a dedicated website, the RNAi Stock Validation and Phenotypes Project (RSVP, http://www.flyrnai.org/RSVP.html), and stocks are available from three stock centers, the Bloomington *Drosophila* Stock Center (United States), National Institute of Genetics (Japan), and TsingHua Fly Center (China).

KEYWORDS RNAi; *Drosophila*; screens; phenotypes; functional genomics

A striking finding from the genomic revolution and whole-genome sequencing is the amount of information missing on gene function. Although *Drosophila* is arguably the best-understood multicellular organism and a proven model system for human diseases, mutations mapped to specific genes with readily detectable phenotypes have been isolated for ~15% of the >13919 annotated fly coding genes (http://flybase.org/; FlyBase R6.06). The lack of information on the majority of genes (the "phenotype gap") suggests that researchers have been unable to either assay their roles experimentally and/or resolve issues of functional redundancy. In addition, some phenotypes may be only detected on specific diets and environments. Further, our understanding of the function of many genes for which we have some information is limited by pleiotropy, whereby an earlier function of the gene prevents analysis of later functions.

The availability of *in vivo* RNAi has revolutionized the ability of *Drosophila* researchers to disrupt the activity of single genes with spatial and temporal resolution (Dietzl *et al.* 2007; see review by Perrimon *et al.* 2010), and thus address the phenotype gap. Motivated by the power of the approach and the needs of the community, three large-scale efforts, the Vienna *Drosophila* RNAi Center (VDRC, http://stockcenter.vdrc.at/control/main), the National Institute of Genetics (NIG, http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp),

doi: 10.1534/genetics.115.180208
Manuscript received July 8, 2015; accepted for publication August 24, 2015; published Early Online August 27, 2015.
Supporting information is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.180208/-/DC1.
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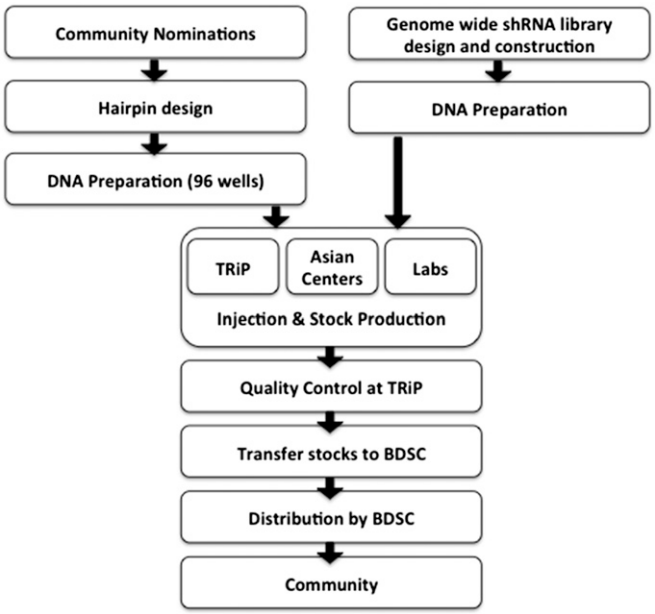

Figure 1 The TRiP Platform: production pipeline flowchart. Details are included in the text.

Table 1 Summary of RNAi and overexpression vectors from the TRiP

Vectors	Features	Maps	References
	RNAi vectors		
VALIUM1	Long dsRNA, vermilion	Figure S1	Ni *et al.* (2008)
VALIUM10	Long dsRNA, vermilion	Figure S1	Ni *et al.* (2009)
VALIUM20	shRNA, vermilion	Figure S1	Ni *et al.* (2011)
VALIUM21	shRNA, vermilion	Figure S1	Ni *et al.* (2011)
VALIUM22	shRNA, vermilion	Figure S1	Ni *et al.* (2011)
WALIUM10	Long dsRNA, mini-white	Figure S1	This work
WALIUM20	shRNA, mini-white	Figure S1	This work
WALIUM22	shRNA, mini-white	Figure S1	This work
Q-UAS-WALIUM20	shRNA, mini-white	Figure 1	This work
	Overexpression vectors		
VALIUM10-roe	Vermilion	Figure S1	This work
VALIUM10-moe	Vermilion	Figure S1	This work
WALIUM10-roe	Mini-white	Figure S1	This work
WALIUM10-moe	Mini-white	Figure S1	This work

All vectors are available from the DF/HCC DNA Resource Core PlasmID repository at Harvard Medical School. The order page for the TRiP vectors can be found at http://plasmid.med.harvard.edu/PLASMID/GetVectorsByType.do?type=drosophila%20in%20vitro%20and%20in%20vivo%20expression. Maps with attributes, complete sequences, and detailed cloning protocols can be found on the TRiP website (http://www.flyrnai.org/TRiP-HOME.html) under Reagents, Maps, and Protocols.

and the *Drosophila* Transgenic RNAi Project (TRiP) at Harvard Medical School (HMS) (http://www.flyrnai.org/TRiP-HOME.html) have over the years generated large numbers of RNAi lines that aim to cover all *Drosophila* genes. These resources are proving invaluable to address a myriad of questions in various biological and biomedical fields including but not limited to research in the areas of cell biology, signal transduction, cancer, neurodegeneration, metabolism, and behavior.

The TRiP was initiated in 2008 with the goals of developing efficient vectors for RNAi, generating and distributing a genome-scale collection, and providing validation of the fly stocks. Below, we describe details on our TRiP production pipeline (Figure 1), reagents generated, state of the collection, and validation efforts as of May 2015. Note that the project is still ongoing. Regular updates are provided on the TRiP website as well as at FlyBase and stock center websites.

Materials and Methods

Vector construction

Knockdown vectors: Construction of the first-generation TRiP knockdown vectors, pVALIUM1 and pVALIUM10, are described in Ni *et al.* (2008) and Ni *et al.* (2009), respectively. The second-generation TRiP knockdown vectors, pVALIUM20 and pVALIUM22, are described in Ni *et al.* (2011). To construct pWALIUM10, pVALIUM10 was first cut by *Hin*dIII to remove the *vermilion* gene and then annealed DNA oligos carrying an *Asc*I cutting site (forward primer, 5′-AGCTTCACGACCTGAGGCGCGCCA-3′, reverse primer, 5′-AGCTTGGCGCGCCTCAGGTCGTGA-3′) were inserted into the linearized vector. The *mini-white* gene was cut from pUAST using *Asc*I and was then cloned into the *Asc*I site. The orientation of the *mini-white* gene was confirmed by restriction enzyme digestion and DNA sequencing, and the resulting vector with the correct orientation was named pWALIUM10. pWALIUM20 and pWALIUM22 were constructed in a similar way based on pVALIUM20 and pVALIUM22, respectively, using the same DNA oligos.

Overexpression vectors: To construct pVALIUM10-roe, pVALIUM10 was cut by *Xba*I to remove a fragment containing the *ftz* intron and attR. The resulting vector was named as pVALIUM10-roe. pVALIUM10-roe was further cut by *Eco*RI and *Xba*I, and then an annealed DNA product (forward primer, 5′-AATTCGCAGATCTCCATATGAGCTAGCTACTAGTGTC-3′, reverse primer, 5′-CTAGACACTAGTAGCTAGCTCATATGGAGATCTGCG-3′) was inserted to generate pVALIUM10-moe. pWALIUM10-roe and pWALIUM-moe were constructed in the same way as described above, using pWALIUM10 and the same DNA oligos.

Construction of a QUAS vector for RNAi: From the TRiP RNAi vector, WALIUM20, the two copies of loxp-5xUAS sequences were deleted and replaced with the 94-bp QUAS sequence (five copies of the QF binding site). Briefly, the QUAS fragment was obtained by PCR from the pQUAST vector (Potter *et al.* 2010). The linearized WALIUM20 backbone was generated by PCR from the WALIUM20 vector to exclude the two copies of loxp-5xUAS. Then the QUAS fragment was inserted into the linearized WALIUM20 backbone using an in-fusion reaction (Clontech).

All of the TRiP vectors described above are available from the PlasmID repository of the Dana Farber/Harvard Cancer Center (DF/HCC) DNA Resource Core at Harvard Medical School (http://plasmid.med.harvard.edu/PLASMID/).

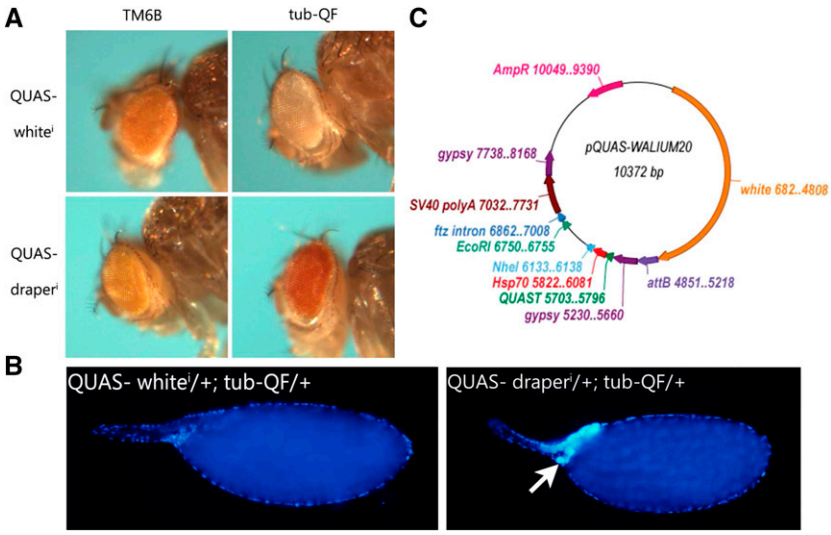

Figure 2 The pW20-QUAST vector for RNAi. (A) *Drosophila* eyes from flies expressing *QUAST-white-RNAi* (*QUAST-white-i*) (top) or *QUAST-draper-i* (bottom) with the *tubulin-QF* driver or *TM6B* controls. Eye pigmentation is reduced in flies expressing *white-i* but not *draper-i*. The difference in eye pigmentation between *QUAST-draper-i*/+; *tubulin-QF*/+ and *QUAST-draper-i*/+; *TM6B*/+ flies is due to the number of transgenes. (B) Stage 14 egg chambers from flies expressing *QUAST-white-i* (left) or *QUAST-draper-i* (right) with the *tubulin-QF* driver. Nurse cells have been cleared normally in the *white-i* egg chamber, but nurse cells persist (arrow) in the *draper-i* egg chamber. (C) Map of the QUAS vector.

Generation of Transgenic RNAi lines

dsRNA lines: Long dsRNA hairpins were cloned into VALIUM series vectors (VALIUM1 or VALIUM10) and injected into embryos for targeted phiC31-mediated integration at genomic attP landing sites on the second (attP40) or third (attP2) chromosomes as described by Ni *et al.* (2008) and Ni *et al.* (2009). All transgenic lines were sequenced to confirm the identity of the dsRNA hairpin.

shRNA lines: shRNAs (21 bp) were cloned into VALIUM series vectors (VALIUM20, VALIUM21, or VALIUM22) and injected into embryos for targeted phiC31-mediated integration as described by Ni *et al.* (2011). All transgenic lines were sequenced to confirm the identity of the shRNA and miR-1 scaffold.

RNA Isolation, Reverse Transcription, and Real-Time qPCR

Detailed protocols are provided in the Extended Materials and Methods of Sopko *et al.* (2014). Briefly, RNA was isolated by guanidinium thiocyanate-phenol-chloroform extraction using TRIzol (Life Technologies) and glass-bead-based cell disruption. Genomic DNA was eliminated by incubation with DNase (QIAGEN), and samples were processed for cleanup with an RNeasy MinElute Cleanup Kit (QIAGEN). One microgram of purified RNA was incubated with a mix of oligo(dT) and random hexamer primers and with iScript RT (iScript cDNA Synthesis Kit, Bio-Rad) for complementary DNA (cDNA) synthesis. cDNA was used as the template for amplification, using validated primers in iQ SYBR Green Supermix with a CFX96 real-time PCR detection system (Bio-Rad). Query gene expression was relative to a control sample, normalized to the expression of three reference genes: *ribosomal protein L32*, *α-tubulin*, and either *nuclear fallout* or *Gapdh1*, using the $^{\Delta\Delta}C_T$ analysis method.

Rescue constructs

Fosmid rescue constructs: *D. persimilis* clones were obtained from the DGRC and then retrofitted for site-specific insertion into *D. melanogaster* as described in detail in Kondo *et al.* (2009). Fosmids appropriate for testing rescue of specific genes were identified using the RNAi Rescue online tool (http://www.flyrnai.org/cgi-bin/RNAi_find_rescue_compl.pl) (Kondo, *et al.* 2009).

C911 constructs: Fifteen efficient shRNAs were chosen for generation of C911 versions: *aurora* (*aur*, FBgn0000147), *Tao-1* (FBgn0031030), *wee* (FBgn0011737), *grapes* (*grp*, FBgn0261278), *PAK-kinase* (*Pak*, FBgn0014001), *Sak kinase* (*SAK*, FBgn0026371), *Cyclin-dependent kinase 8* (*Cdk8*, FBgn0015618), *loki* (*lok*, FBgn0019686), *hopscotch* (*hop*, FBgn0004864), *dropout* (*dop*, FBgn0036511), *gilgamesh* (*gish*, FBgn0250823), and *slipper* (*slpr*, FBgn0030018). Twenty-one-basepair sequences identical to the original targeting shRNA but with complementary nucleotides at positions 9–11 were synthesized, cloned into pVALIUM20 or pVALIUM22 (Supporting Information, Table S3), and injected into embryos for targeted phiC31-mediated integration as described by Ni *et al.* (2011). Injection was at the same attP site as the original efficient targeting shRNA. All transgenic lines were sequenced.

Data availability

All TRiP stocks are available at the BDSC (LINK), NIG (LINK) and THFC (LINK). All vectors are available from the DF/HCC DNA Resource Core PlasmID repository at Harvard Medical School. The order page for the TRiP vectors can be found at http://plasmid.med.harvard.edu/PLASMID/GetVectorsByType.do?type=drosophila%20in%20vitro%20and%20in%20vivo%20expression. Maps with attributes, complete sequences, and detailed cloning protocols can be found on the TRiP website (http://www.flyrnai.org/TRiP-HOME.html) under Reagents, Maps, and Protocols.

Results and Discussion

The TRiP Vectors

Vectors for RNAi: Over the years, the TRiP has generated a series of 22 knockdown vectors (Vermilion-AttB-Loxp-Intron-UAS-MCS; VALIUM), to facilitate the incorporation

Pool #	# shRNAs in pool	attP	# embryos injected	% larvae recovered	% fertile G0s	% G0s giving transformants	# transformants	# sequenced	# shRNAs recovered
1	9	P2	776	50	19	6	7	7	4 (44%)
2	15	P40	856	32	32	14	78	78	13 (87%)
3	15	P40	933	42	32	14	112	112	15 (100%)
4	15	P40	919	44	24	17	123	123	14 (93%)
5	12	P40	988	65	27	14	115	115	10 (83%)
6	4	P40	535	26	17	21	31	31	4 (100%)

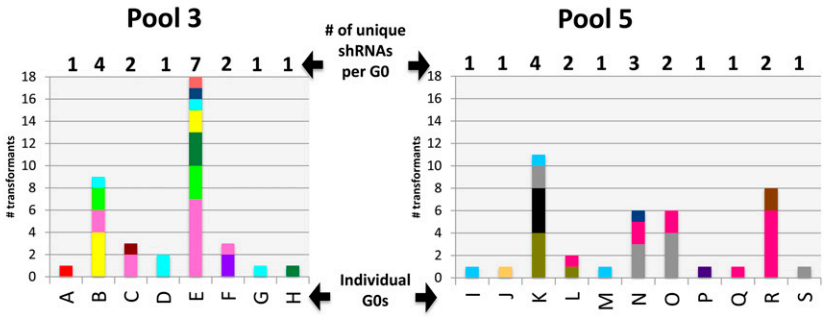

Figure 3 Pooled injection results. Examples of the frequencies of independent shRNA transgenic lines recovered from six different pools of individual constructs. Details from single G0's from Pools 3 and 5 are shown. Note that seven different shRNA transformants were recovered from a single G0 from pool 3 that contains 15 different constructs.

of RNAi hairpins into attP landing sites (Ni *et al.* 2008, 2009, 2011; Table 1; Figure S1). In Table 1 and Figure S1 we list the most commonly used vectors as well as those not previously reported. All VALIUM vectors contain a wild-type copy of *vermilion* as a selectable marker and an attB sequence to allow for phiC31 targeted integration at genomic attP landing sites (Groth *et al.* 2004). *vermilion* was chosen rather than *mini-white* as the proper gene dosage of *white* has been found to be important in behavioral studies (An *et al.* 2000). The VALIUM vectors were also designed with two pentamers of upstream activation sequence (UAS) sequences, one of which can be removed using the Cre/loxP system. Thus, based on findings that UAS sites promote transcription in an additive fashion, if Gal4 is not limiting, the modular number of UAS copies allows for generation of a phenotypic series. Moreover, because the attP chromosome is usually homozygous viable, it is possible to generate 5X, 10X, 15X, and 20XUAS combinations (Ni *et al.* 2008). In addition to manipulating the number of UAS sequences, the level of RNAi knockdown can also be altered by using Gal4 lines of various strengths, rearing flies at different temperatures, or via coexpression of *UAS-Dicer2* (Dietzl *et al.* 2007). Note that Dicer2 (Dcr2) is only effective with VALIUM1 and VALIUM10-series RNAi fly stocks, as Dcr2 processes long double-strand RNAs (dsRNAs) but not the short hairpin RNAs (shRNAs) used with other TRiP vectors (see below).

The first-generation knockdown vectors chosen by the TRiP for RNAi stock production were VALIUM1 and VALIUM10 (Table 1 and Figure S1). Both allow expression of long dsRNA hairpins, usually between 400 and 600 bp. These are very effective for RNAi in somatic tissues but are not as effective in the female germline (Ni *et al.* 2008, 2009). Subsequently, we showed that shRNAs containing a 21-bp targeting sequence embedded into a micro-RNA (miR-1) backbone are very effective for gene knockdown in both the germline and soma (Ni *et al.* 2011). For shRNA expression we developed the second-generation knockdown vectors, VALIUM20, VALIUM21, and VALIUM22 (Table 1 and Figure S1)(Ni *et al.* 2011). All subsequent TRiP lines were generated with shRNAs in VALIUM20 (for knockdown in germline or soma) or VALIUM22 (germline only).

Since some researchers prefer to use *mini-white* as the selectable marker for transgenesis, we also generated new versions of the VALIUM vectors in which *vermilion* is replaced with *white* (WALIUM10, WALIUM20, and WALIUM22; Table 1 and Figure S1). Except for the selectable marker, the WALIUM vectors have the same attributes as their *vermilion* containing counterparts, VALIUM10, VALIUM20, and VALIUM22, respectively. We, and others, have used these WALIUM vectors and found that they function as well as the VALIUM vectors for transgenic RNAi.

A QUAS vector for RNAi: The Q system (Potter *et al.* 2010) provides an alternative to the GAL4/UAS system. The Q system is particularly valuable when the expression of two different genes needs to be targeted to two different cell types in the same fly, which is made possible by combining the Q and Gal4/UAS systems. For example, to express *geneX* in the germline and *geneY* in follicle cells, flies of genotype *germline-Gal4*, *UAS-geneX*; *follicle cell-QF*, *QUAS-geneY*, can be generated. To allow such applications, we built and tested a QUAS vector. The new vector, pW20-QUAST, is effective for knockdown, as shown for the genes *white* and *draper* (Figure 2), and should be effective when combined with Gal4/UAS.

Vectors for overexpression: For many genes, overexpression phenotypes can provide valuable information on gene function and provide tools for epistasis experiments and genetic screens. After using TRiP RNAi lines for gene knockdown, members of the fly community asked the TRiP to develop a vector that allowed them to overexpress genes using a comparable strategy. Thus, we generated *vermilion* and *mini-white* versions of VALIUM10 for overexpression experiments (Table 1 and Figure S1). Specifically, we generated

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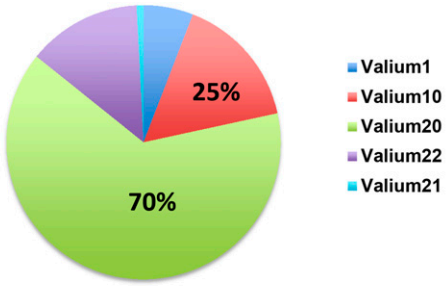

B

	1st generation		2nd generation		
TRiP KD vectors	**VALIUM1**	**VALIUM10**	**VALIUM20**	**VALIUM22**	**VALIUM21**
# TRiP Stocks	**661**	**1,784**	**7,437**	**1,513**	**93**
TRiP ID nomenclature	**JF**xxxxx[1]	**JF**xxxxx[1] **HM**xxxxx[2]	**HMS**xxxxx[3] **HMC**xxxxx[4] **HMJ**xxxxx[5]	**GL**xxxxx[6]	**GLV21**xxxxx[7]
relative efficiency	*	**	***	***	**
works best in	**soma**	**soma**	**soma & germline**	**germline**	**germline**
hairpin	**long dsRNA**	**long dsRNA**	**shRNA**	**shRNA**	**shRNA**
vector size	**6,214bp**	**11,142bp**	**8,357bp**	**8,973bp**	**7,904bp**
selectable marker	**vermilion**	**vermilion**	**vermilion**	**vermilion**	**vermilion**
cloning method	**MCS**	**recombination**	**MCS**	**MCS**	**MCS**
promoter	**HSP70**	**HSP70**	**HSP70**	**P-transposase**	**P-transposase**
terminator	**SV40**	**SV40**	**SV40**	**K10**	**K10**
insulator	**yes**	**yes**	**yes**	**yes**	**no**

Figure 4 Summary of available TRiP fly stocks. (A) The percentage of RNAi fly stocks available in each vector is indicated. (B) Main features of the vectors and details on the stocks available: (*) Still in production, accepting nominations; (1) JF: generated at Janelia Farm; (2, 3, and 7) HMS: generated at the TRiP at Harvard Medical School; (4) HMC: generated at the TRiP (HMS) in collaboration with the TsingHua Fly Center (THFC), China; (5) HMJ: generated at the TRiP in collaboration with the National Institute of Genetics (NIG), Japan; (6) Generated at the TRiP, the TRiP and THFC, or the TRiP and NIG.

pVALIUM10-roe, pVALIUM10-moe, pWALIUM10-roe, and pWALIUM10-moe. These vectors allow cloning for overexpression by recombinational cloning ("roe" versions for recombination overexpression) or into a multicloning site ("moe" for multicloning overexpression). Note: that all of the TRiP vectors described above are available from the DF/HCC Plasmid Resource Core in Boston (http://plasmid.med.harvard.edu/PLASMID/).

The TRiP collection

Generation of the collection: All fly stocks generated at the TRiP are inserted into one of two attP sites, *attP40* on the left arm of the second chromosome at 25C6 or *attP2* on the left arm of the third chromosome at 68A4. These sites were selected for their abilities to provide high levels of induced expression of the transgenes, yet maintain low basal expression when the transgenes are not induced (Markstein *et al.* 2008). The landing site chosen by the TRiP for hairpin insertion is guided first by the preference of the community member nominating the gene and second by the TRiP. If a TRiP stock for a particular gene is available in one location a second TRiP stock for the same gene will be generated in the second location. In May 2015, of the 11,491 total TRiP stocks, 5232 (45%) are in attP40 (II) and 6259 (54%) are in attP2 (III).

We used both single-construct cloning and pooled library approaches to generate the TRiP plasmid constructs. Either dsRNA or shRNA constructs were generated individually in 96-well plates, or, in the case of shRNAs, they were selected from the shRNA libraries generated in VALIUM20 and VALIUM22 starting from a pool of 83,256 unique shRNA oligonucleotides synthesized on glass slide microarrays (Ni *et al.* 2011). For the TRiP constructs that were constructed individually in 96-well plates, we used two different approaches to generate transformed fly stocks. Early in the project, constructs were injected individually into attP40- or attP2-bearing lines and transformants were recovered. As only one attB insert can integrate by phiC31-mediated recombination into an attP site (Groth *et al.* 2004), we later injected pools of constructs, established transgenic lines, and then subsequently characterized the inserted DNA by sequencing. This approach proved to be extremely efficient and quickly became the method of choice (Figure 3). Typically 20–25 constructs per pool were injected, although in some cases more complex pools from the VALIUM20 and VALIUM22 libraries were injected (see above).

From the shRNA libraries, we initially injected pools derived from the VALIUM20 library, established transgenic lines, and then determined the sequence of the inserted vector for each line. However, we found that approximately one-third of the lines carried either an empty vector or a vector with an incorrect shRNA sequence, which likely reflected the error frequency of the original library. Thus, we decided to first sequence individual clones and pool only those with a correct sequence. We retransformed the library into bacteria and sequenced 31,953 clones. Of those, 19,239 had a correct shRNA sequence, representing 8694 unique clones targeting 4856 different genes. To generate fly stocks from these "quality selected" libraries, we pooled and then injected 3903 clones targeting genes for which no VALIUM20 stocks had been made.

State of the collection: To date the TRiP has generated 11,491 stocks and has ~3386 in final production stages, and ~53 additional genes have been nominated by the *Drosophila* community or are part of the Gene Groups and Hu-Dis Projects described below (Figure 4 and Figure 5). Altogether, the collection covers 9803 unique FBgns or 71% of the genes in the fly genome (Flybase Release 6.05) with 81% of highly conserved genes represented as determined using the

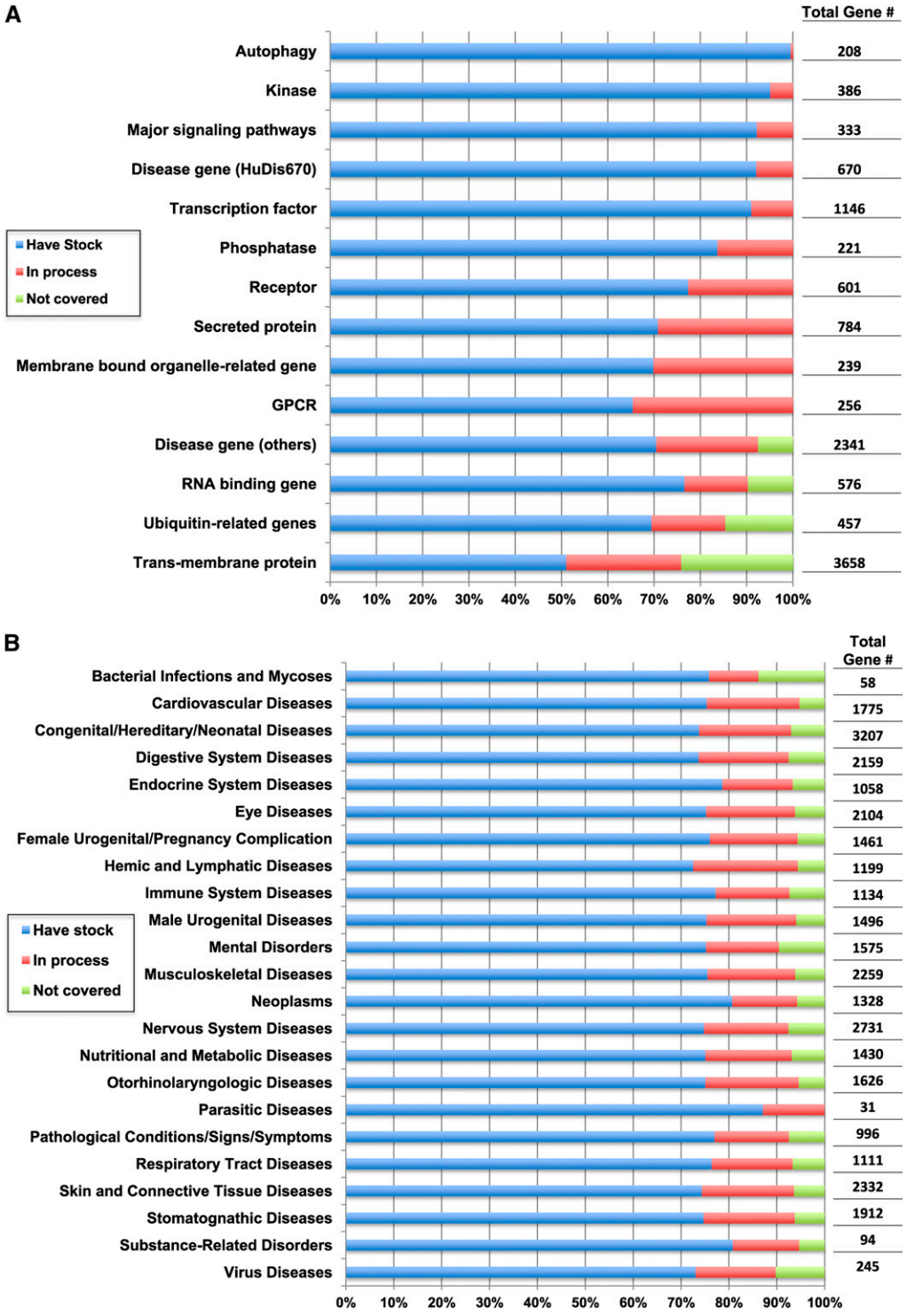

Figure 5 TRiP fly stocks corresponding to specific gene categories. (A) TRiP coverage of specific gene groups. (B) TRiP coverage of human disease orthologs. List of genes in each category is available at http://www.flyrnai.org/glad as well as the TRiP website at http://www.flyrnai.org/TRiP-HOME.html. The number of genes in each category is indicated next to the name category. Note that the lists on GLAD are updated periodically so the numbers may not match perfectly.

DRSC Integrative Ortholog Prediction Tool (DIOPT), (Hu *et al.* 2011).

Approximately 2445 TRiP stocks generated with long dsRNA hairpins were inserted into first-generation VALIUM vectors, VALIUM1 and VALIUM10 (Figure 4). Primers for the TRiP double-strand hairpins were designed using the amplicon design tool SnapDragon at the *Drosophila* RNAi Screening Center (DRSC; http://www.flyrnai.org/DRSC-HOME.html) (Flockhart *et al.* 2012). When possible, sequences common to all splice forms of the gene of interest were chosen as long as they did not include 19-bp matches to other sequences in the genome. Specific and detailed descriptions of the generation of long dsRNA hairpins can be found in Ni *et al.* (2008, 2009).

All remaining TRiP stocks (>9000) were generated with shRNAs inserted into the second-generation VALIUM vectors,

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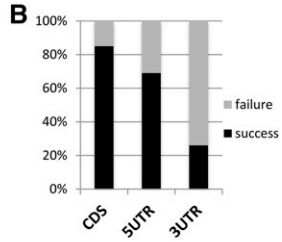
	0-25%	25-50%	50-75%	75-99%	No KD
# lines	214	111	67	36	72
percent	43	22	13	7	14



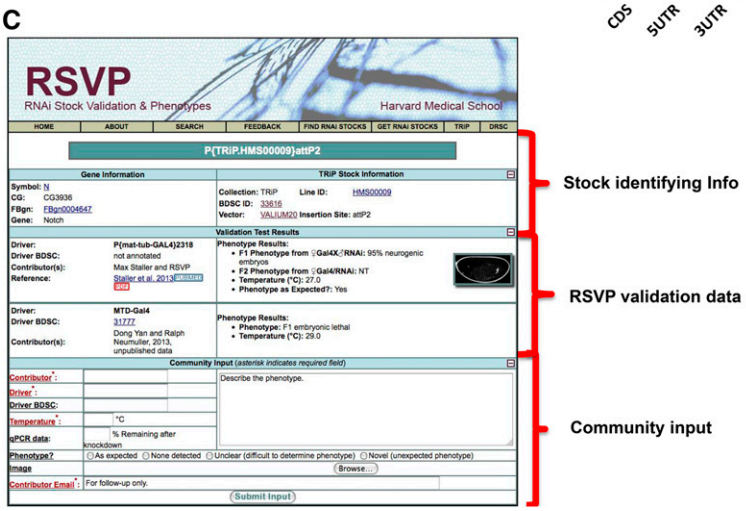


Figure 6 The validation of TRiP lines. (A) Knockdown efficiency of shRNAs analyzed from 0- to 4-hr embryos derived from maternal-Gal4, shRNA females. (B) Percentage of shRNA lines generating >50% knockdown and their targeting regions (CDS, coding sequence; 5′ and 3′ UTR). (C) Snapshot of RSVP webpage. Example RSVP "details page" layout for a TRiP line targeting Notch (Line ID: HMS00009). "Validation Test Results" are curated from publications, TRiP data, online community input, or personal communications to the TRiP. Bottom, text boxes for further input by the community. At RSVP there is a details page for each TRiP line generated, as well as for RNAi fly stocks in other major public collections.

VALIUM20, VALIUM21, and VALIUM22 (Figure 4). Our experience and elsewhere has shown that, in general, shRNAs are more effective than long dsRNAs (Ni *et al.* 2011). For design of the shRNAs, sequences common to all splice forms of a gene are identified, and any subsequences 16 bp or longer that match other genes are not considered in the selection of the 21-bp targeting sequence. Each subsequence is given a score (calculated as described by Vert *et al.* 2006) and the highest scoring sequences are selected. Top- and bottom-strand oligos are designed using an automated Perl program developed internally, and the hairpins were cloned into the vector (see Ni *et al.* 2011).

Approximately 4595 genes have been nominated by the *Drosophila* community and over the years >260 different investigators nominated lists of genes. In addition, we prioritized specific categories of genes to help researchers perform targeted screens. We defined lists of 23 major gene categories (kinases, transcription factors, secreted proteins, etc.; 14 categories are shown in Figure 5A) and generated shRNA lines to provide comprehensive sets of RNAi stocks. We have generated a web resource, named GLAD (for Gene List Annotation at the DRSC; http://www.flyrnai.org/glad) where lists of major gene categories, as well as subcategories, can be found and downloaded (Hu *et al.* 2015). Of special interest, we assembled a TRiP collection representing *Drosophila* orthologs of genes associated with human diseases (Human Disease TRiP Project, HuDis-TRiP, http://www.flyrnai.org/HuDis). There are currently TRiP fly stocks in the HuDis-TRiP collection for 1575 *Drosophila* orthologs of human disease-associated genes (Figure 5B). These include 85% coverage for 670 high-confidence *Drosophila* orthologs of high-confidence disease-associated human genes.

The "TRiP Toolbox": In addition to the TRiP RNAi lines, the TRiP provides a set of TRiP Toolbox stocks, including injection stocks for labs wishing to generate their own RNAi lines (*e.g.*, isoform-specific lines or other custom lines that would not be appropriate for the TRiP community nomination and production pipeline), as well as commonly used Gal4 lines combined in most cases with *UAS-Dcr2* to enhance knockdown using long dsRNAs (*i.e.*, in VALIUM1 or VALIUM10). A list of available lines is available at http://www.flyrnai.org/TRiP-TBX.html and in Table S1, as well as at stock center websites (*e.g.*, http://flystocks.bio.indiana.edu/Browse/TRiPtb.htm).

Distribution of TRiP fly stocks

All completed stocks are annotated on the TRiP website and on FlyBase and made immediately available to the research community through the Bloomington *Drosophila* Stock Center (BDSC), the NIG (http://www.shigen.nig.ac.jp/fly/nigfly/), and the TsingHua Fly Center (http://center.biomed.tsinghua.edu.cn/public/eq-category/all/modelanimalfacility). Currently, the BDSC is distributing 10,434 RNAi stocks, the NIG has 5961, and the THFC has 8591. As of May 2015, 286,658 TRiP RNAi stocks have been distributed from the BDSC to 1271 different user groups at 640 different organizations in 39 countries. A total of 1347 TRiP stocks have been distributed from the NIG, Japan, and 34,000 from the THFC, China.

Validation of the TRiP lines

RNAi Stock Validation and Phenotypes Project: Challenges with RNAi include evaluation of the efficiency (level of knockdown) and specificity (potential off-target effects, OTEs). To facilitate the selection of the best available RNAi fly stocks, we initiated the RSVP to evaluate the performance of existing

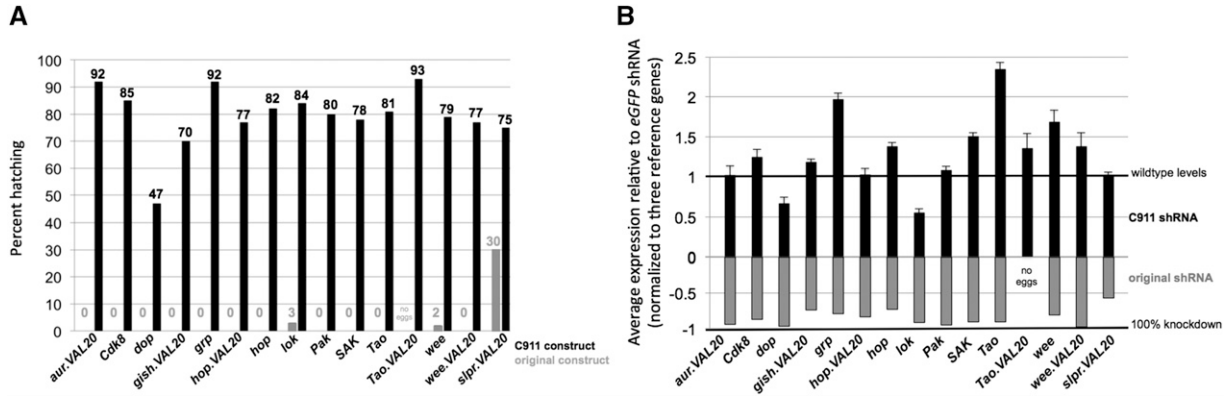


Figure 7 Addressing OTEs with C911 shRNAs. (A) Hatch rate examination of efficient shRNAs and their C911 counterparts. (B) Target expression levels for original shRNA and their C911 counterparts, relative to an EGFP-targeting shRNA.

TRiP fly stocks. We performed validation experiments (phenotype analyses and RT–qPCR) for a number of lines, collected available published information, and established a web resource (http://www.flyrnai.org/RSVP.html) to provide the community with all available information relevant to performance of the lines. Importantly, on the RSVP web pages, community members have the opportunity to share their own data relevant to TRiP stocks (Figure 6).

To date we have performed RT–qPCR validation analysis for >500 TRiP fly stocks. Most RT–qPCR analyses were performed in early embryos from *MTD-Gal4*; *UAS-shRNA* animals as described in Sopko *et al.* (2014). On average, 65% of TRiP stocks display knockdown efficiencies of >50% (Figure 6A). To facilitate searching for primers appropriate for RT–qPCR analysis, we assembled FlyPrimerBank (http://www.flyrnai.org/FlyPrimerBank) (Hu *et al.* 2013b). Relevant to long dsRNA reagents, the tool indicates if a given primer pair should be avoided as the primers are predicted to amplify the dsRNA itself.

RSVP also collects information relevant to the phenotypes observed with specific Gal4 lines. In particular, results from various large-scale screens have been included. Among these are germline and maternal effect screens using the MTD–Gal4 driver (Staller *et al.* 2013; Sopko *et al.* 2014; Yan *et al.* 2014), a muscle screen using the muscle Dmef2–Gal4 line (Perrimon and Randkelv, unpublished results), a screen in gut stem cells using the esg-Gal4 (Zeng *et al.* 2015), and a screen of maternally expressed genes involved in embryonic patterning (Liu and Lasko 2015). As of May 2015, and specific for the TRiP stocks, the RSVP contains 8334 data entries for 5202 TRiP stocks representing 3735 fly genes. This information is particularly helpful for selecting lines that have strong knockdown phenotypes; moreover, if multiple RNAi lines targeting the same gene have the same phenotypes with similar Gal4 lines, then it suggests that these RNAi reagents are likely on target. Finally, this information will help cull the lines that are not optimal for knockdown efficiencies or that are associated with OTEs.

Interestingly, based on RT–qPCR and phenotypic analyses, not all regions of a gene appear optimal for hairpin design. Specifically, shRNAs designed to target 5′ or 3′ UTRs of genes produce effective knockdown in ~60 or ~30% of fly stocks, respectively. By contrast, up to 85% of TRiP lines with shRNAs designed to the coding region of a gene produce effective RNA knockdown as determined by RT–qPCR and phenotypic analyses (Figure 6B).

Most recently, the RSVP has incorporated from FlyBase data entries for RNAi lines generated by the NIG and the VDRC making it possible for researchers to choose stocks from among the various RNAi collections. In total the RSVP now also contains 23,451 data entries for 17,782 RNAi lines representing 11,346 genes.

Rescuing with fosmids: A critical issue when a phenotype is observed with a specific RNAi line is to evaluate whether the phenotype reflects knockdown of the intended target gene or results from an OTE. The OTE issue and various approaches to addressing the problem have been discussed at length (*e.g.*, see review by Perrimon *et al.* 2010). To test for OTEs, the gold standard method, although relatively cumbersome, is a rescue approach, such as rescue using genomic DNA from a different species (Kondo *et al.* 2009; Langer *et al.* 2010). An alternative is to rescue using an ORF from a UAS construct, although interpretation of the result using this approach could be complicated if overexpression of the ORF alone in a wild-type background generates a phenotype. To date we have generated 38 fosmid lines with DNA from *D. persimilis* (Table S2). We selected *D. persimilis* as we have at the DRSC access to a fosmid library of 70,000 clones, and this species is closely related to *D. pseudoobscura*, which we have shown previously to be ideal for such rescue experiments (Kondo *et al.* 2009).

By combining shRNA and fosmid-bearing chromosomes we tested whether providing a *D. persimilis* ortholog could rescue the presumed on-target gene and were able to rescue shRNA-induced phenotypes about two-thirds of the time. This moderate failure rate may be attributed to the DNA excision method used for fosmid clone library generation, which may inadvertently truncate gene promoter and termination sequences. Additionally, the large blocks of cloned *D. persimilis* DNA may no longer be regulated like native chromatin and consequently this might affect transcription factor accessibility and general transcription.

Table 2 Online resources for search and view of TRiP stocks and corresponding information

Information and Screening Centers		
The Transgenic RNAi Project	TRiP	http://www.flyrnai.org/TRiP-HOME.html
Drosophila RNAi Screening Center	DRSC	http://www.flyrnai.org/DRSC-HOME.html
TRiP Stock Centers		
Bloomington *Drosophila* Stock Center	BDSC	http://flystocks.bio.indiana.edu/
National Institute of Genetics, Japan	NIG	http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp
TsingHua Fly Center, China	THFC	http://center.biomed.tsinghua.edu.cn/public/eq-category/all/modelanimalfacility
Lists		
Human Disease-Fly Ortholog Pairs	HuDis-TRiP	http://www.flyrnai.org/HuDis
GLAD-Gene List Annotation at DRSC	GLAD-TRiP	http://www.flyrnai.org/glad
Tools		
RNAi Stock Validation and Phenotypes	RSVP	http://www.flyrnai.org/RSVP.html
Updated Targets of RNAi Reagents	UP-TORR	http://www.flyrnai.org/up-torr/
PCR Primers in *Drosophila*	FlyPrimerBank	http://www.flyrnai.org/FlyPrimerBank
DRSC Ortholog Prediction Tool	DIOPT	http://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl
DRSC Disease Gene Query Tool	DRSC-DGQT	http://www.flyrnai.org/cgi-bin/DRSC_DG_query.pl

Addressing OTEs with C911 constructs: An alternative method for assessment of OTEs is to examine C911 versions of the targeting shRNA (Buehler *et al.* 2012). C911 versions are near identical shRNAs but with mismatches at positions 9–11. The mismatches are predicted to disrupt on-target binding but preserve seed sequence-mediated OTEs, since the anti-sense and sense seed sequences remain intact. We generated C911 versions of 15 shRNAs for which expression in the germline results in defective hatching (see Table S3). As expected for on-target shRNA constructs, C911 mutations restored hatching to wild-type rates for nearly all shRNAs. The one exception was for a construct targeting *drop out (dop)*, which only partially rescued the phenotype (Figure 7A). We further verified by RT–qPCR that mutation of these three residues eliminates knockdown of on-target transcript for 13 targets. For the exceptions, *dop* and *loki*, the C911 construct only partially restored mRNA to wild-type levels (Figure 7B). These data suggest that C911 constructs may serve as more suitable controls as compared with common negative control constructs, *e.g.*, constructs expressing eGFP or shRNAs targeting *white*.

Online search of TRiP stocks and information

We have assembled a number of resources for online search of existing RNAi lines and to view corresponding information, *e.g.*, on efficiency (see TRiP website http://www.flyrnai.org/TRiP-HOME.html and Table 2). We provide two online tools for direct search and view of RNAi fly stock information (UP-TORR and RSVP), we provide links between these tools and from other results pages to these tools, and we provide organized groups of TRiP stocks, *e.g.*, based on gene function. UP-TORR (http://www.flyrnai.org/up-torr/) allows the search of TRiP and other RNAi fly stocks based on up-to-date gene annotation information (Hu *et al.* 2013a) and links to RSVP. As described above, RSVP (http://www.flyrnai.org/RSVP.html) allows users to search and view information about knockdown efficiency (RT–qPCR data) and phenotypes (in text format and when available, supplemented with images) for specific RNAi fly stock/Gal4 driver combinations. Moreover, RSVP includes results curated by FlyBase for other major stock collections, such as phenotypes associated with VDRC fly stocks.

To start an RNAi fly stock search with a list of orthologs from other species or with a disease term, results pages from the ortholog search tool DIOPT and the disease-gene ortholog search tool DIOPT-DIST (Hu *et al.* 2011) include a link that allows the user to carry results to UP-TORR. In addition, a list of TRiP stocks corresponding to ~670 high-confidence fly orthologs of high-confidence human disease-associated genes (*i.e.*, the HuDis-TRiP stocks; see above) is available for view as a table including BDSC stock IDs and links to RSVP, or as a download (URL at Table 2). Moreover, the TRiP home page provides links that prepopulate the UP-TORR search page with gene groups (*e.g.*, kinases) to facilitate identification of stocks for focused studies (URL at Table 2).

Concluding remarks

Transgenic RNAi fly stocks have become essential tools in the *Drosophila* molecular genetic toolbox, as illustrated by the number of requests for RNAi fly stocks from stock centers, and the observation that a very large proportion of current *Drosophila* publications include studies using one or more RNAi fly stocks. On the production side, our own and other efforts are approaching coverage of all *Drosophila* genes (in particular, coverage of all community-nominated genes). Thus, production efforts might shift to specific needs such as production of replacements for ineffective shRNAs and production of fly stocks targeting specific gene isoforms. Moreover, now that such a large number of RNAi fly stocks are available and being tested, there is an increasing need for curation of the information regarding performance of the fly stocks and inclusion of information in a centralized database such as RSVP. Because researchers often know that a given RNAi fly stock does or does not perform in a manner consistent with expectation (*e.g.*, based on mutant phenotype data)

well in advance of publication, the most timely approach is for researchers to provide community feedback on RNAi efficiency independent of publication of research results. More than ever, community feedback is needed to annotate the various RNAi collections.

Acknowledgments

G.J.H., R.L., and N.P. are investigators of the HHMI. The Transgenic RNAi Project (TRiP) at Harvard Medical School (HMS) thanks Aram Comjean for website oversight and Linda Jiang for stock maintenance in the early stages of the TRiP. We thank Kevin Moses and Chonnettia Jones and the HHMI Visitor's Research Program for support of the work performed at Janelia Farm. We also thank Monti Mercer, Megan Hong, Grace Zheng, Dona Fetter and Jessica Ketting at Janelia Farm for helping to process the transformant lines. R.U. and S.K. thanks Satoru Yoshida and Fumiwo Ejima for their help with DNA sequencing at NIG. We also thank Kathy Matthews and Kevin Cook for their advice, support, and enthusiasm while making the TRiP stocks available to the fly community. Finally, the TRiP at HMS thanks the *Drosophila* community for their nominations, encouragement, and collegiality. Work at the TRiP and *Drosophila* RNAi Screening Center is supported by National Institutes of Health (NIH) R01GM084947 (N.P.), R01GM067761 (N.P.), and R24RR032668 (N.P.). Work in Japan is supported by the National Institute of Genetics. Work at the Tsinghua Fly Center is supported by the National Key Technology Research and Development Program of the Ministry of Science and Technology of the People's Republic of China (no. 2015BAI09B03), the National Basic Research Program (973 Program) (no. 2013CB35102), and the National Natural Science Foundation of China (no. 31371496). Work at Boston University is supported by NIH R01GM060574 (K.M.) and RO1GM094452 (K.M.) and we thank Jon Iker Etchegaray who suggested the idea of the QUAS-RNAi vector. Work at Yale University is supported by NIH RO1GM043301 (L.C.). Work at CSHL was supported by NIH R37GM062534 (G.J.H.). Work at New York University School of Medicine Medical was supported by HHMI and an administrative supplement (American Recovery and Reinvestment Act) to R01HD14900-09 (R.L.). Work at the Bloomington Drosophila Stock Center is supported by the Office of The Director, National Institutes of Health under Award no. P40OD018537.

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Communicating editor: J. Sekelsky

GENETICS

Supporting Information
www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.180208/-/DC1

The Transgenic RNAi Project at Harvard Medical School: Resources and Validation

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DOI: 10.1534/genetics.115.180208

Table S1 The TRiP Toolbox Stocks

TRiP Toolbox Stocks	Genotype	BL Stock#
Injection Stocks		
y sc v nanos-integrase; attP40	y[1] sc[1] v[1] P{y[+t7.7]=nos-phiC31\int.NLS}X; P{y[+t7.7]=CaryP}attP40	
y v nanos-integrase; attP40	y[1] v[1] P{y[+t7.7]=nos-phiC31\int.NLS}X; P{y[+t7.7]=CaryP}attP40	25709
y sc v nanos-integrase; attP2	y[1] sc[1] v[1] P{y[+t7.7]=nos-phiC31\int.NLS}X; P{y[+t7.7]=CaryP}attP2	25710
Gal4, UAS dcr2 Stocks		
w, elav-Gal4; UAS-dcr2	w[1118], P{w[+mC]=GAL4-elav.L}; P{w[+mC]=UAS-Dcr-2.D}2	25750
w, ms1096-Gal4; UAS-dcr2	w[1118], P{w[+mW.hs]=GawB}Bx[MS1096]; P{w[+mC]=UAS-Dcr-2.D}2	25706
w, UAS-dcr2; twist-Gal4	P{w[+mC]=UAS-Dcr-2.D}1, w[1118]; P{w[+mC]=GAL4-twi.2xPE}1	25707
w, UAS-dcr2; actin-Gal4/CyO	P{w[+mC]=UAS-Dcr-2.D}1, w[1118]; P{w[+mC]=Act5C-GAL4}25FO1 / CyO, Cy[1]	25708
w, UAS-dcr2; nanos-Gal4	P{w[+mC]=UAS-Dcr-2.D}1, w[1118]; P{w[+mC]=GAL4-nos.NGT}40	25751
w, UAS-dcr2; engrailed-Gal4, UAS-GFP	P{w[+mC]=UAS-Dcr-2.D}1, w[1118]; P{w[+mW.hs]=en2.4-GAL4}e16E, P{w[+mC]=UAS-2xEGFP}AH2	25752
w, UAS-dcr2; blistered-Gal4/CyO	P{w[+mC]=UAS-Dcr-2.D}1, w[1118]; P{w[+mC}=bs-GAL4.Term}G1	25753
w, UAS-dcr2; nubbin-Gal4	P{w[+mC]=UAS-Dcr-2.D}1, w[1118]; P{w[+mW.hs]=GawB}nubbin-AC-62	25754
w, UAS-dcr2; spalt-Gal4	P{w[+mC]=UAS-Dcr-2.D}1, w[1118]; P{w[+mW.hs]=GawB}salm[LP39]	25755
w, UAS-dcr2; Dmef2-Gal4	P{w[+mC]=UAS-Dcr-2.D}1, w[1118]; P{w[+mC]=GAL4-Mef2.R}R1	25756
w, UAS-dcr2; C96-Gal4	P{w[+mC]=UAS-Dcr-2.D}1, w[1118]; P{w[+mW.hs]=GawB}bbg[C96]	25757
w, UAS-dcr2; pannier-Gal4/TM3, Ser	P{w[+mC]=UAS-Dcr-2.D}1, w[1118]; P{w[+mW.hs]=GawB}pnr[MD237] /TM3, Ser[1]	25758

Gal4 Stocks		
MTD-Gal4	P{otu-GAL4::VP16.R}1, w[*]; P{GAL4-nos.NGT}40; P{GAL4::VP16-nos.UTR}CG6325[MVD1] (also known as MTD-GAL4)	31777
Mapping Stocks		
y sc v; Gla Bc/CyO	y[1] sc[1] v[1]; wg[Gla-1], Bc[1] / CyO, Cy[1]	
y v; Sco/CyO	y[1] v[1]; noc[Sco] / CyO, Cy[1]	
y v; TM3, Sb/TM6, Tb	y[1] v[1]; TM3, Sb[1] / TM6, Tb[1]	
y v; Ly/TM3, Sb	y[1] v[1]; sens[Ly-1] / TM3, Sb[1]	
y v; Sb/TM3, Ser	y[1] v[1]; Sb[1] / TM3, Ser[1]	
y v; Dr, e/ TM3, Sb	y[1] v[1]; Dr[1] e[1] / TM3, Sb[1]	
y sc v; Dr, e/ TM3, Sb	y[1] sc[1] v[1]; Dr[1] e[1] / TM3, Sb[1]	32261

Table S2 ***D. persimilis*** **rescue fosmids**

Fosmid clone ID	*D. melanogaster* genes covered	FBgn	Construct	Stock	Chromosome Location(s)	Rescue Observed
G727P8862D2	esg	FBgn0001981	Yes			
	CG15258	FBgn0032563				
G727P8482E4	E(spl)mdelta-HLH	FBgn0002734	Yes			
	E(spl)mgamma-HLH	FBgn0002735				
	Nf1	FBgn0015269				
	CG42261	FBgn0259146				
	CG42261	FBgn0259146				
G727P8542A9	B52	FBgn0004587	Yes	Yes		
	Task6	FBgn0038165				
	CG9588	FBgn0038166				
	lkb1	FBgn0038167				
	omd	FBgn0038168				
G727P8957F10	Sep5	FBgn0026361	No	Yes	on III	
	CSN4	FBgn0027054				
	nito	FBgn0027548				
	CG2906	FBgn0033240				
	CG2915	FBgn0033241				
	CG14763	FBgn0033243				

	CG8726	FBgn0033244				
	pcs	FBgn0033988				
	CG7639	FBgn0033989				
	CG7544	FBgn0033994				
G727P8116G11	ND75	FBgn0017566	No	Yes	on II	
	CG2147	FBgn0030025				
	sni	FBgn0030026				
G727P8171G2	CG14694	FBgn0037845	Yes			
	CG6574	FBgn0037846				
	CG14866	FBgn0038315				
	CG6276	FBgn0038316				
	CG6236	FBgn0038318				
G727P8220G11	zfh1	FBgn0004606	No	Yes		
G727P8229F12	Cbp53E	FBgn0004580	No	Yes	on III	
	CG12917	FBgn0033490				
	ste24b	FBgn0034175				
	ste24a	FBgn0034176				
	gem	FBgn0050011				
	CR30461	FBgn0050461				
	ste24c	FBgn0050462				
G727P8237E7	BBS4	FBgn0033578	Yes	Yes		
	CG13229	FBgn0033579				

	CG13231	FBgn0033580				
	CG12391	FBgn0033581				
	CG13230	FBgn0040764				
	Cyp12d1-p	FBgn0050489				
	Cyp12d1-d	FBgn0053503				
G727P8252D3	sd	FBgn0003345	Yes			
G727P8310A11	unk	FBgn0004395	No	Yes	on III	
	Hmu	FBgn0015737				
	CG3368	FBgn0039508				
	bigmax	FBgn0039509				
	CG3348	FBgn0040609				
G727P8508C2	G9a	FBgn0040372	Yes			
	CG3038	FBgn0040373				
G727P8552G3	Mipp1	FBgn0026061	Yes	No		
	CG13033	FBgn0036638				
	nxf2	FBgn0036640				
	Smn	FBgn0036641				
	mbf1	FBgn0262732				
G727P8133D3	mei-W68	FBgn0002716	Yes	Yes	on II	
	Spn55B	FBgn0028983				
	CG2064	FBgn0033205				
	CG12042	FBgn0033206				

	TBCB	**FBgn0034451**				
G727P8149E1	**cno**	**FBgn0259212**	**No**	**Yes**	**on III**	
G727P8209C9	**baz**	**FBgn0000163**	**Yes**	**Yes**	**on II**	**Yes**
	CG8918	**FBgn0030823**				
	CG32563	**FBgn0052563**				
G727P8331F10	**wbl**	**FBgn0004003**	**No**	**Yes**	**on II & III**	
	cora	**FBgn0010434**				
	CG7137	**FBgn0034422**				
G727P8333C5	**CG6788**	**FBgn0030880**	**No**	**Yes**	**on II**	
	CG15629	**FBgn0031630**				
	CG3225	**FBgn0031631**				
	CG5043	**FBgn0032636**				
	CG5050	**FBgn0032637**				
	CG5050	**FBgn0032637**				
	dl	**FBgn0260632**				
G727P8345D6	**shg**	**FBgn0003391**	**Yes**	**Yes**	**on II**	
	CG9350	**FBgn0034576**				
	cpa	**FBgn0034577**				
	RIC-3	**FBgn0050296**				
G727P8359B3	**l(2)gl**	**FBgn0002121**	**Yes**	**Yes**	**on II**	**Yes**
	CG3164	**FBgn0025683**				
	Ir21a	**FBgn0031209**				

	CG4822	**FBgn0031220**				
G727P8401F10	**CG8311**	**FBgn0034141**	**No**	**Yes**	**on II**	
	CG3829	**FBgn0035091**				
	zip	**FBgn0265434**				
G727P8423G11	**B52**	**FBgn0004587**	**No**	**Yes**	**on III**	
	Task6	**FBgn0038165**				
	CG9588	**FBgn0038166**				
	lkb1	**FBgn0038167**				
	omd	**FBgn0038168**				
G727P8446F2	**Arpc1**	**FBgn0001961**	**Yes**			
	DNApol-gamma35	**FBgn0004407**				
	Orc5	**FBgn0015271**				
	RpII33	**FBgn0026373**				
	CG7968	**FBgn0028532**				
	CG7953	**FBgn0028533**				
	CG7916	**FBgn0028534**				
	CG9008	**FBgn0028540**				
	TM9SF4	**FBgn0028541**				
	CG8997	**FBgn0028920**				
	CG13083	**FBgn0032789**				
	CG10194	**FBgn0032790**				
	CG18094	**FBgn0032791**				

	CG10189	**FBgn0032793**				
	CG10188	**FBgn0032796**				
	Top3alpha	**FBgn0040268**				
	CG33306	**FBgn0053306**				
	CG33307	**FBgn0053307**				
	CG33649	**FBgn0064115**				
	mRpS23	**FBgn0260407**				
G727P8461B1	**Tim8**	**FBgn0027359**	**No**	**Yes**	**on II**	
G727P8479H2	**Nab2**	**FBgn0028471**	**No**		**Yes**	
	CG9173	**FBgn0035218**				
	CG5715	**FBgn0039180**				
	crb	**FBgn0259685**				**Yes**
G727P8481B7	**yrt**	**FBgn0004049**	**No**	**Yes**	**on II**	
	yellow-e3	**FBgn0038150**				
	yellow-e2	**FBgn0038151**				
	Ir87a	**FBgn0038153**				
	yellow-e	**FBgn0041711**				
G727P8730B8	**par-6**	**FBgn0026192**	**No**	**Yes**	**on II**	**Yes**
	CG8188	**FBgn0030863**				
	CG8173	**FBgn0030864**				
	gce	**FBgn0261703**				
	chas	**FBgn0263258**				

G727P8733H3	**alpha-Cat**	**FBgn0010215**	**Yes**	**Yes**	**on II**	
G727P8776E4	**CG10257**	**FBgn0033985**	**Yes**	**Yes**	**on II**	
	ckn	**FBgn0033987**				
	aPKC	**FBgn0261854**				
G727P8779F6			**No**	**Yes**	**on III**	
G727P880D1	**nrv2**	**FBgn0015777**	**No**	**Yes**		
G727P883B7	**l(3)neo18**	**FBgn0011455**	**No**	**Yes**	**on III**	
	Nrx-IV	**FBgn0013997**				
	CG5645	**FBgn0036254**				
	Atg12	**FBgn0036255**				
	RhoGAP68F	**FBgn0036257**				
	CG5642	**FBgn0036258**				
	CG9760	**FBgn0036259**				
	Rh7	**FBgn0036260**				
	CG11534	**FBgn0046296**				
G727P8137E6	**ref(2)P**	**FBgn0003231**	**Yes**			
	osm-6	**FBgn0031829**				
	CoVb	**FBgn0031830**				
	CG11043	**FBgn0031831**				
	CG9596	**FBgn0031832**				
	CG13082	**FBgn0032803**				
	CG13081	**FBgn0032804**				

	CG10337	FBgn0032805				
	Tep4	FBgn0041180				
	CG33116	FBgn0053116				
	Ent2	FBgn0263916				
G727P8197B12	mys	FBgn0004657	No	Yes	on III	
	Upf2	FBgn0029992				
	CG1571	FBgn0029993				
	CG2254	FBgn0029994				
	Rph	FBgn0030230				
	Atg8a	FBgn0052672				
	Tango5	FBgn0052675				
G727P8470C11	CG17666	FBgn0036311	No	Yes	on III	
	CG10754	FBgn0036314				
	Atg1	FBgn0260945				
	CG42588	FBgn0260965				
	CG42709	FBgn0261674				
	Sap130	FBgn0262714				
G727P8823D9	CG6912	FBgn0038290	Yes			
	CG3984	FBgn0038291				
	CG3984	FBgn0038291				
	CG3987	FBgn0038292				
	CG6904	FBgn0038293				

G727P8387G2	**CG31609**	**FBgn0051609**	**Yes**			
	dm	**FBgn0262656**				
G727P8475E8	**His3.3B**	**FBgn0004828**	**Yes**			
	His3.3A	**FBgn0014857**				
	Ost48	**FBgn0014868**				
	CG7065	**FBgn0030091**				
	fh	**FBgn0030092**				
	dalao	**FBgn0030093**				
	Zpr1	**FBgn0030096**				
	CG9034	**FBgn0040931**				
	His3:CG31613	**FBgn0051613**				
	His3:CG33803	**FBgn0053803**				
	His3:CG33806	**FBgn0053806**				
	His3:CG33809	**FBgn0053809**				
	His3:CG33812	**FBgn0053812**				
	His3:CG33815	**FBgn0053815**				
	His3:CG33818	**FBgn0053818**				
	His3:CG33821	**FBgn0053821**				
	His3:CG33824	**FBgn0053824**				
	His3:CG33827	**FBgn0053827**				
	His3:CG33830	**FBgn0053830**				
	His3:CG33833	**FBgn0053833**				

	His3:CG33836	**FBgn0053836**				
	His3:CG33839	**FBgn0053839**				
	His3:CG33842	**FBgn0053842**				
	His3:CG33845	**FBgn0053845**				
	His3:CG33848	**FBgn0053848**				
	His3:CG33851	**FBgn0053851**				
	His3:CG33854	**FBgn0053854**				
	His3:CG33857	**FBgn0053857**				
	His3:CG33860	**FBgn0053860**				
	His3:CG33863	**FBgn0053863**				
	His3:CG33866	**FBgn0053866**				
G727P8550H9	**ash1**	**FBgn0005386**	**Yes**			
	Max	**FBgn0017578**				
	nes	**FBgn0026630**				
	CG6888	**FBgn0036490**				
	mRpL21	**FBgn0036853**				
	CG9666	**FBgn0036856**				
	CG9629	**FBgn0036857**				
	CG14085	**FBgn0036859**				
	CG14086	**FBgn0036860**				
	CG14089	**FBgn0036861**				
	Gbs-76A	**FBgn0036862**				

	rept	**FBgn0040075**				
	CG42374	**FBgn0259720**				
	Bet1	**FBgn0260857**				
G727P8896D3	**Arl1**	**FBgn0000115**	**Yes**			
	brm	**FBgn0000212**				
	CG10516	**FBgn0036549**				
	CG10516	**FBgn0036549**				
	CG17026	**FBgn0036550**				
	CG17029	**FBgn0036551**				
	CG17028	**FBgn0036552**				
	CG17027	**FBgn0036553**				
	CG5830	**FBgn0036556**				
	Hip14	**FBgn0259824**				
	DNApol-delta	**FBgn0263600**				
G727P8254G11	**His2Av**	**FBgn0001197**	**No**	**Yes**	**on III**	
	ro	**FBgn0003267**				
	Rb97D	**FBgn0004903**				
	DIP1	**FBgn0024807**				
	BM-40-SPARC	**FBgn0026562**				
	ball	**FBgn0027889**				
	IntS12	**FBgn0039459**				
	CG5500	**FBgn0039461**				

G727P8779A12	**os**	**FBgn0004956**	**No**	**Yes**	**on III**	
	upd3	**FBgn0053542**				
G727P8106D5	**sei**	**FBgn0003353**	**Yes**			
	tsr	**FBgn0011726**				
	RpL39	**FBgn0023170**				
	Rap2l	**FBgn0025806**				
	gammaSnap	**FBgn0028552**				
	ppk29	**FBgn0034965**				
	CG13563	**FBgn0034966**				
	eIF-5A	**FBgn0034967**				
	RpL12	**FBgn0034968**				
	yki	**FBgn0034970**				
	CG3209	**FBgn0034971**				
G727P8372D3	**Poc1**	**FBgn0036354**	**Yes**			
	skap	**FBgn0037643**				
	CG11964	**FBgn0037644**				
	CG10038	**FBgn0038013**				
	CG10041	**FBgn0038014**				
	MBD-R2	**FBgn0038016**				
	CG4115	**FBgn0038017**				
G727P8486C3	**ImpL2**	**FBgn0001257**	**Yes**	**Yes**	**on II & III**	
	pav	**FBgn0011692**				

	CG14997	FBgn0035515				
	CG1265	FBgn0035517				
	CG15011	FBgn0035518				
	CG1309	FBgn0035519				
	CG11586	FBgn0035520				
	VhaM9.7-a	FBgn0035521				
	ago	FBgn0041171				
G727P8127C5	car	FBgn0000257	No	Yes	on II	
	car	FBgn0000257				
	Tao	FBgn0031030				Yes
G727P8143G8	cdc2c	FBgn0004107	No	Yes	on II	Yes
	CG17267	FBgn0038821				
	CG31199	FBgn0051199				
G727P8228G2	mei-41	FBgn0004367	Yes	Yes	on III	Yes
	CG1434	FBgn0030554				
	CG6847	FBgn0030884				
G727P8383H8	wee	FBgn0011737	No	Yes	on II	Yes
	nop5	FBgn0026196				
	neuroligin	FBgn0031866				
	CG13773	FBgn0042092				
G727P871G11	Zip3 gish	FBgn0038412 FBgn0250823	No	Yes	on II	 Yes

G727P88F12	**Cdk8**	**FBgn0015618**	**No**	**Yes**	**on II**	
	CG1271	**FBgn0035392**				
	CG16753	**FBgn0035393**				
	CG10566	**FBgn0037050**				
	CG10565	**FBgn0037051**				
	Sk2	**FBgn0052484**				
	CG32485	**FBgn0052485**				
G727P8275B7	**cora**	**FBgn0010434**	**No**	**Yes**	**on III**	
	CG7137	**FBgn0034422**				
G727P8543F8	**Atpalpha**	**FBgn0002921**	**No**	**Yes**	**on III**	
G727P8822E6	**CG18577**	**FBgn0037870**	**Yes**	**Yes**		
	SdhC	**FBgn0037873**				
	Tctp	**FBgn0037874**				
	CG6672	**FBgn0037875**				
	CG4820	**FBgn0037876**				
	RpS25	**FBgn0086472**				

Table S3 C911 short hairpin designs

CG	FBgn	Gene	Hairpin ID	TRiP Stock ID	sense_C911	antisense_C911
CG3068	FBgn0000147	aurora	C911_SH01244.N2	C911_GL00047	ATGACGAGACGATCAAGAAGA	TCTTCTTGATCGTCTCGTCAT
CG14217	FBgn0031030	Tao-1	C911_SH03285.N	C911_HMS02205	CAGAGCTACATTGTAGACGAA	TTCGTCTACAATGTAGCTCTG
CG4488	FBgn0011737	wee	C911_SH01410.N2	C911_GL00231	CAAGAACGAGTATCTGATGAA	TTCATCAGATACTCGTTCTTG
CG17161	FBgn0261278	grapes	C911_SH01605.N2	C911_GL00220	ACGATGGGATCTGACTATCAA	TTGATAGTCAGATCCCATCGT
CG10295	FBgn0014001	PAK-kinase	C911_SH01885.N	C911_HMS01609	CACGACGACGGACGACAAGAA	TTCTTGTCGTCCGTCGTCGTG
CG7186	FBgn0026371	Sak kinase	C911_SH01169.N2	C911_GL00067	AAGGCTAGCTTACTTATCCAA	TTGGATAAGTAAGCTAGCCTT
CG14217	FBgn0031030	Tao-1	C911_SH00709.N	C911_HMS00761	CACGAGAAGCTATCTAAAGAA	TTCTTTAGATAGCTTCTCGTG
CG4488	FBgn0011737	wee	C911_SH01544.N2	C911_GL00305	CAGCAGCTGGTGTGTAAGATA	TATCTTACACACCAGCTGCTG
CG10572	FBgn0015618	Cyclin-dependent kinase 8	C911_SH01168.N2	C911_GL00020	CAAGGTGTTCGACTTGATCGA	TCGATCAAGTCGAACACCTTG
CG10895	FBgn0019686	loki	C911_SH01221.N2	C911_GL00008	CAGGATGCGATAGCTAAAGAA	TTCTTTAGCTATCGCATCCTG
CG1594	FBgn0004864	hopscotch	C911_SH01406.N2	C911_GL00244	TCCGAACATACAGAAGTTCAA	TTGAACTTCTGTATGTTCGGA
CG1594	FBgn0004864	hopscotch	C911_SH01252.N2	C911_GL00051	CAGCGAATGTGCTGTGATCTA	TAGATCACAGCACATTCGCTG
CG6498	FBgn0036511	dropout	C911_SH04743.N	C911_HMS02333	CACGATGATGTATCCAGTTAT	ATAACTGGATACATCATCGTG
CG6963	FBgn0250823	gilgamesh	C911_SH01171.N2	C911_GL00015	CGAAGACTGTAAGAAGACTTA	TAAGTCTTCTTACAGTCTTCG
CG2272	FBgn0030018	slipper	C911_SH05304.N	C911_HMC03331	ACCACCCGATCATCTAGTCAA	TTGACTAGATGATCGGGTGGT

Figure S1. The TRiP Valium series vectors

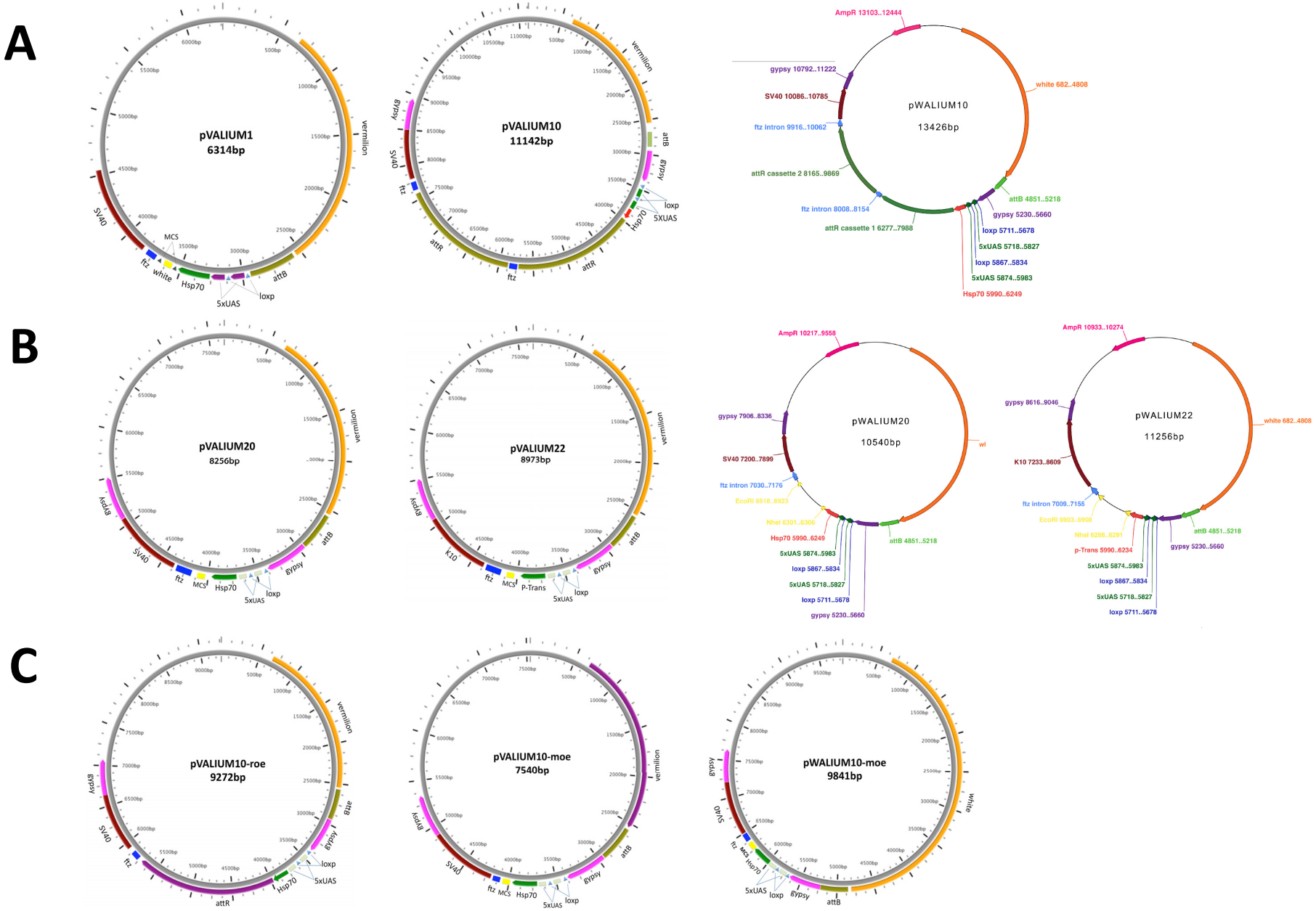

Figure S1 The TRiP vectors.

A. First generation of TRiP RNAi vectors. The first generation of commonly used TRiP RNAi knockdown vectors commonly used, VALIUM1 and VALIUM10, are based on long dsRNAs.

VALIUM1 contains a multiple cloning site (MCS) that allows a single PCR product to be cloned in both orientations to generate the hairpin construct. Additionally, VALIUM1 contains two introns: the *white* intron, located between the inverted DNA repeats, which has been shown to reduce toxicity in bacteria; and the *ftz* intron, followed by the SV40 polyA tail to facilitate hairpin-RNA processing and export from the nucleus. VALIUM1 is an effective vector for RNAi knockdown, however, its strength can be weak but knockdown phenotypes can be boosted by using a higher experimental temperature (27-29^{o}C) and having *UAS-Dicer2* in the genetic background (Ni et al., 2008). Based on the results with VALIUM1, we generated VALIUM10, the best performing vector from among 12 first generation vectors (Ni et al., 2009).

VALIUM10 differs from VALIUM1 in a number of ways: 1. it contains insulator sequences that increase significantly the level of expression of the hairpins; 2. instead of the MCS sites of VALIUM1, VALIUM10 contains a recombination system that facilitates the cloning of the hairpins, and 3. VALIUM10 contains two *ftz* introns. While increased temperature can increase the effectiveness of knockdown with VALIUM10, the presence of UAS-Dicer2 makes less of a difference than with VALIUM1.

We generated versions of VALIUM10, pWALIUM10, in which *vermilion* is replaced with *white*. Except for the selectable marker, the WALIUM vector has all of the same attributes of their *vermilion* containing counterparts.

B. Second generation TRiP RNAi vectors. The second generation knockdown vectors used by the TRiP for RNAi stock production, VALIUM20 and VALIUM22 (variant: VALIUM21), carries short interfering RNA (siRNAs) hairpins embedded in a modified

scaffold of the microRNA *miR-1* that uses the endogenous microRNA pathway to deliver the short hairpin into the genome (Haley et al., 2008; 2010; Ni et al., 2011). Note, that in our design (Ni et al., 2011), unlike in Haley et al. (2008), the siRNAs do not include mis-matches at positions 2 and 11. These vectors were used for generating most of the TRiP lines as they work effectively both in the germline and the soma.

VALIUM20, contains *vermilion* as a selectable marker; an attB sequence to allow phiC31-targeted integration at genomic attP landing sites; two gypsy sequences to enhance hairpin DNA transcription; two pentamers of UAS, one of which can be excised using the Cre/loxP system to generate a 5XUAS derivative; the *hsp70* basal promoter; a multiple cloning site (MCS) for cloning the short hairpins in the microRNA scaffold, and a *ftz* 3'UTR intron followed by a SV40 3'UTR as a source for a polyA signal sequence. Data from the TRiP and others show that VALIUM20 produces a more effective knockdown than VALIUM10 in the soma, and works well in the female germline (Ni et al., 2011).

VALIUM22 has each of the attributes of VALIUM20 but differs in having the *P-transposase* core promoter instead of the hsp70 basal promoter and the *ftz* 3'UTR intron is followed by a *K10* polyA instead of the SV40 3'UTR. These unique attributes make VALIUM22 particularly effective for RNA knock down in the female germline. However, the P-element transposase promoter is less effective than the *hsp70* basal promoter to drive expression in somatic cells. VALIUM21, a variant of VALIUM22, differs only in that it lacks the *ftz* intron and gypsy sequences found in VALIUM22, however it is still highly effective in the germ line.

As for VALIUM10, we generated versions of VALIUM20 and VALIUM22, where *vermilion* is replaced with *white*, pWALIUM20 and pWALIUM22.

C. Overexpression vectors: We generated pVALIUM10-roe, pVALIUM10-moe, pWALIUM20-roe and pWALIUM10-moe. With

the latter vectors, researchers have the option to clone their genes for over-expression by recombination (”roe” versions) or in a multi-cloning site (”moe”).